Aspirin for the Treatment of Nonalcoholic Fatty Liver Disease

Title: Aspirin for the Treatment of Nonalcoholic Fatty Liver Disease

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ORIGINAL STUDY PROTOCOL

Aspirin for Nonalcoholic Fatty Liver Disease (NAFLD):
A Randomized Clinical Trial

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I. BACKGROUND AND SIGNIFICANCE

A. Background.

Nonalcoholic steatohepatitis (NASH) is an aggressive form of nonalcoholic fatty liver disease (NAFLD) that affects an estimated 18 million Americans, and can lead to cirrhosis, hepatocellular carcinoma (HCC) and death[1, 2]. Despite the growing prevalence and mortality of NASH cirrhosis[2, 3], there are currently no effective FDA-approved therapies for the treatment of NAFLD and NASH. Considerable recent preclinical and observational data[4, 5 6] demonstrate that long-term aspirin use is associated with significantly reduced risk for adverse hepatic events, including HCC and liver-related death, in unselected populations without established liver disease. However, whether aspirin improves liver fat and markers of inflammation in patients with established NAFLD is unknown. Further, the mechanisms underpinning these putative associations are undefined. Addressing these knowledge gaps has been highlighted as a key priority "to realize the full potential of aspirin in precision chemoprevention" [7].

It has been recommended by subspecialty societies, including the American Association for the Study of Liver Disease (AASLD), that changes in liver fat content as quantified by magnetic resonance spectroscopy (MRS) or MRI proton density fat fraction (MRI-PDFF), can serve as an appropriate and clinically relevant primary outcome in early, phase 1 and 2 clinical trials of novel NAFLD therapeutics. Specifically, recent advances in MR-based imaging techniques now permit very accurate and validated means of quantifying intrahepatic lipids, via MRS and MRI-PDFF. Results of intrahepatic lipids (%) by MRS correlate closely with biochemical and histological assessment of liver triglyceride content, with an area under the receiver operating characteristic curve that exceeds 90%. Moreover, MRS measures global hepatic fat content across the entire liver (rather than from a small sample, from a liver biopsy), which is relevant as lipid content may be heterogeneous and thus liver biopsy could result in sampling error. Collectively, this robust prior evidence demonstrates that MRS (and MRI-PDFF) are robust and accurate means of quantifying steatosis for the primary endpoint assessment, in early-phase NAFLD interventional trials. In addition to these established MRI endpoints, the present study also includes an optional enrollment and month 6 liver biopsy, as part of the optional biopsy sub-study, but liver biopsy is not required for the main study.

II. SPECIFIC OBJECTIVES & ENDPOINTS

We will conduct a randomized, placebo-controlled trial to assess the feasibility and efficacy of aspirin for reducing liver fat, inflammation and fibrosis, in 80 adults aged 18-65 with established nonalcoholic fatty liver disease (NAFLD), confirmed by validated imaging modalities or by a clinical liver biopsy within 12 months of the screening visit.

Primary Efficacy Endpoint: 6-month absolute difference in % hepatic fat fraction (HFF), measured by 1H-MRS.

Key Secondary Endpoints:

1. Relative 6-month percentage change in HFF %, by 1H-MRS.
2. Proportion of patients with 30 percentage point or greater relative reduction in HFF, by 1H-MRS.
3. Absolute change in liver fat %, as measured by MRI-PDFF.
4. Relative percentage change in liver fat %, as measured by MRI-PDFF.

Non-Key Secondary Endpoints:

5. 6-month change in alanine aminotransferase (ALT) and aspartate aminotransferase (AST).
6. 6-month change in the composite of inflammation and fibrosis, measured by the cT1 score, using Liver*MultiScan*
7. 6-month change in liver stiffness measurement (LSM) of fibrosis, assessed by vibration controlled transient elastography
8. 6-month changes in body weight (kg) and body mass index (BMI).

Exploratory Endpoints:

9. Changes in circulating biomarkers of aspirin target engagement
10. Changes in levels of pro- and anti-inflammatory lipid species and plasma metabolites, and changes in gut microbial composition and function, that may be impacted by aspirin therapy

Safety Endpoints:
Safety endpoints will be assessed according to the following schedule, and will be closely monitored by the IRB and by the Data Safety Monitoring Board (DSMB). Criteria for individual and/or study discontinuation based on safety assessments are outlined in detail below (see Safety Monitoring).

1. CBC: assessed at screening, baseline, and 6 months (and hematocrit additionally assessed at 3 months)
2. Pregnancy testing at all visits

III. SUBJECT SELECTION

A. Eligibility

Inclusion criteria:

1. Ages 18 to 65 years
2. NAFLD defined as confirmed hepatic steatosis (>5% intrahepatic triglycerides) by liver biopsy (within 12 months of screening) or by validated imaging modality (non-contrast CT scan, 1H-MRS, MRI-PDFF, ultrasound), in the absence of significant alcohol consumption (>2 drinks daily for women, >3 drinks daily for men) and other causes of hepatic steatosis. If relevant liver imaging (defined above) or liver biopsy has not been performed clinically within the prior 12 months before the screening date, liver ultrasound will be performed as part of the screening visit.

Exclusion criteria:

1. Other chronic liver disease, including viral hepatitis B or C infection, alpha-1 antitrypsin deficiency, Wilson's disease, hemochromatosis, autoimmune hepatitis, primary biliary cholangitis, primary sclerosing cholangitis, or HIV infection, as defined by medical record review or by patient self-report, and confirmed with the treating medical provider.
2. Alcohol intake greater than or equal to two drinks per day in women or three drinks per day in men.

