Study Title: Does inhibition of liver specific 11β-hydroxysteroid dehydrogenase type 1 enzyme lower liver fat in NAFLD or NASH?

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Protocol Version date: 4-8-19 Edited by R.Basu,MD

Dr Rita Basu (PI) has relocated to University of Virginia Health System (UVa). The study will be conducted at both Mayo Clinic and UVa.We have completed enrollment at Mayo Clinic and are currently enrolling at UVA only.

Background and Rationale:

11β-hydroxysteroid dehydrogenase type 1 enzyme pathway:

Glucocorticoids are potent regulators of glucose, fat and protein metabolism. Tissue specific conversion of cortisone to cortisol via 11β-hydroxysteroid dehydrogenase type 1 enzyme pathway (11β-HSD-1) results in high local cortisol concentrations (1). 11β-HSD-1 is present in multiple tissues including the liver and adipose tissue with activity being greater in omental than subcutaneous fat (2-5). The 11β-HSD-1 pathway has attracted considerable attention both as a therapeutic target and a potential contributor to the pathogenesis of diabetes, obesity and the so called “metabolic syndrome” (1; 6; 7). Our observation that the splanchnic bed of obese humans (8) produces large amounts of cortisol with all of it occurring within the liver strongly implies that the resultant high local cortisol concentrations within the liver could be important determinants of hepatic insulin action. If so, inhibitors of hepatic 11β-HSD-1 could have dramatic effects on hepatic insulin action and could become an important new therapy for type 2 diabetes.

The role of high circulating intra-hepatic cortisol on modulation of insulin action in humans is an area of active investigation. Glucocorticoids induce glucose intolerance in humans in a dose and duration dependent manner (9-12) by inducing hepatic and extra hepatic insulin resistance (12-21). However, the exact mechanism of this effect was unknown. In vitro studies have reported that glucocorticoids increase11β-HSD-1 enzyme activity and mRNA expression in rat liver cells (3; 22). Our laboratory has developed a non-invasive technique to measure hepatic cortisone to cortisol conversion in humans (23). We have recently reported that liver cortisol production is higher in obese humans (8; 23; 24) through increased activity of hepatic 11β-HSD-1 enzyme. We also have recently completed a series of experiments and concluded that glucocorticoid excess stimulates hepatic cortisone to cortisol conversion via 11β-HSD-1 enzyme pathway and is associated with hepatic and extrahepatic insulin resistance in nondiabetic humans (25 ; 26). This implies targeting hepatic 11β-HSD-1 enzyme could ameliorate steroid induced changes in lipid and glucose metabolism.

That the liver is an important regulator of the diabetogenic effects induced by GC use is borne by the fact that liver selective GC receptor antagonists have been shown to reduce fasting glucose and improve hepatic insulin action during a hyperinsulinemic euglycemic clamp (27). A recent clinical trial has shown that 11β-HSD inhibition for 12 weeks can reduce liver fat (~14%), and total body weight without any effects on insulin induced suppression of glucose production (28). However, to our knowledge liver specific inhibitors of 11β-HSD have not been rigorously tested in the manner we currently propose.

Hepatic fat:

Under physiologic conditions, the liver is exposed to free fatty acids (FFA) derived from food and adipose tissue depots. These FFA are taken up by hepatocytes, where they undergo esterification to

form triglycerides (TG) that are ultimately stored in vacuoles or released to the bloodstream as very-low-density lipoproteins (VLDL). When equilibrium is disrupted, uptake and/or de novo synthesis of FFA is increased relative to fatty acid oxidation; if the rate of TG production is greater than that of VLDL synthesis and secretion, TGs accumulate in macrovacuoles within the hepatocyte (29) (termed *hepatic steatosis*). Clinically, hepatic steatosis is defined as macrovesicular fat accumulation in ≥ 5% of hepatocytes (30). NASH (non-alcoholic steatohepatitis) is an advanced form of fatty liver disease associated with inflammation and fibrosis. Liver enzymes (AST and ALT) are likely to be elevated in more than 90% of cases of NASH. In patients with NAFLD (non-alcoholic fatty liver disease), approximately 30% with isolated steatosis will progress to NASH. Of those with NASH, approximately 20% will develop cirrhosis, and of those with cirrhosis, 30–40% will decompensate and succumb to liver-related death over a 10-year period (31).

