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The use of CHS-131, a selective PPAR-gamma modulator to treat NAFLD/NASH

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BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

**THE USE OF CHS-131, A SELECTIVE PPAR- γ MODULATOR TO
TREAT NAFLD/NASH**

by

ADITYA JOSHI

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Approved by

First Reader _____

Christos Mantzoros, M.D., D.Sc., Ph.D., *h.c. mult.*

Professor of Medicine, Harvard Medical School

Chief, Endocrinology Section, VA Boston Healthcare System

Adjunct Professor of Medicine, BUSM

Second Reader _____

Mina Moussavi, Ph.D.

Assistant Professor of Physiology and Biophysics

Department of Physiology and Biophysics

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ADITYA JOSHI

ABSTRACT

Background: Non-alcoholic fatty liver disease (NAFLD) and its progression to non-alcoholic steatohepatitis (NASH) is a spectrum of diseases that is rising in prevalence and is strongly associated with obesity, diabetes, and insulin resistance. There has been much research into potential therapeutics, as the current recommended treatment, thiazolidinediones (TZDs) present a host of negative side effects such as fluid retention and weight gain. CHS-131 is a selective PPAR- γ modulator with antidiabetic effects and less side effects compared to TZDs. The aim of this study was to investigate the effects of CHS-131 on metabolic parameters and liver pathology in a diet-induced obese (DIO) and biopsy-confirmed mouse model of non-alcoholic steatohepatitis.

Methods: Male C57BL/6JRj mice were fed an AMLN diet (40% fat with trans-fat, 20% fructose and 2% cholesterol) for 33 weeks prior to a liver biopsy procedure. Animals that were biopsy-confirmed to have steatosis and fibrosis were stratified into 3 treatment groups: 1) Low dose CHS-131 (10mg/kg), 2) High dose CHS-131 (30mg/kg), 3) Vehicle. Metabolic parameters, liver pathology including NAFLD activity score,

metabolomics/lipidomics, markers of liver function and liver, subcutaneous and visceral adipose tissue gene expression was assessed.

Results: CHS-131 did not affect body weight, fat, lean or water mass, or food intake in DIO-NASH mice with fibrosis. CHS-131 improved fasting insulin levels and insulin sensitivity as assessed by intraperitoneal insulin tolerance test. CHS-131 improved total cholesterol, ALT, AST and increased adiponectin levels in plasma. CHS-131 improved NAS in liver histology and tended to reduce markers of hepatic fibrosis. Diet induced NASH mice treated with CHS-131 demonstrated a hepatic shift to diacyl- and triacyl-glycerol's with shorter chains, increased expression of genes stimulating fatty acid oxidation and browning and decreased expression of genes promoting fatty acid synthesis, triglyceride synthesis and inflammation in adipose tissue.

Conclusion: CHS-131 improves liver histology in a diet-induced obese and biopsy confirmed mouse model of NASH by affecting the hepatic lipidome, reducing insulin resistance and altering lipid metabolism and inflammation in adipose tissue.

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LIST OF ABBREVIATIONS

a-SMA	Alpha- smooth muscle actin
AASLD	American Association for the Study of Liver Diseases
ALT	Alanine aminotransferase
AMLN	Amylin liver NASH
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AST	Aspartate transaminase
AUC	Area under the curve
BAX	BCL-2 associated X protein
BCL-2	B-cell lymphoma 2
BMI	Body Mass Index
Ccl2	C-C Motif Chemokine Ligand 2
CIDEA	Cell death inducing DFFA like effector A
CNS	Central nervous system
Colla1	Collagen, type I, alpha 1
CVD	Cardiovascular disease
DAG	Diacylglycerols
EASD	European Association for the Study of Diabetes
EASL	European Association for the Study of the Liver
EASO	European Association for the Study of Obesity

Elov13	ELOVL Fatty Acid Elongase 3
Fasn	Fatty acid synthase
FFA	Free Fatty Acid
GI	Gastrointestinal
GLP-1	Glucagon-Like Peptide 1
IHC	Immunohistochemistry
IL	Interleukin
Insr	Insulin receptor
IPITT	Intraperitoneal insulin tolerance test
Irs1	Insulin receptor substrate 1
Lepr	Leptin receptor
MRI	Magnetic resonance imaging
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEFA	Non-esterified fatty acid
NF- κ β	Nuclear factor kappa-light-chain-enhancer of activated B cells
OGTT	Oral glucose tolerance test
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PGC-1a	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PO	Per os (orally)

PPAR	Peroxisome proliferator activated receptor
RANKL	Receptor activator of NF- κ B Ligand
sPLS-DA	Sparse partial least squares discriminant analysis
SPPAR γ M	Selective PPAR- γ modulator
T2DM	Type 2 Diabetes Mellitus
TAG	Triacylglycerol
TC	Total cholesterol
TG	Triglyceride
TNF- α	Tumor necrosis factor – alpha
TZD	Thiazolidinedione
UCP1	Uncoupling protein -1
VLDL	Very Low Density Lipoproteins