3. Cirrhosis or any prior liver decompensation event (i.e., ascites, spontaneous bacterial peritonitis, hepatic encephalopathy, esophageal varices, and/or hepatorenal syndrome), defined by review of medical record (including clinical notes, prior liver imaging, procedures and prior liver biopsies where available), and by confirmation from treating medical provider.
4. Severe cardiovascular disease (i.e. myocardial infarction within the last year, left ventricular ejection fraction of less than 40% or active angina), defined by medical record review or by patient self-report
5. Severe renal disease (defined as eGFR less than 30)
6. Pregnancy or desire to become pregnant or breastfeeding.
7. Contraindications to aspirin use, including aspirin intolerance, prior peptic ulcer disease, prior GI or non-GI bleeding
8. Thrombocytopenia
9. Aspirin use within the preceding 3 months
10. Current use of other antithrombotics, anticoagulants, or antiplatelet agents (not including non-aspirin nonsteroidal anti-inflammatory drugs, NSAIDs)
11. Routine MRI exclusion criteria (i.e. metal implants, etc.)
12. Bariatric surgery within the past 2 years
13. Active cancer (i.e. currently undergoing treatment), except for non-melanoma skin cancer.

B. Recruitment:

<u>We will use the following potential techniques for recruitment:</u>

1. Advertisements in newspapers
2. MGH and BWH's "Research Studies Seeking Volunteers" email
3. RSVP for health emailing
4. Internet posting (Craig's List, Facebook)
5. Mass mailings, based on demographic and geographic area
6. Advertise in local newspapers
7. Postering throughout the Boston metropolitan area, with close attention to recruiting minorities
8. Letters to patients from Massachusetts General Hospital, Brigham and Women's Hospital, North Shore Medical Center, Salem Hospital and Faulkner Hospital ("Partners Healthcare System") who have been diagnosed with obesity, fatty liver disease and/or related conditions, based on Partners Healthcare System-approved search of billing records "RPDR" search. Potential subjects will be contacted after signed permission from a provider OR contacted directly if the potential subject has previously signed up for this feature.
9. Recruitment through the MGH Fatty Liver Clinic, The Gastrointestinal Surgery Clinic at MGH and other clinics in which current study staff provide care.
10. Recruitment through the MGH NAFLD Tissue Repository Studies (IRB #: 2009P002081 and IRB #: 2009P002098)
11. Recruitment through a list of research subjects who have previously been recruited by our study team and have agreed to recontact for future studies.
12. Recruitment through materials distributed to providers who care for NAFLD patients outside of the Partners System.
13. Recruitment through the Research Match (Vanderbilt Recruitment Tool)

Subjects who express interest will be pre-screened via a REDCap questionnaire or phone.

IV. SUBJECT RECRUITMENT

A. Trial Population

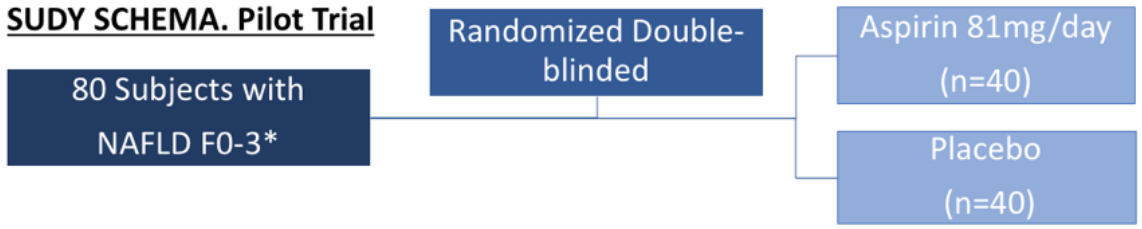
Subjects responding to the initial recruitment advertisements will undergo initial screening and if deemed potentially eligible for participation, they will be contacted by telephone and invited to participate in a screening visit. Based on a conservative assumption of 60% rate of either screening failure or subjects declining to participate in a research study, we expect to screen 200 adult subjects and enroll a total of 80 subjects with confirmed NAFLD without cirrhosis.

B. Consent

All subjects will receive the consent form for the study as well as the Human Subject's Bill of Rights. These documents will be read by the study subjects and also reviewed carefully by the study subject with a clinician on the research staff, prior to participating in the study. Any questions, concerns, or ambiguities will be appropriately clarified by the study clinician prior to the patient signing consent. Potential study subjects will sign informed consent and only then will they begin participation in the study. If new information is ascertained during the study, we will modify our consent and re-consent our patients accordingly.

C. Randomization

After baseline testing, 80 subjects who meet all eligibility criteria will be randomized to one of two treatments for 6 months: 1) low-dose aspirin (81mg per day), or 2) identical, blinded placebo pills.



Study Endpoints	Month 0	Month 6
Intrahepatic Lipids (1H-MRS)	✓	✓
Inflammation / Fibrosis (Liver*Multi*Scan, Fibroscan)	✓	✓
Specialized Proresolving Mediators (SPMs)	✓	✓
Untargeted Eicosanoids, Inflammatory Markers	✓	✓
PNPLA3 rs738409 genotype	✓	

*All subjects will have NAFLD with fibrosis stage 0-3 (F0-3) confirmed by prior clinical liver biopsy or by validated imaging modality.

V. STUDY PROCEDURES:

A. Overview of Study Design.

An overview of the trial design is shown in the figure. This study will examine the effect of low-dose aspirin administration on intrahepatic lipid accumulation (by ^{1}H-MRS) in 80 subjects with confirmed NAFLD without cirrhosis.

The MGH Research Pharmacy will prepare and dispense blinded, identical aspirin or placebo capsules to the randomized subjects, thereby maintaining double-blinding.