Currently, the favored technique for estimating the liver fat fraction (LFF) is quantitative magnetic resonance (MR) imaging (32). MR imaging in general ignores the multispectral nature of hepatic TG that can be used to distinguish between saturated fat, monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA). MR spectroscopy (MRS) of liver fat has been performed, and although it suffers from sampling error due to the small voxel size relative to liver size, the wealth of spectral information obtained can be used to determine the composition and quantity of hepatic fatty acids. Previous MRS studies of the liver (32-37) have used manual fitting techniques that are time intensive and operator biased (38-43). LC Model is a semi-automated, widely available MRS fitting program that can quantify up to 10 different fat resonances in the liver (38). We have developed a reliable, reproducible method for acquiring liver spectra on a 3T MRI scanner to calculate liver fat composition. This method was evaluated with 6 nondiabetic subjects scanned during 2 separate sessions, 10 days apart. In each session, 4 separate liver fat measurements were taken (8 scans/subject). Spectra were preprocessed (phased, averaged) and then analyzed using LC Model software. Quantified spectral data were combined into 4 liver fat indices: total fatty acids (TFA), polyunsaturated fatty acid index (PUFAI), total unsaturated fatty acids (TUFA), and the standard fat water ratio (FWR), similar to the LFF. Calculations were performed to evaluate intraclass correlation coefficient (ICC) for each index and variability within the cohort (ie, intrascan, interscan, inter-session, intersubject). The FWR varied from 0.02 to 0.26, indicating a wide range of liver fat in this healthy cohort (normal was defined as FWR≤0.05). Liver fat index ICCs for intrascan, interscan, and intersession reliability ranged from 0.92 (PUFAI interscan) to 0.99 (FWR intrascan and interscan), indicating that our method is highly reproducible. Variance components models (SAS PROC NESTED) showed that the majority of variance in the indices resulted from differences between subjects (FWR=98%, TFA=90%, TUFA=96%, PUFAI=89%). TFA had a small intersession variance (4%), whereas TFA and PUFAI had small scanner-related variance (6% and 9%, respectively). The remaining variance components were negligible (39). Mayo Clinic is implementing this scanning method for patients being evaluated for liver fat. We recently concluded a nutritional intervention trial in which this method was used to measure hepatic fat in subjects with IFG (impaired fasting glucose) (40; 41).

Magnetic resonance elastography (MRE) is a non-invasive MRI based technique that measures tissue stiffness (42). The widely applied clinical MRE technique was developed at the Mayo Clinic and is available at more than 300 centers worldwide. MRE provides a new quantitative parameter "tissue stiffness" useful for tissue characterization and differentiation and found useful in the detection and staging of liver fibrosis with high technical success and excellent accuracy (43-47) . Liver stiffness is affected by the presence of inflammation and fibrosis. MRE by way of measuring elevated liver

stiffness can differentiate nonalcoholic steatohepatitis characterized by inflammation and or fibrosis from simple steatosis or just fatty liver. Chen et al (45) showed that MRE had excellent accuracy (AUROC 0.93, sensitivity 94%) for differentiating simple fatty liver from steatohepatitis. Two studies have shown that MRE has excellent accuracy (92-94%) for distinguishing advanced fibrosis and cirrhosis in NASH patients from those with lesser degree of fibrosis in patients with NASH study (48; 49). Preliminary clinical experience has shown that MRE is useful in assessing treatment response in cases that included patients with fibrosis (50). MRE is therefore a useful noninvasive technique for detection, staging and assessment of treatment response. Since moving to UVA we are now using a Siemens scanner instead of GE so data are slightly different. The Liver fat % is comparable but the fibrosis scores are not the same from each scanner. Hence, we are opting to study some people with liver fibrosis stage 0 up at UVA.

Glucose tolerance:

The most frequently described conditions associated with NAFLD are obesity and T2DM, with insulin resistance (IR) as the underlying key factor for development of nonalcoholic fatty livers. Cusi et al have conducted elegant studies in patients with NAFLD and have shown reduced insulin sensitivity (Si) (eg, in liver and muscle), regardless of glucose tolerance status (51). People with impaired fasting glucose (IFG; fasting glucose concentration, 100-125 mg/dL) or impaired glucose tolerance (IGT; 2-hour glucose, 140-199 mg/dL after the standard 75-g oral glucose tolerance test [OGTT]) have a 25% to 30% chance of T2DM development within the next 5 to 10 years (52-55). Numerous studies (56-58) have shown that IGT, either alone or in combination with IFG (IFG/IGT), is associated with defects in insulin secretion and action. We will phenotype the subjects to gain better understanding of their metabolic status.

