Research Plan

Kimberly Liao

Identification of a Model Agnostic Disease Driver in Non-alcoholic Steatohepatitis; Implications for Drug Development

A. Rationale:

With the recent rise in diabetes, obesity, and metabolic syndrome, non-alcoholic fatty liver disease (NAFLD) has reached epidemic proportions; one in three Americans are diagnosed with the disease [1-2]. Currently, a stage of NAFLD – non-alcoholic steatohepatitis (NASH) – has even greater implications. NASH, characterized by steatosis and inflammation, is predicted to become the primary cause for liver transplants in the next five years [2]. This disease could further progress to NASH with fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [2-4]. Evidence [4] also suggests that NASH can also progress to HCC prior to cirrhosis, indicating the NASH stage as a pivotal point in the NAFLD progression. To date, there are no approved drugs or therapies proven to treat NASH with fibrosis [3, 5]. In part, the lack of treatments can be accredited to the clinical trials punctuated with failures [7-8]. The weak translational data can be explained by the inherent disconnect between animal models wherein the drug is evaluated and human liver disease where the drug is expected to work and the inconsistency exhibited in how biochemical/histopathological endpoints are governed in each unique patient [2, 6]. It is abundantly clear that NASH is an area of tremendous unmet medical need and given the sheer size of this burgeoning epidemic, a treatment strategy is urgently required that can prevent its transition to scarring and the sequelae of scarring.

B. Research Question:

Can a model agnostic functional transcriptomic analysis approach be utilized to identify a disease driver in NASH?

Hypothesis:

The observed failure of clinical trials in NASH and lack of robust translational success suggest that the current model systems do not fully recapitulate human disease and the hallmark pathological features of NASH may not be caused by the same pathway in animal models and human subjects. Therefore, it is hypothesized that a model agnostic (model independent) disease driver is likely to play a role in human NASH. Identification of such a disease-driver can spur development of drugs that neutralize the driver and bridge the disconnect between animal models and human subjects.

C. Procedure:

The experiment will be performed at Angion Biomedica Corp. under the supervision of Dr. Prakash Narayan.

Banked hematoxylin-eosin-stained (H&E) and picrosirius red-stained (PSR) liver slides from 3 distinct murine models of NASH will be obtained. Banked frozen liver homogenates of these models will also be obtained for the purposes of RNA isolation, cDNA synthesis, and quantitative real-time polymerase chain reaction (qPCR) (IACUC #2019-014). All animals (n =4 per cohort) had been previously sacrificed and liver samples had been previously collected for histopathological (10% formalin) or transcriptomic (liquid N_2) analysis for an unrelated study.

I. Animal Models:

Sham (control) cohort: Adult male C57BL/6 mice (18-20 g) had been randomized to standard rodent diet (sham) for 12 weeks. Drinking water was provided *ad libitum*.

Model 1 – FFD cohort: Adult male C57BL/6 mice (18-20 g) had been randomized to standard rodent diet (sham) or a fast food diet (FFD - rodent diet with 40 kcal% fat, 20 kcal% fructose and 2% cholesterol; D09100301, Research Diets, NJ) for 12 weeks [9-10]. Drinking water was provided *ad libitum*.

Model 2 – FFD + TAA cohort: Adult male C57BL/6 mice (18-20 g) had been randomized to

standard rodent diet (sham) or a fast food diet (FFD - rodent diet with 40 kcal% fat, 20 kcal% fructose and 2% cholesterol; D09100301, Research Diets, NJ) for 12 weeks. Drinking water was provided *ad libitum*. Additionally, the FFD cohort was administered thioacetamide (TAA, 100 mg/kg, IP X 3/week; TAA is a liver scarring agent) for 12 weeks [9]. Drinking water was provided *ad libitum*.

Model 3 – FFD + CCL_4 + glucose: Adult male C57BL/6 mice (18-20 g) had been randomized to standard rodent diet (sham) or a fast food diet (FFD - rodent diet with 40 kcal% fat, 20 kcal% fructose and 2% cholesterol; D09100301, Research Diets, NJ) for 12 weeks. Drinking water was provided *ad libitum*. Additionally, the FFD cohort was administered carbon tetrachloride (CCl_4) (0.32 µg/g, IP x 1/week; CCL_4 is a liver scarring agent) +18.9 g/L d-glucose in the drinking water for 12 weeks [5].

II. Liver Histopathology

- Histopathological analysis will be utilized to verify presence of disease and for semi-quantitation.
- Hematoxylin-eosin (H&E)-stained liver sections will be studied under a microscope (blinded to group) and scored on basis of the NAFLD Activity Score (NAS) [5].
- Picrosirius red (PSR) staining of liver sections will be semi-quantified for extracellular fibrillar collagen using Bioquant Image Analysis [10].
- Quadruplicates for liver slide will be scored for NAS and fibrosis to ensure the quantitation encompasses the whole liver.

III. Gene Targeting and Primer Design

- A literature-based gene campaign will be conducted to identify candidate genes of interest in NASH.
- Based on the campaign, gene-specific forward and reverse primers will be generated based on sequencing data from NCBI and designed using Primer3Plus primer-design tools.
- Designed primer sequences will be produced using Oligo Sigma services (Millipore-Sigma; Massachusetts, U.S).

IV. Transcriptomic Analysis

- The level of gene expression in all liver tissue samples will be measured by two-step quantitative polymerase chain reaction (qPCR).
- The Qiagen RNeasy Mini Kit will be used to extract total RNA from sham (control) and toxin-induced NASH (experimental) liver samples according to manufacturer's instructions and stored at -80°C.
- cDNA will then be synthesized from the extracted RNA using the Thermofisher High-Capacity cDNA Reverse Transcription Kit and a BioRad S1000 Thermocycler following manufacturer protocol.
- Samples will be diluted 1:5 with nuclease free H20, and stored at -20°C for qPCR, and the threefold diluted products will be used for quantitative real-time PCR (qPCR).
- SYBR-Green qPCR will be performed with Thermofisher Power-Up SYBR Green Master-Mix qPCR and on a Thermofisher Quant-Studio 3 Real-Time PCR system.
- Each qPCR reaction will be performed in triplicate for all tissue samples following Power-Up SYBR Green manufacturer protocol for Fast qPCR for a total volume of 10 µl.

Risk and Safety:

- A senior supervising scientist, before experimentation, will assess certain risks.
- The potentially hazardous agents that will be used are mouse liver and Thermofisher Power-Up SYBR Green Master-Mix.
- During the course of experimentation, gloves, goggles, and a lab coat will be worn.
- All handling of these agents will be according to the manufacturer's directions and conducted per the guidelines of Angion Biomedica Corp's Chief Safety Officer. These guidelines include standard operating procedures, use of personal protective equipment and proper disposal of potential biohazards.
- Excess liver tissue, excess master mix, and used gloves will be disposed in closed containers in marked biohazard bins which are disposed by a professional waste disposal company.
- The Angion Biomedica Safety Manual for all hazardous agents being used during experimentation will be read and reviewed before experimentation.

Data Analysis:

- All data (NAFLD Activity Score (NAS), scarring, mRNA) will be recorded and organized on Microsoft Excel.
- NAS data will be presented as mean scores in each group.
- Fibrosis scoring (PSR) data will be presented as fold-change in relation to the sham cohort.
- mRNA levels will be presented as fold-change in relation to the sham cohort as well.
- A student's T-test (p-value) will be used to compare data diseased cohorts to sham cohort.
- Microsoft Excel will be used for the NAS vs. mRNA correlation and to calculate Pearson product moment (r) and the p-value.

D. Bibliography

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NO ADDENDUMS EXIST