Screening Visit: (n= an estimated 200 subjects)
Pre-screening for eligibility will be performed, including obtaining confirmation from treating medical provider regarding potential eligibility, if indicated. At the screening visit, a history and physical (including measurement of vital signs, height and weight, and calculation of BMI) will be performed. A creatinine, CBC and a PT/INR will be performed. A pregnancy test will be performed in all premenopausal women. Individuals will be queried regarding alcohol use and frequency of use, as well as for history of other chronic liver disease or cirrhosis or hepatic decompensation. Hepatitis B surface antigen and/or Hepatitis C Ab testing will be performed in study subjects who have not had this, as part of their clinical care. Individuals will be carefully queried for all inclusion and exclusion criteria and for their eligibility to undergo MRI scanning.

Finally, histology slides from prior clinically-indicated liver biopsies will be requested if/when available. Record requests will be initiated for slides to be obtained outside of MGH. Slides will be reviewed at MGH for a central pathology read. Slides will be reviewed for grading of NASH activity score and other NAFLD/NASH-related pathology assessments. All histology slides will be returned after review.

Study Visits:
1. Baseline and 6-month visit:
The baseline visit will occur within 16 weeks after the screening visit. The following assessments will be made during the baseline visit and repeated at the month 6 visit:

- Interview to determine changes in medical history since the prior visit.
- Measurement of blood pressure, weight, BMI, waist-to-hip ratio
- Serum or urine pregnancy testing in women of childbearing age and potential.
- Fasting blood samples:
    - Comprehensive metabolic panel, complete blood count, PT/INR, insulin/glucose, hemoglobin A1c, inflammatory markers, lipid panel and liver function testing. These laboratory assessments are consistent with current recommendations for early-phase clinical intervention trials of NAFLD/NASH[8]
    - NAFLD Fibrosis Score will be calculated from standard labs and subject characteristics (age, BMI, blood glucose, platelet count and albumin).
    - Serum and Plasma will be collected for future measurement of lipids, bile acids, other metabolites including adipokines and inflammatory markers.
- Additional peripheral blood (serum, plasma, and whole blood) from which we will extract DNA and RNA. This will permit testing (among other things) for the *PNPLA3* rs738409 genotype, as this is an established NAFLD risk locus, and testing of PNPLA3 is currently recommended in NAFLD/NASH clinical trials[8].
- Stool samples that have been collected at home will be brought with the patient on the Baseline Visit. A collection kit provided during the Screening Visit will allow minimal contact with the stool and allow easy storage of the sample until the day of the Baseline Visit. If the patient forgets or is unable to complete the sample collection prior to their appointment, they may be given the materials to mail their sample to the study staff.
- MRI: 1H-MRS of the right and left hepatic lobe will be performed to quantify hepatic lipid content and other fat depots. LiverMultiscan sequences will also be performed for noninvasive analysis of hepatic inflammation and fibrosis. During the same MRI session,

MRI-PDFF will also be performed. MRI will be repeated at the 6-month visit (for a total of 2 MRI sessions per subject).

- Fibroscan to measure liver stiffness via transient elastography (ultrasound). Fibroscan will be repeated at the 6-month visit (for a total of 2 Fibroscans per subject).
- Standard bionutrition assessment by trained research nutritionist.
- Assessment of adherence (month 6 visit) by returned pill count and by self-report.
- Three validated questionnaires will be administered to subjects:
    - The Paffenbarger Physical Activity questionnaire
    - The SF-36 health survey questionnaire
    - The semi-quantitative Food Frequency Questionnaire (FFQ)

Total Blood Drawn: Total blood volume from the participant is below 200ml for this Baseline Visit and for the Month 6 visit. As this is below 300ml over at least 6 hours, this does not result in added risk. Normal blood donation volume is 450-500ml.

2. Follow-up 3-month visit: (n=80)
This visit will be performed during a +/- 2-week interval around the planned date, to accommodate potential scheduling conflicts. A physical examination and measurements of body weight and waist-to-hip ratio will be completed. Study subjects will be assessed for interim medical history, and any side effects to study drug. We will measure a hematocrit.

3. Twice monthly telephone calls or voicemail messages (+/- 1 week):
The trained clinical research coordinator, working directly with the PI, will conduct twice-monthly check-in telephone calls to enrolled subjects on the months when the subject does not have a study visit (i.e. at month 2, month 4 and month 5), using a Partners IRB-approved telephone script. The purpose of these calls will be to ask about medication compliance and to inquire about any concerns or side effects related to the study drug. All subjects will be reminded during this call or voicemail message that the PI (Dr. Simon) will be available by pager at all times during the study to answer any questions or concerns that arise.

4. Early Termination Visit:
Subjects who discontinue the study early due to an adverse event, toxicity, or personal reasons will be asked to complete an Early Termination visit. This visit will include all assessments that are performed at the final 6-month visit described above. Participants must have been on the study medication for at least 11 weeks in order to be eligible for completing an Early Termination visit.

5. Remote Visits: Subjects who do not live locally will have the option to perform the 3-month follow-up visit remotely. This will be performed with remote labs (via Quest Diagnostics Locations) and medical history via phone call with a study staff member. For these remote visits, the study staff member will speak to the subject over the phone to review the medical history. If during a phone-medical history, the study staff member believes the subject may be experiencing side-effects related to the study medication, the PI will be notified and the subject will be informed that they need to come to MGH for a physical exam. For these remote visits, the study team will FedEx study medication to the subject in a manner approved by the research pharmacy as needed for resupply. The subject would bring back unused study medication and empty vials to the study team at the next in person visit to maintain standard study drug accountability logs.

B. DRUGS

Aspirin: Aspirin 81mg tablets (generic; purchased directly by the MGH Research Pharmacy and made into blinded aspirin capsules that are identical to placebo capsules).