Approach:

We propose to combine the triple tracer cortisol technique with voxel matched MRS and MRE liver (pre vs. post) to evaluate hepatic cortisol production and hepatic fat and hepatic fibrosis. We will conduct baseline testing in subjects to estimate baseline hepatic cortisone to cortisol conversion. We will also establish glucose tolerance status by our established labeled OGTT (6,6 $^{2}H_2$ glucose). Following baseline evaluation subjects with NASH, NAFLD, will be randomized to one of two groups and treated either with active drug (liver specific 11β-HSD type 1 inhibitor**)** or placebo for 12 weeks +/- 1 week. OGTT, liver MRS, MRE and triple tracer cortisol study will be repeated. Liver enzymes (AST, ALT, ALP) as well as other safety tests (CPK,TSH,INR,T bilirubin) will be measured before, monthly during therapy and at one month following therapy. Details in the DSMP.

Specific Aim: Determine if inhibition of hepatic 11β-HSD type 1 activity lowers hepatic fat in subjects with NAFLD or NASH.

I) Primary Hypotheses:

- **a)** AZD4017 decreases hepatic fat as compared to placebo.
- **b)** AZD4017 decreases hepatic conversion of [^{13}C] cortisone to [^{13}C] cortisol as compared to placebo.

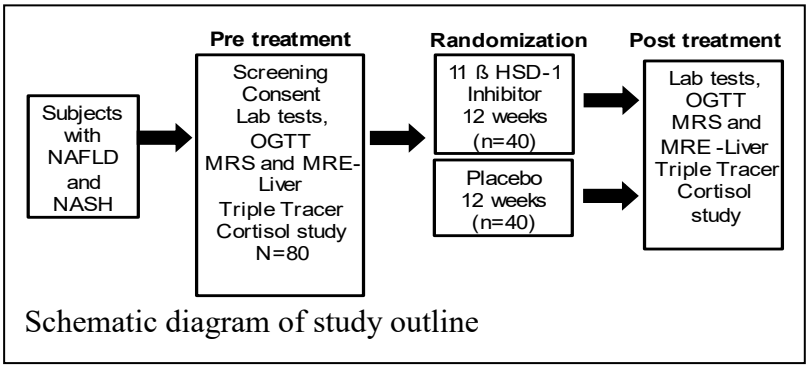
II) Secondary Hypotheses:

- **a)** Liver enzymes (AST, ALT) will be reduced in subjects treated with AZD4017 as compared to placebo.

b) Liver fibrosis measured with MRE will be reduced in subjects treated with AZD4017 as compared to placebo.
c) Total insulin sensitivity (Si) and hepatic insulin sensitivity (Si liver) will be improved after treatment with AZD4017 than at baseline.
d) Total insulin sensitivity (Si) and hepatic insulin sensitivity (Si liver) will be improved in subjects treated with AZD4017 as compared to placebo.

Exploratory Aim (future plan): We wish to analyze biomarkers of fibrosis and 11 β HSD polymorphisms since polymorphisms in the 11β-HSD1 gene are also associated with components of the metabolic syndrome (59; 60). We wish to test if polymorphisms are likely associated with who may or may not respond to the 11 β HSD inhibitor.

Biomarkers: NAFLD, NASH and Inflammation: NAFLD and NASH are increasing in incidence and prevalence in the United States and worldwide, in parallel with the increasing rates of obesity (61).