INTRODUCTION

Definition/Progression to NASH

Non-alcoholic fatty liver disease (NAFLD) is defined as a condition in which there is excess of fat accumulation in the liver, not due to an excessive consumption of alcohol. Specifically the European Association for the Study of the Liver (EASL), European Association for the Study of Diabetes (EASD), and European Association for the Study of Obesity (EASO) define it as the presence of steatosis in greater than 5% of hepatocytes with no alcohol, drug or viral induced steatosis.¹ It includes a spectrum of manifestations that range from non-alcoholic fatty liver (NAFL) and its progression to non-alcoholic steatohepatitis (NASH). NAFLD, the accumulation of hepatic fat, progresses to NASH when there is development of hepatic inflammation and hepatic fibrosis. This occurs in approximately 30-40% of patients and leads to increased liver related complications such as cirrhosis, liver failure, and hepatocellular carcinoma.²

Diagnosis

Diagnosis of NAFLD is often difficult using non-invasive measures and it is usually diagnosed incidentally. Patients are often evaluated when they present with insulin resistance, obesity, or factors associated with metabolic syndrome. While the gold standard for diagnosis and staging is liver biopsy which can quantitatively differentiate simple steatosis from NASH, it presents several limitations as an invasive procedure that

can present with severe complications (major pain, bleeding requiring a transfusion, death in .3% of patients).³ The use of biomarkers, fibrosis scores, and elastography are acceptable non-invasive techniques to identify cases.⁴ Diagnosis is based on the presence of hepatic steatosis via imaging or histology, after ruling out other causes for chronic liver disease or alcohol consumption. However, the problem remains that the disease is often not discovered as patients are often asymptomatic until liver damage begins to occur. Further research is needed to determine tools and methods to predict groups of people that are at the highest risk of developing NAFLD/NASH and to predict the progression of NAFLD/NASH in patients.

Prevalence/Incidence

NAFLD is the most common liver disease in Western countries and is expected to become the most common reason for liver transplantation by 2030.⁵ Epidemiological studies find that it affects 25% of adults, more commonly men than women (30-40% vs. 15-20%).²⁶ However, these numbers must be considered with the fact that incidence rates are uncertain because many patients remain undiagnosed due to the difficulty in diagnosing the disease.

NAFLD has long been viewed from the lens of hepatologists and has been seen primarily as a liver disorder but growing evidence has shown that it is a multisystem disease that affects other extrahepatic organs and regulatory pathways.²⁴ In groups with known risk

factors, the prevalence of NAFLD is highly elevated and it has been strongly associated with diabetes, obesity, and cardiovascular disease (CVD).⁷ While the majority of studies have used anthropometric and biochemical measurements which are not definitive markers for assessing NAFLD, even studies using non-invasive imaging techniques have nearly all shown that NAFLD increases a patients' risk of having type 2 diabetes mellitus (T2DM).² A survey of 2,830 patients with T2DM found that 70% of patients with T2DM also had NAFLD.^{4,8,9} Likewise, a study of 3000 patients found that NAFLD was present in 25% of patients with a normal BMI (20.0 – 20.49 kg/m²), 67% of patients with an overweight BMI (25.0 – 29.9 kg/m²), and in 94% of patients with an obese BMI (>30.0 kg/m²).⁴ Although uncertainty exists whether NAFLD is a coexisting condition in people with an increased risk for CVD or is an independent risk factor, it remains that there is a strong relationship between NAFLD and an increased prevalence of CVD. Evidence shows that CVD influences the outcomes in NAFLD patients to a greater extent than the progression of liver disease.¹⁰ Moreover, while only 6-15% of patients that have NAFLD are thought to have NASH¹¹, studies have found that NASH is prevalent in 25-30% of patients with obesity or T2DM and over 35% of patients with severe obesity and T2DM.⁴

Pathophysiological Aspects of NAFLD

NAFLD has often been thought of as the hepatic manifestation of insulin resistance, and it was thought at one point that a “single-hit”, insulin resistance, could explain the whole

spectrum of NAFLD to NASH.¹² Recently, the view on NAFLD has shifted toward a “multiple parallel hit” model of factors, including genetic and environmental factors, obesity and insulin resistance, dysregulation of adipokines, lipotoxicity, endoplasmic reticulum stress, oxidative stress, dysbiosis of gut microbiota, and endocrine disruptors.¹³