Placebo: Blinded placebo capsules that are identical to the aspirin capsules will be prepared by the MGH Research Pharmacy (purchased directly by the MGH Research Pharmacy).

Rationale for dose: Dosing is based on U.S. Preventive Services Task Force prior recommendations for consideration for primary CV and colorectal cancer (CRC) prevention[9]. Use of 81mg/day is also consistent with another active RCT of aspirin for CRC prevention (NCT02394769)[10]. Aspirin is not contraindicated in NAFLD. Nevertheless, bleeding with aspirin is dose-dependent[11], so the choice to use low-dose aspirin (81mg per day daily dose) will optimally balance safety and efficacy.

Rationale for duration: 6 months is recommended for early-phase NAFLD clinical trials by the AASLD[8], is widely used[12-14], and is sufficient to reveal changes in fat[15-21], thus this trial duration appropriately balances efficacy and compliance.

The risks of low-dose (81mg) aspirin treatment include aspirin allergy or intolerance, with or without associated bronchospasm, gastrointestinal (GI) upset, risk for peptic ulcer disease, and bleeding, including GI bleeding, intracranial bleeding and bleeding from other sites[11, 22-24]. We have taken several steps to minimize these potential risks. First, we have selected a low daily aspirin dose (81mg per day), which minimizes potential bleeding or ulcer-related risks, compared to higher doses. Second, we will exclude any subject with a personal history of aspirin intolerance, and anyone with a personal history indicating an increased risk for bleeding, including: personal history of bleeding or stroke, and/or history of coagulopathy and/or thrombocytopenia, and/or any subject with current use of any other anti-coagulant or antithrombotic medications. Third, we will conduct a 3-month visit with assessment of safety labs (hematocrit), as well as an interval history and physical examination to assess for any interval bleeding. Finally, we will conduct twice-monthly telephone check-ins that will further enhance safety by giving subjects additional opportunities to further discuss any issues or concerns. Importantly, we will ensure that all subjects are aware of and understand these risks, through our informed-consent process.

It should be emphasized that bleeding-related risks are not felt to be elevated in individuals with non-cirrhotic NAFLD (defined by fibrosis stage <= 3) compared to the general population. In fact, recent preliminary data suggest that patients with NAFLD may have an increased risk for thrombotic events for which an anti-thrombotic medication may offer important benefits[25]. Thus, we do not expect daily aspirin 81mg to increase the risk of bleeding more in a population with NAFLD without cirrhosis, compared to the general population. Overall, we believe that the potential overall benefits low-dose aspirin for adults with non-cirrhotic NAFLD/NASH outweigh the potential risks.

C. DEVICES AND PROCEDURES:

Intrahepatic fat: We will perform proton MR spectroscopy (1H-MRS) and MRI-PDFF of the right and left hepatic lobe to determine hepatic fat fraction, using a 3Tesla MRI device (Siemens Trio, Siemens Medical Systems, Erlangen, Germany) as previously described[26]. Advantages of higher magnetic field strength are an increase in signal-to-noise ratio resulting in improved spatial resolution and increased chemical shift dispersion, potentially improving assessment of

other fat resonances. MRI sequences are also performed for assessment of other fat depots and LiverMultiScan analysis within the same session.

LiverMultiScan: *LiverMultiScan* uses the same scanner as ^{1}H-MRS, producing a validated, non-invasive assessment of inflammation and fibrosis called the Liver Inflammation Fibrosis (LIF) score, which has increasing severity from 0-4, and which correlates with NAFLD histological severity[27], and further predicts outcomes.[28] Image processing and analysis of 1H-MRS and *LiverMultiScan* data will be completed without charge through an established collaboration that is already in place between the PI (Dr. Simon) and Perspectum Diagnostics, who also work closely with the MGH Department of Radiology.

Liver function tests: A standard liver function test panel consisting of ALT, AST, GGT, alkaline phosphatase, total bilirubin, direct bilirubin, indirect bilirubin, ferritin and albumin will be measured using a standard clinical platform.

Lipids and Lipoproteins: A fasting lipid profile, including total cholesterol, LDL, HDL and triglycerides, will be determined.

The NAFLD fibrosis score: This score predicts the probability of fibrosis in patients with NAFLD using a score that uses the patient's age, body mass index, blood glucose levels, aminotransferase levels, platelet count, and albumin. A high NAFLD fibrosis score of >0.676 is associated with a probability of advanced fibrosis (F3-F4) of 82 percent (sensitivity 43 percent, specificity 96 percent), and a score of <-1.455 is associated with a negative predictive value of 88 percent (sensitivity 77 percent, specificity 71 percent) based on prior studies.[29, 30]

Fibroscan: Elastography (Fibroscan) is a rapid, non-invasive ultrasound-based modality that measures liver stiffness by transmission of mild amplitude and low frequency (50Hz) through the intercostal space using a vibrator at the skin surface. The vibration induces a shear wave velocity through the hepatic tissue, which is directly related to tissue stiffness and is expressed in kilopascals (kPa). Transient Elastography is an inexpensive, reproducible, painless, rapid <10 minutes and easy to perform tool. It has been proven in many studies to correlate well with degree or severity of fibrosis and or cirrhosis.

Additional Blood Samples: After the subject has signed the informed consent form, peripheral blood will be collected for serum, plasma and whole blood, from which we will extract DNA and RNA. The blood draw will occur on the day of the Baseline Visit (Visit 1). Raw samples will be transferred at room temperature within 24 hours of collection to the laboratory of Dr. Tracey Simon for sample processing and storage at -80^{0} C.