Schematic diagram of study outline

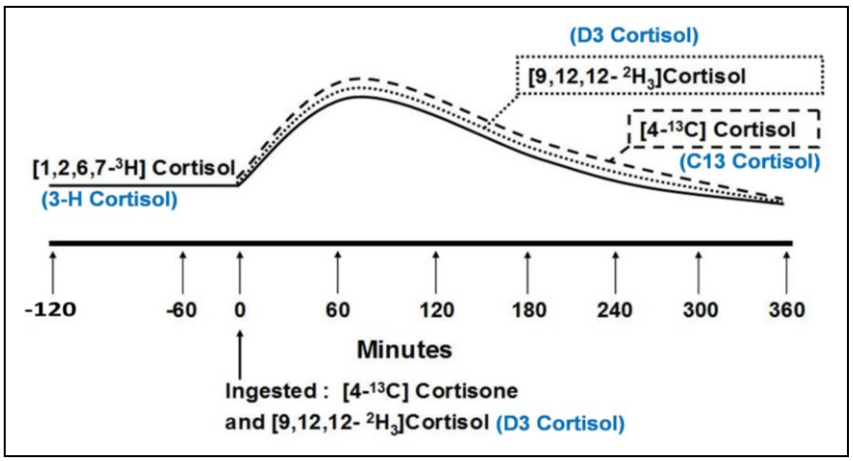
Both disorders are characterized by the presence of hepatic steatosis. NASH is distinguished from NAFLD on the basis of liver injury and liver inflammation (62). One disease pathogenesis paradigm holds hepatocyte cell death via lipoapoptotic pathways as a central event in the onset of progressive forms of NASH; furthermore, hepatocyte apoptosis correlates with fibrosis stage in NASH patients (63; 64). There are many circulating surrogate markers for fibrosis in NASH. The enhanced liver fibrosis (ELF) score is a function of serum levels of hyaluronic acid (HA), tissue inhibitor of metalloproteinases-1 (TIMP-1), and procollagen III N-terminal peptide (PIIINP) is a validated tool for assessing serum fibrosis (65). Other clinical surrogates include the AST/platelet ratio index (APRI) and age-spleen-platelet ratio index (ASPRI); however, the ELF test is unique in its incorporation of three molecules involved in the synthesis and breakdown of extracellular matrix. Newer serum based scores such as APRI (AST to platelet ratio index), FIB-4 (Age, ALT,AST, platelet count), NAFLD fibrosis score (age,ALT,AST,platelet count,BMI,albumin, impaired fasting glucose/diabetes) and BARD (ALT/AST ratio,BMI,diabetes) score may be calculated using the data gathered. In this study we will be able to correlate MRE results with circulating levels of all proposed biomarkers.

Subjects: Detailed information is provided in the DSMP. However, in brief, otherwise healthy (age 21-75 years), subjects (BMI >19kg/m^2) with NAFLD (MRS liver fat ≥ 5%, total bilirubin (TB) <1.5 xULN, INR <1.3) will be enrolled following IRB approval. Subjects with previous biopsy proven and/or MRE proven NASH will be included. Subjects with history suggestive of NAFLD/NASH per recent guidelines published by Loomba et al (66) will be invited to participate. If they meet criteria following initial screening they will be included in the study if their MRE shows F0 or greater fibrosis. Glucose tolerance status will be determined by OGTT. We will include subjects with type 2 diabetes who are on stable doses of medications (except pioglitazone) to control hyperglycemia and have baseline HbA1c of 10% or lower. Subjects will be chosen from UVa databases and will be contacted by phone or in writing and invited to participate in the study.. If subjects recently (within 3 months) had any of the exams, tests or procedures involved in this study conducted as part of their clinical care or as part

of another study that are accessible they may not need to be repeated. It will be up to the study doctor to review and consider if previous findings are appropriate to use. Healthy will indicate that they generally are in good health and have no chronic medical condition including hepatic disease, stroke, Alzheimer's disease, alcoholism or increased alcohol consumption over the ADA guidelines or any disorder that may potentially impact the outcome measures. Thyroid medications, statins and antihypertensive medications that are metabolically neutral (e.g. low dose thiazides) will be permitted. At this time based on a prior human study done using a similar 11-βHSD1 inhibitor (Ref: Stefan et al, lancet, May 2014) suggests that usage of drugs potentially associated with non-alcoholic fatty liver disease (NAFLD) such as amiodarone, methotrexate, perhexiline, estrogens, tamoxifen, nifedipine, diltiazem, choloroquine and other hepatotoxic agents will need to be reviewed if used for more than 2 consecutive weeks in the 2 years preceding the screen visit. Subjects taking TZD's, Atazanavir, Indinavir, Ketoconazole, Valproic acid, Silybum marianum and Valeriana officinalis will be excluded. Additionally if subjects are taking drugs such Vit E, Ursodeoxycholic acid, Gemfibrozil, anti TNF therapies and probiotics will be allowed if on stable doses in the preceding 2 weeks and continued throughout the study. However as with all concomitant medication usage the study doctors will review on case by case basis prior to inclusion in trial. The final decision for enrollment will be based on the clinical judgement, discretion of the study doctors.
Subjects that engage in vigorous physical exercise programs may be included. The level of exercise will be assessed by PI to determine if eligible to participate in study. Those actively losing weight will be excluded. Dietary advice will be provided by the research dietician to ensure that subjects maintain a constant body weight within 2% of their baseline during the trial period. Body composition may be measured during the screen visit in the body composition core using Lunar iDXA or similar machine at UVa to measure body composition. Mayo uses software version 6.10 (GE Healthcare Technologies, Madison, WI) to measure total body fat, and waist–hip measurements will be done to look at abdominal adiposity. Screen visit will also include blood and urine tests, height, weight, vital signs, ECG, a physical exam and review of their medical history. Hemoglobin must be greater than or equal to 12.0 in males and 11.0 in females. Subjects will report to UVa campus for screen visit and monthly outpatient visits.