The association between NAFLD, obesity, and T2DM is evident, and increasing studies show that the risk varies according to NAFLD severity.² A study that compared the impact of obesity, insulin resistance, and NAFLD to the risk of T2DM found that the risk factors independently all predicted T2DM and when the risk factors were all present, the risk of T2DM increased fourteen-fold. Furthermore, the study found that ultrasound-detected fatty liver improvements were associated with a decrease in risk for T2DM and fatty liver deterioration was associated with an increased risk of T2DM.¹⁴

Despite these multiple factors, insulin resistance continues to play a fundamental role in NAFLD and its consequences have a multifold of effects on many metabolic pathways. Peripherally, insulin loses its ability to inhibit hormone-sensitive lipase, resulting in an increased hydrolysis of triacylglycerol to glycerol and free fatty acids (FFAs). This results in an increase in FFAs circulating in the plasma which consequently reach the liver. Furthermore, in the liver, insulin resistance causes an increase in fatty acid oxidation and gluconeogenesis. However, the compensatory hyperinsulinemia of insulin resistance causes an increase in fatty acid synthesis by the liver (*de novo* lipogenesis) and a decrease in triglyceride export via very low density lipoproteins (VLDLs).

The result of the increased FFAs from adipose lipolysis, and *de novo* lipogenesis is an excessive accumulation of triglycerides in the liver.¹² Peripheral insulin resistance has been shown in patients with NAFLD to be the main contributor to this, with circulating FFAs, dietary fat supply, and *de novo* lipogenesis accounting for 59%, 15%, and 26% of hepatocellular triglycerides, respectively.¹⁵

The liver is also responsible for the β -oxidation of hepatic lipids, which takes place mostly within mitochondria. The influx of FFA into the mitochondria is controlled by carnitine O-palmitoyl transferase 1 which activates peroxisome proliferator activated receptor-alpha (PPAR α) and initiates the breakdown of FFA. Normally, an increase in lipid circulation would cause the upregulation of hepatic mitochondrial oxidative capacity. However, carnitine O-palmitoyl transferase 1 is inhibited by insulin, and the development of insulin resistance impairs this process, resulting in a deposition of fat which further intensifies insulin resistance. Studies have found that this impairment is not identical in obesity and NAFLD. In one study, obese individuals showed five times the mitochondrial activity than lean individuals, while the rates of mitochondrial activity was 40% lower in patients with NASH, favoring lipid deposition and insulin resistance.¹⁶ In addition to decreased mitochondrial activity, patients with NAFLD have also been shown to have increased oxidative stress paired with an increase in mitochondrial uncoupling and the leakage of protons across the electron transport chain. This results in the

generation of reactive oxygen species, which accelerates inflammation and progression to NASH.¹⁷

The development of hepatic steatosis can lead to the activation of various inflammatory pathways that have also been identified as critical in the development of insulin resistance. Animal studies in mice with diet induced steatohepatitis, have shown that FFA's stimulate the expression of NF- κ B mediated TNF- α , which can promote insulin resistance via IKK- β activation and *c-jun*-N-terminal kinase activation, creating a self-perpetuating pathway.¹⁸ The IKK complex, which then activates the NF- κ B pathway by phosphorylating the inhibitory molecule I κ B α , has been shown in animal studies to induce hepatic inflammation along with systemic insulin resistance.¹⁹ Receptor activator of NF- κ B (RANKL), which is produced by various tissues such as skeletal muscle, immune cells, and adipose tissue has also been shown to play a role in the formation of hepatic insulin sensitivity. RANKL binds to the RANK receptor in the liver and activates the NF- κ B pathway leading to inflammation and insulin resistance.

In addition to NF- κ B signaling, the release of certain pro-inflammatory adipocytokines such as IL-1 β , or IL-6 can induce insulin resistance in the liver.²⁰ Through the aforementioned proinflammatory pathways, hormones, FFA's and adipocytokines, the liver functions as both a source and target organ for the development of insulin resistance and inflammation.

Treatment of NAFLD

Lifestyle Modifications

There is currently no pharmacological therapy that has been approved for the treatment of NAFLD. However, due to the common pathophysiological mechanisms shared by T2DM and NAFLD, it is not surprising that there is overlap in therapies used in their treatment. The goal of treatment is to prevent or reverse the progression of NAFLD and avoid the development of cirrhosis and liver failure associated with NASH. The first line of treatment is often lifestyle modifications via changes in diet and exercise targeted towards a weight loss of 7-10%¹ and reducing the intake of saturated fat.¹ Aerobic exercising increases whole body lipid oxidation, improves steatosis and cardio-metabolic risk profiles, and can protect patients from developing T2DM.²¹ It has been observed that patients who achieve >7% of weight loss have a 65-90% resolution of NASH, however a meta-analysis of trials studying weight loss in NAFLD found that less than 50% of patients are able to achieve this.^{21,22} Furthermore, weight reduction of >3% has been found to improve steatosis, >5% to improve inflammation, and >10% to improve fibrosis, implying that the prevention of steatosis may be a viable target to prevent the development of NASH.²³