Some examples of the type of analyses that we may perform with the specimens collected are provided in the following paragraphs:

Lipid Mediators, Inflammatory Markers and Metabolites Serum and plasma samples for measurement of circulating metabolites, inflammatory markers and lipids relevant to aspirin target engagement, including eicosanoid lipid species, SPMs, untargeted lipid metabolites and bile acids, will be quantified using validated and well-established liquid chromatography tandem mass spectrometry (LC-MS-MS) platforms for metabololipidomics profiling. Metabolomics profiling will be performed with Metabolon, Inc. Lipidomics profiling will be performed with our collaboratirs, Drs. Maddipati and Charles Serhan, with data acquired in negative ionization mode, and further eicosanoid and SPM quantification by multiple reaction monitoring (MRM)[31,

[32], according to published criteria[33, 34]. Dr. Maddipati together with Co-Investigator Dr. Charles Serhan have shown excellent reproducibility of this method for quantifying eicosanoid derivatives, including SPMs and other circulating lipid mediator species[32, 34], with a lower limit of detection of ~0.1pg[34]. Notably, this method will also permit measure of SPM precursors (e.g. erythrocyte docosahexaenoic acid [DHA], eicosapentaenoic acid [EPA]), which also reflect dietary intake, aspirin use and hepatic DHA/EPA[35, 36], and which may impact hepatic steatosis[37].

Stool Metagenomics: Stool samples will be collected at home on two instances (before the Baseline Visit and Before Visit #4). A collection kit will allow minimal contact with the stool and allow easy storage of the sample until the day of the study visit. If the patient forgets or is unable to complete the sample collection prior to their appointment, they may be given the materials to mail their sample to the study staff at a later date. Stool samples will be collected on a spoon and placed into two tubes. The tubes will be included in stool collection kit and will contain 95% ethanol for both future DNA/RNA extractions and metabolomic analysis. Upon receiving the samples, the stool in the first tube will be centrifuged and separated from the fixative. Then, both stool samples will be stored at -80°C in the lab of Dr. Tracey Simon and Dr. Raymond Chung.

Genotyping

Genomic DNA samples will be used for the identification and/or genotyping of polymorphisms that may predict the pathogenesis and progression of NAFLD. Additionally, polymorphisms in any one of many genes, including those encoding drug receptors, drug transporters, and cell signaling pathways, can be important determinants of clinical response. An advantage of developing a DNA-based diagnostic test as a clinical biomarker is that only a small sample of tissue (i.e. blood from a finger prick or buccal cells) is required for genotyping of a large number of polymorphisms. DNA-based diagnostic assays also provide a clinician the ability to perform high-throughput and accurate diagnostic tests for a reasonable cost.

Transcriptional Profiling

Whole-genome analyses are beneficial to research studies where the end goal is to focus on a small number of genes. Transcriptional profiling is an efficient tool to sort through the expression of thousands of genes, and to identify significant associations with disease severity or drug response. Another advantage of performing whole-genome analyses is that novel biological pathways related to the disease could be more clearly elucidated, and mechanisms of drug action can be revealed in a genomic context.

Proteomics

Although genotyping and transcriptional profiling can yield important pharmacogenomic results, a major disadvantage of these techniques is the lack of correlation between gene expression at the mRNA level with the amount of actual expressed protein within the cell. Proteomics technologies allow for the characterization of protein expression, protein-protein interactions, and post-translational modifications and processing that are important in disease states. Moreover, the ideal platform for a clinical diagnostic test is an ELISA assay to detect serum levels of a particular secreted protein biomarker.

Serum and plasma samples will be used for proteomic and/or ELISA analysis to identify protein or peptide biomarkers that are associated with NAFLD outcomes. Typically, 2-D gel electrophoresis will be used for initial detection and location of protein differences, in addition to liquid chromatography-mass spectrometry and tandem mass spectrometry for detailed peptide

mapping, characterization and identification of proteins. Multiple ELISA assays for proteins from biological fluids will subsequently be developed for marker validation.

VI. APPROACH AND BIOSTATISTICAL ANALYSIS

A. Experimental approach:

A total of approximately 80 adult subjects with NAFLD (F0-3) will be enrolled after a screening visit at which study eligibility will be determined and confirmed. We expect to screen up to 200 subjects in order to enroll a total of 80 subjects. Eligible subjects will be randomized to receive aspirin or placebo. Subjects will be studied for 6 months.

B. Data Integrity:

To ensure data entry accuracy, peer review of source documentation and case report forms in REDCap will be completed following MGB Study Data Management and Quality Assurance Plan Guidelines. Research coordinators will exchange subject files and review informed consents, case report forms, and adverse event logs for accuracy and completeness. Additionally, eCRFs in REDCap will be reviewed for accuracy and completeness using source data verification.

C. Statistical Analysis and Power

Data Analysis Plan:

This statistical analysis plan will be finalized before database lock. Baseline data between groups will be compared using chi-squared test or Fisher's exact test for categorical variables, and using ANOVA for continuous variables, with any non-normally distributed data log-transformed prior to analysis. If significant baseline group imbalance is detected on any particular variable, that variable will be included as a covariate in the analyses.

Absolute and relative changes in continuous outcomes between treatment groups will be calculated using analysis of covariance (ANCOVA), with treatment group at randomization included as a fixed effect, and the baseline value of the relevant outcome included as a covariate. Logistic regression will be used for binary outcomes (i.e., achievement of ≥30% relative HFF reduction). For the primary analysis, all study endpoints will be analyzed using an intention-to-treat approach, using data from all randomly assigned participants in the full analysis set. The primary analysis will assume that missing data were missing at random[38], and data augmentation for missing observations will be conducted via multiple imputation, using all available non-missing data of the outcome measurement from the same treatment arm.