Study testing: Includes out-patient visits for iDXA (screen and 3 month), blood (screen/monthly) and urine tests (screen and 3 month), monthly prescription pick up visits, labeled OGTT (screen and 3 month), MRS, MRE (screen and 3 month) and in-patient visits (see experimental design) to measure hepatic cortisone to cortisol conversion using our established triple tracer cortisol technique in subjects with NAFLD or NASH.

Treatment: Subjects will be randomized to receive either AZD4017 800 mg/day for three months in two divided doses morning (400 mg) and evening (400 mg); matched placebo for three months. Pill counting will be done at monthly visits to monitor compliance and adherence to therapy. Unused pills will be returned and discarded by the research pharmacy. Both subjects and investigators will be masked to the type of treatment. We anticipate that 50 subjects in each of the two groups (with ~5 anticipated drop outs per group) will be sufficient to answer our primary hypotheses.



Once enrolled in the study, subjects will be asked not to donate blood 12 weeks before and 12 weeks after the study. Per Institutional Review Board guidelines and blood bank the total amount of blood withdrawn in any 12 week period will not exceed 1 unit of blood (~550 ml) and this includes all screening bloods (~24 ml), monthly safety bloods (~8 ml) , OGTT (~60 ml), Triple tracer cortisol study (~150 ml).

Experimental design:

MRS and MRE for liver fat and fibrosis will be done if not done previously following enrollment into study. For flexibility of scanning we will allow subjects to undergo MR scans at any time prior to the screening visit. A standard 75 gm labeled (6,6-$^2H_2$ glucose) OGTT will be performed over 240 mins to determine glucose tolerance status. OGTT: The labeled OGTT will enable us to concurrently measure various indices of insulin action, insulin secretion, and DI (composite measure of insulin secretion accounting for the prevailing level of IR). Furthermore, this protocol also allows measurement of EGP; this method was validated (41; 67-73) in various population cohorts in the US and Europe. OGTT will not be performed on subjects taking insulin for management of hyperglycemia. Subjects will be required to fast overnight and report to the CRTU at Mayo Clinic or UVa campus on the morning of testing. Only sips of water will be allowed until testing is complete. An 18-gauge cannula will be inserted in a forearm for blood withdrawal. After baseline sampling (–15 and 0 minutes), a dextrose solution (Limeondex [Fisher]) will be consumed within a few minutes; this solution will contain 3 g of 6,6- $^2H_2$] glucose (~4% enrichment) in 72 g of dextrose. Blood samples will be withdrawn at 10, 20, 30, 60, 90, 120, 150, 180, 210, and 240 minutes for measurement of glucose, insulin, C-peptide, glucagon, and [6,6- $^2H_2$] glucose enrichment. At the end of the study, subjects will be provided a snack and dismissed.

Cortisol study:

Subjects will return on a different day for the in-patient visit. Subjects will be admitted to the CRTU between 4 pm and 6 pm on the evening before the study and will be provided a standard supper (carbohydrate: protein: fat in the ratio of 50:20:30). consisting of ~33% of the subject's total estimated calorie intake based on Harris Benedict + 20% calorie requirements. Urine pregnancy test will be done in women of child bearing potential. Subjects will be awakened the following morning and a catheter will be placed in a forearm vein for tracer infusion and in the dorsum of the opposite hand which will be placed in a heated box (~55^{0}C) to permit sampling of arterialized venous blood. A bladder catheter will be offered on the morning of study if subjects are unable to use a bedpan, urinal or bedside commode. A primed (24μCi) continuous (0.24 μCi/min) infusion of [1,2,6,7–^{3}H] cortisol will