We will also utilize data from all participants who adhere to therapy as assigned and complete the final, 6-month visit for study outcomes (i.e., the per protocol analysis set).

We will conduct the following subgroup and sensitivity analyses for the primary and key secondary endpoints:

To address potential confounding due to meaningful weight loss between baseline and month 6, we will exclude any participant who loses ≥3% body weight during the 6-month study period. We will construct separate, multivariable-adjusted regression models accounting for *a priori* defined clinical confounders, including age, sex, race/ethnicity, type 2 diabetes status at baseline, fasting insulin level at baseline, body weight and visceral adipose tissue (VAT) volume, as previously recommended for NAFLD clinical trials[8]. We will also repeat the analyses with missing data for primary and key secondary endpoints considered as nonresponses, by carrying forward the baseline values. We will also conduct subgroup analyses for the endpoints of

absolute change in HFF by MRS (primary endpoint) and relative HFF change by MRS (key secondary endpoint) in participants with significant, stage 2-3 fibrosis at baseline.

Sample Size, Statistical Power & Multiplicity Testing:
We anticipate a conservative drop-out rate of approximately 15%, thus we estimate an effective sample size of 68 patients. Based on prior studies of a dietary intervention for hepatic steatosis (Ryan et al. [39]), and data from a meta-analysis of oral omega-3 supplementation and hepatic steatosis (Parker et al.[40]) we expect an effect size of 3% with the standard deviation of the change in liver fat of 2.45, by ^{1}H-MRS. Applying these estimates, the probability is >90% that the study will detect a treatment difference at a two-sided 0.05 significance level[41]. The Holm procedure to control for multiple testing will be applied to the 4 key secondary outcomes, as follows: P=0.0125 for relative change in HFF by MRS, P=0.0167 for proportion achieving ≥30 percentage point reduction in HFF by MRS, P=0.025 for absolute change in HFF by MRI-PDFF, and P=0.05 for relative change in hepatic fat by MRI-PDFF. For the other non-key secondary outcomes no multiplicity adjustments will be employed. For exploratory outcomes, no multiplicity adjustments will be used.

Definitions of Analysis Sets:
The full analysis set includes all 80 randomized subjects. Subjects in the full analysis set will contribute to the "intention-to-treat" analysis. The as-treated set will include all participants who adhered to therapy as assigned and completed the final, 6-month study visit for study outcomes. Finally, an additional analysis set for a sensitivity analysis will be constructed with missing data for study endpoints treated as nonresponses, by carrying forward baseline values.

VI. RISKS AND DISCOMFORTS

A. Potential Risks to the Subjects

Human Subjects Involvement and Characteristics: This study will enroll 80 adults with NAFLD without cirrhosis (F0-3) between the ages of 18 and 65 years. Rationale for exclusions is discussed in the study protocol; in brief, these exclusions are designed to 1) exclude persons at greatest risk for significant adverse events, and 2) avoid confounding effects of other clinical comorbidities and/or medications.

We may send de-identified samples to a third party for analysis, such as to Metabolon, or to Qiagen, GenomeScan, or others. These specimens will not be linked to any individual identifiers associated with the patients. If we send de-identified samples to a third party, we will first generate a contract for this purpose through the hospital supply chain.

Potential risks from study drug:

- ***Aspirin***: Subjects will be randomized to receive aspirin in the form of an 81mg capsule or blinded, identical placebo capsule, to be taken once daily. The risks of aspirin treatment include aspirin allergy or intolerance, with or without associated bronchospasm, gastrointestinal (GI) upset, risk for peptic ulcer disease, and bleeding, including GI bleeding, intracranial bleeding and bleeding from other sites[11, 22-24]. We have taken several steps to minimize these potential risks. First, we have selected a low daily aspirin dose (81mg per day), which minimizes potential bleeding or ulcer-related risks, compared to higher doses. Second, we will exclude any subject with a personal history of aspirin intolerance or aspirin allergy, and anyone with a personal history indicating an increased risk for bleeding, including: personal history of bleeding or stroke, and/or history of coagulopathy and/or thrombocytopenia, and/or any subject with current use of any other anti-coagulant or antithrombotic medications. Finally, we will conduct a 3-month visit with assessment of

safety labs (hematocrit), as well as an interval history to assess for any interval bleeding. Moreover, we will conduct twice-monthly telephone check-ins that will further enhance safety by giving subjects additional opportunities to further discuss any issues or concerns. Importantly, we will ensure that all subjects are aware of and understand these risks, through our informed-consent process. However, it should be noted that bleeding-related risks are not felt to be elevated in individuals with non-cirrhotic NAFLD/NASH, compared to the general population. In fact, recent preliminary data suggest that patients with NAFLD/NASH may have an increased risk for thrombotic events for which an anti-thrombotic medication may offer important benefits[25]. Thus, we do not expect daily aspirin 81mg to increase the risk of bleeding more in a population with non-cirrhotic NAFLD, compared to the general population. Overall, we believe that the potential overall benefits low-dose aspirin for adults with non-cirrhotic NAFLD to outweigh the potential risks.

- **Placebo:** The risks associated with a placebo capsule include very minor intolerance or GI upset, however this is very infrequent and placebo pills are generally well-tolerated.

Risks from blood draws:
Blood sampling is performed in this study and there is always a very minor risk of infection, bruising, or syncope during a blood draw. There is also the discomfort of having one's blood drawn. The blood draws will be performed by trained staff who have certificates documenting their ability to draw blood.

Risks from Fibroscans:
Fibroscans will be performed using FDA approved devices. There are no known foreseeable risks associated with exposure to Fibroscan.

Risks from MRI/MR Spectroscopy:
MRI and MR spectroscopy will be performed twice during this prospective study (baseline and final 6-month visit) using FDA-approved devices and pulse sequences. A standard MRI questionnaire will be administered to all potential study subjects, and no study subject with a contraindication to MR testing will be admitted to the study. There are no known foreseeable risks associated with exposure to MRI, provided patients are appropriately screened and there are no metallic implants (i.e. vascular clamps or pacemakers). All potential subjects will be carefully screened for the presence of such implants or any other contraindication to MRI, prior to each exam. No study subject with a contraindication to MR testing will be admitted to the study. Some subjects report some claustrophobia during MRI scans. If a patient expresses any discomfort whatsoever during the scan, the procedure will be aborted and not repeated without his/her full consent.

Risks of Stool Collection:
Stool will be collected using a stool collection kit that contains some special preservatives to make sure they are stable over time. If skin comes into contact with these preservatives, they could irritate the skin. The preservative can simply be washed off with soap and water.

Risks of Genetic Testing
Genetic information that results from this study does not have medical or treatment importance at this time. However, there is a risk that information about taking part in a genetic study may influence insurance companies and/or employers regarding a subject's health. To safeguard the privacy of our subjects, genetic information obtained in this study will not be shared or made available to be placed in the medical record. Taking part in a genetic study may also have a negative impact on family or other personal relationships.

We will in no way discourage use of any medications that are clinically indicated for the subject. Subjects will be discontinued from the study if they start a medication that is known to affect liver fat, including methotrexate, amiodarone, oral steroids or SERMs (such as tamoxifen). The subject will be able to continue in the study if they initiate other medications that are not known to affect liver fat content.

B. Protection Against Risk
In addition to the formal safety monitoring plan described below, the procedures to protect against or minimize potential risks include the following: (1) the assignment of unique study subject numbers to patients, (2) the use of these primary identifiers throughout the study, (3) storage of information in locked file cabinets, and (4) access limited to study personnel for these file cabinets and data. The blood draws will be performed by trained staff who are certified to draw blood. We are performing a physical examination and routine laboratory tests prior to allowing anyone to enter the protocol to ensure that subjects are medically stable. Close monitoring of patients throughout the study will ensure that adverse effects from our interventions. All study subjects will be instructed on how to contact study clinicians in the case of an emergency. The PI (Dr. Simon) will be available by pager at all times during the study to answer any questions or address any concerns that a subject may have.

Participants will also be required to sign a consent form prior to entering into the study. All recruitment materials will be approved by the MGH Institutional Review Board prior to initiating the study.

C. Anticipated Benefits to Subjects
There is no expected benefit to participation in this study. If one is assigned to receive aspirin therapy, it is possible that their amount of liver fat may decrease and their cardiovascular risk and/or waist fat may decrease during the study. However, those effects are not promised or guaranteed. If benefits occur, there is no way to know how long they will last.
Participants will have thorough physical exams while in this study. If a medical condition is discovered, one will be referred to your physician. It is possible that information learned from this study may help improve therapy for people who are obese. Further, all participants will receive information about their metabolic and nutritional health at the screening visit, consistent with current recommendations for clinical trials in patients with NAFLD/NASH[8]. The risk involved in study participation is thus thought to be balanced by the possible benefits to participants.

IX. INDEPENDENT MONITORING OF SOURCE DATA:

Monitoring of Source Data:
The principal investigator will be reviewing each subject's data periodically throughout the duration of the study for quality, validity and integrity assurance and for adherence to the IRB approved protocol. In addition, the study coordinators will monitor subject documents on an ongoing basis. All source data will be organized and filed into subject binders. Any pertinent notes to file regarding the subject will be documented by the study coordinator and kept in the applicable subject binder. All subjects will be assigned a study identification number.

Data Quality Control Monitoring:
Data will be recorded on specified Case Report Forms retained at Massachusetts General Hospital through REDCap. REDCap is a secure, web-based application designed to support data capture for research studies. The system was developed by a multi-institutional consortium initiated at Vanderbilt University and designed to comply with HIPAA regulations. Data forms from REDCap can be automatically exported into a spreadsheet to which additional data

(laboratory studies, etc) can be added. Forms that have missing or inconsistent data will also be entered into the database, except that a missing value indicator will take the place of the missing or inconsistent information. A missing information form will be filled out to indicate the items that are missing. Any paper data forms will be filed in a dedicated locked filing cabinet organized by subject code number. To ensure data entry accuracy, peer review of source documentation and case report forms in REDCap will be completed following MGB Study Data Management and Quality Assurance Plan Guidelines. The data will reside on the secure Massachusetts General Hospital secure file server which is within the secure Partners firewall, and which is backed up every day, with periodic secure back-ups off-site.

A physician will be available at all times during the study by pager to answer any questions a patient might have. The physician will arrange to immediately see every patient with a concern. All efforts will be made to protect the confidentiality rights of the study subjects who will be referred to by code numbers only. Confidentiality of the patients will always be of paramount importance to study investigators. No data on patients will be shared with persons other than those directly involved in the study, except at the documented request of the patient. Samples that are sent to laboratories outside of MGH will be labeled with a non-identifying numeric code.