be started at minus 120 minutes and blood will be collected for measurement of [^{3}H] cortisol concentration at timed intervals in each subject to calculate basal production rate of cortisol. At time 0, subjects will ingest 1.0 mg of [4-^{13}C] cortisone and 1.0 mg of [9,12,12-$^{2}H_3$] cortisol. At time 0, the [^{3}H] cortisol infusion rate will be increased in order to mimic the temporal pattern of change as previously published (23). Arterialized venous blood then will be sampled at periodic intervals (suggested timelines are -30, 0, 20, 40, 60, 75, 90, 120, 150, 180, 210, 240 minutes) for measurement of [^{2}H] cortisol, [^{2}H] cortisone, [^{13}C] cortisone and [^{13}C] cortisol enrichment, and [^{3}H] cortisol [^{3}H] cortisone specific activity (23). At UVa subjects will come in to CRU morning of study and all other interventions will be similar as outlined above.

Analytical methods
All blood samples will be immediately placed on ice, centrifuged at 4^{0}C, separated and stored at -80^{0}C until analyses. Plasma glucose will be analyzed using YSI (yellow springs Ohio), plasma insulin will be measured using a chemiluminescence method with the Access Ultrasensitive Immunoenzymatic assay system (Beckman, Chaska, MN) and C-peptide and glucagon measured using a radioimmunoassay; Linco Research. Plasma [6,6-$^{2}H_2$] glucose enrichment will be measured using gas chromatographic mass spectrometry (74). Plasma cortisol, cortisone, D3 cortisol, D3 cortisone, 13C cortisone and C13 cortisol enrichments will be measured using liquid chromatography tandem mass spectrometry as previously described (23; 75; 76). [^{3}H] cortisol radioactivity will be measured using high performance liquid chromatography followed by liquid scintillation counting (23; 77). Body composition will be measured using Lunar dual energy X-ray absorptiometry software (GE Healthcare Technologies).

Calculations:
[^{3}H] cortisol will be used to trace the systemic rate of appearance of both [^{13}C] cortisol and [^{2}H] cortisol. Thus, the systemic rate of appearance (ug/min) of [^{13}C] cortisol derived from the ingested [^{13}C] cortisone will be calculated by using Steele's steady state equation. Hepatic [^{13}C] cortisol production will be calculated as the area under the curve for the rate of appearance of [^{13}C] cortisol divided by one minus the hepatic extraction of [^{2}H] cortisol (23; 78). Insulin sensitivity and secretion indices will be calculated using unlabeled and labeled models established by us previously (41; 61-67)

Statistical Analysis
Demographics and baseline characteristics will be described by treatment group and the groups compared using two-sample t-tests.

Primary analysis
We will first compare the change in LFF from baseline (time 0) to post treatment (12 week) between the two treatment groups, one with patients treated with active drug and the other with patients treated with placebo, using analysis of covariance (ANCOVA) including baseline LFF as a covariate. This primary analysis will be based upon the intention to treat principle, including all patients randomized who have both baseline and post treatment measures of LFF.

Next we will compare rates of hepatic conversion of [^{13}C] cortisone to [^{13}C] cortisol between the groups, again using ANCOVA, analogous to the analysis of LFF.

Power for Primary Analysis
A sample size of 45 in each group will have 80% power to detect a difference in means of 3.1 assuming a mean square error (MSE) of 4.6 using an ANCOVA and a two-sided test with alpha 0.05. The MSE of 4.6 is estimated based upon the study of Stefan et al. (28) where an MSE of 4.6 and a difference in group means of 2.6 were observed. We anticipate that as many as 5 patients could drop out of each treatment group and so will recruit additional patients to each treatment group for a total of 100 patients. Based upon this our study is adequately sized to achieve our aims. We will now also include subjects with fibrosis score 0 up but with liver fat % >5 % as we believe data from these subjects will also be valuable and of interest in the final analyses. Since fibrosis scores are unlikely to change in the short duration of this trial and change in fibrosis is a secondary endpoint we believe this approach does not distract from our primary endpoint related to lowering of liver fat %.

Secondary Analysis
The data for the primary outcomes will be explored estimating treatment effects by sex. These too will be tested in the ANCOVA, including interaction terms between treatment and sex.
Outcomes related to secondary hypotheses will be analyzed analogously to the primary outcomes, using ANCOVA.

Detailed human subjects protection plan/DSMP prepared as a separate document

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