Safety Monitoring:
A Data Safety Monitoring Board (DSMB) will be established at Massachusetts General Hospital consisting of independent members, who will review all study procedures, adverse events, protocol violations, protocol exceptions and deviations as well as study inclusion and exclusion criteria. The DSMB will also review safety monitoring blood tests and study data as available. The DSMB members will consist of independent experts in the field with experience in the conduct of clinical trials, statistical knowledge and independence from the direct management of the clinical trial. These members include Drs. Karen Miller MD (Professor of Medicine, Massachusetts General Hospital), Jules Dienstag MD (Professor of Medicine, Massachusetts General Hospital), and Dr. Charles Serhan, PhD (Professor of Anesthesiology, Brigham and Women's Hospital). In addition, the PI (Dr. Simon) and the co-investigator team (Drs. Chan, Chung and Corey) will participate in a portion of the meeting as non-voting members. The DSMB and the study investigators will meet together at least once every 6-9 months, or more often as needed, and the chair (Dr. Miller) will be the contact person for the DSMB and will be responsible for overseeing the meetings, developing the agenda and summarizing the findings of the meeting.

All treatment-related serious adverse events will be reported to the DSMB within 24h of occurrence. In addition, the chair may call ad hoc meetings. The Board will be provided with reports of serious adverse events as they occur and the chair of the committee has the responsibility of calling an ad hoc meeting if the type or frequency of the serious adverse events is of concern. In addition, an ad-hoc meeting may be called to evaluate whether a subject should be unblinded due to safety concerns or a serious adverse event (SAE). If a study subject is unblinded, the PI will not be made aware of the treatment assignment.

The Board may request additional information from the Investigator in the course of its review. The Investigator shall not be present for part of the Board's meeting.

Safety assessments:

3. CBC: at screening, baseline, and 6 months (and hematocrit at 3 months)
4. Pregnancy testing at all visits

The physician investigator will review all laboratory results in a timely manner.

Subjects will be discontinued if they develop:

1. Bleeding event resulting in hospitalization or decrease in hematocrit by >20% from baseline
2. Decrease in hematocrit by >20% from baseline
3. Positive pregnancy test
4. Severe / intolerable side effects of aspirin
5. Development of malignancy
6. Initiation of the following medications: methotrexate, amiodarone, systemic steroids or tamoxifen

Safety Monitoring:

1. Review of all adverse events (expected, unexpected and serious)

Severity, frequency and reporting protocol adherence

2. Review safety assessments described above
3. Review CBC (or hematocrit) results with unblinded monitor
4. Recruitment, enrollment (adherence to eligibility criteria)
5. Dropout rates
6. Protocol changes

Discontinuation Guidelines:

1. Individual:
    a. Positive pregnancy test
    b. Severe / intolerable side effects of aspirin
    c. Bleeding in the gastrointestinal tract
    d. Decrease in hematocrit by >20% from baseline
    e. Development of malignancy
    f. Initiation of the following medications: systemic steroids for >5 days, methotrexate, amiodarone, tamoxifen
2. Study:
    a. Unexpected severe / frequent adverse events

The major outcome choice following the data review by the Board is: 1) continuing the trial unchanged, or 2) modify the protocol and/or consent form, or 3) terminate the trial.

The chairperson (Dr. Miller) will be responsible for overseeing the meetings, developing the agenda and summarizing the meeting. The chairperson is the contact person for the DSMB.

The DSMB responsibilities will include:

1. Review of the research protocol, informed consent documents and plans for safety and data monitoring of the study. This review is to determine the risks and benefits to research subjects, protection and safety of the subjects and to offer suggestions for improving the study design.
2. Review interim data to detect adverse effects to determine if the trial should continue as originally designed, should be changed or should be stopped based on the data.
3. Evaluate the progress of the trial including recruitment goals, accrual and retention of participants and other factors that may affect the study outcome.
4. Protect confidentiality of the study participants, trial data and results of the monitoring.

C. Adverse Events Reporting

All treatment emergent serious adverse events will be documented and reported immediately to the IRB at Partners HealthCare System, as well as to the DSMB. An event that is serious must be recorded on the case record and requires expeditious handling to comply with regulatory requirements. In the event that a patient becomes ill or injured as a direct result of participation in the research study, necessary medical care will be made available. All adverse effects will be reported as per Partners Healthcare System requirements. Potentially serious adverse events (SAEs) will be followed to resolution or stabilization and reported as SAEs if they become serious.

A serious adverse event is defined as one that meets any one of the following criteria:

1. Fatal or life threatening
2. Requires inpatient hospitalization
3. Results in persistent or significant disability or incapacity
4. Congenital anomaly
5. Important medical event that may jeopardize the patient or require intervention to prevent serious outcome
6. Cancer
7. Overdose
8. Results in development of drug dependency or drug use

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Changes to Study Protocol After Trial Initiation Date:

- Version 4 (Amendment 2; approval date: 09/10/2019) updated the informed consent process descriptions.

- Version 5 (Amendment 5; approval date 10/02/2019) increased the upper age limit from 65 to 70 years; changed the frequency of follow-up phone calls from twice monthly to once monthly as recommended by the MGB QI team; and updated the funding information.

- Version 6 (Amendment 8; approval date 01/06/2020) updated the format of the SF-36 questionnaire; changed the compensation scheme for subjects, while keeping the total compensation for the overall trial unchanged; included the option of a serum pregnancy test for women of childbearing potential (in addition to urine pregnancy test); and included updated written instructions for stool collection kits, for subjects.

- Version 7 (Amendment 10; approval date 09/30/2020): added an oral microbiome sample to the baseline and month 6 time points; updated the payment distribution scheme for each study visit.

- Version 8 (Amendment 11; approval date 11/12/2020): clarified the exclusion criteria for thrombocytopenia by including the definition of lower platelet count threshold of 100K/uL.

- Version 9 (Amendment 12; approval date 02/19/2021): added an optional pre-treatment and/or post-treatment liver biopsy.

- Version 10 (Amendment 17; approval date 08/05/2022): added institution-wide research announcements as an additional method of potential recruitment.

Changes to Statistical Analysis Plan After Trial Initiation Date: none