Bariatric Surgery

Often times medical treatment and lifestyle modifications fail to achieve sustained weight loss, and in these cases, surgical therapy is often recommended. Even in morbidly obese patients, where medications often show limited efficacy, bariatric surgery has been found to be effective. The mechanisms that support improvements through bariatric surgery are improvements in T2DM and insulin resistance, reduced hyperlipidemia, improvements in metabolic syndrome, as well as alterations in the route of food delivery resulting in changes in gut/pancreatic hormones, fat distribution, FFA metabolism, and adipocytokine release. A meta-analysis on the effects of bariatric surgery on patients with NAFLD found an improvement in liver histology in more than three-fourths of patients, with a majority of NAFLD patients having a complete resolution of the disease. Patients had the most improvement in liver steatosis, followed by steatohepatitis, and fibrosis.²⁴ However, it is important to note that some cases have been reported where bariatric surgery has resulted in an worsening of fibrosis.²⁵

Metformin

When lifestyle modifications fail to accomplish and maintain the weight loss as necessary, pharmacological treatments are considered to supplement a patient's diet and exercise. Since insulin resistance plays such a major role in the pathogenesis of NAFLD, insulin sensitizing drugs seem to be an apparent therapeutic. Metformin is the most common first-line drug used to treat T2DM and is known to improve both hepatic and peripheral insulin resistance, and decrease hepatic glucose production. Furthermore, it

has been shown to have an anti-tumor effect and reduce rates of hepatocellular carcinoma. However, metformin has not been shown to improve liver histology or reduce hepatic fat and inflammation, and is thus not recommended for the treatment of liver disease in patients with NASH. 26,27

Vitamin E

Vitamin E is known to have strong antioxidant properties which gives it the ability to act on various mechanisms that contribute to NAFLD/NASH. During the progression of NAFLD, oxidative stress results from the formation of reactive oxidative species, due to increased fatty acid oxidation and oxidative phosphorylation. Oxidative stress has been implicated as a key contributor to hepatocyte injury, senescence, and apoptosis, and its pathways are known to play a key role in the pathogenesis of NASH.²⁸ Thus, using antioxidants such as vitamin E can provide a therapeutic benefit by reducing oxidative stress to slow down the progression to NASH. Furthermore, vitamin E has been shown to have effects that can reduce the inflammatory response, leading to a retardation in fibrosis and cirrhosis. Studies show that vitamin E supplementation increases adiponectin mRNA and protein levels, which suppresses hepatic fatty acid synthesis and reduces inflammation. Lastly, vitamin E has been shown to reduce rates of apoptosis by increasing levels of anti-apoptotic proteins (BCL-2) and decreasing levels of pro-apoptotic proteins (BAX, P53, Caspase-9, Cytochrome C), along with suppressing the expression of pro-inflammatory cytokines. Clinical studies have found that treatment

with vitamin E improves steatosis, reduces inflammation and ballooning, decreases liver function test values, and resolves steatohepatitis. However, studies have found that long term vitamin E supplementation has been associated with an increased risk for prostate cancer along with increased risks for cardiovascular related mortality.^{29,30} Based on these findings, the AASLD and EASL recommend vitamin E in non-diabetic patients with biopsy-proven NASH.¹

Incretin Based Therapies (GLP-1 Agonists)

Incretin mimetics, also known as Glucagon-Like Peptide 1 (GLP-1) agonists, function to activate GLP receptors on pancreatic beta cells and stimulate glucose dependent secretion of insulin. Furthermore, they are known to activate GLP receptors within the GI tract, lung, kidney, and CNS where they exhibit metabolic functions such as delayed gastric emptying, appetite suppression, and enhanced liver glucose uptake.²⁸ GLP-1 agonists, have been shown in both animal and human studies to stimulate weight loss, improve insulin sensitivity, and improve lipotoxicity. While they are mainly used to treat diabetes, beneficial effects in NASH patients have been shown, such as decreased serum ALT levels, improvements in hepatic fat content, and fibrosis.³¹ A study where NASH patients were given liraglutide, a GLP-1 agonist, or placebo, found that the liraglutide group achieved a 39% resolution of NASH compared to the placebo.¹⁷ Other randomized controlled trials have found other GLP-1 analogs to reduce hepatic fat, and liver function

tests, however further clinical studies are needed to fully confirm their safety and efficacy.³²

Thiazolidinediones

Thiazolidinediones (TZDs) are another class of drugs used to treat insulin resistance. TZDs are ligands that bind to the peroxisome proliferator activated receptor gamma, (PPAR- γ), a transcription factor that regulates adipocyte differentiation. Binding of TZDs to PPAR- γ activates processes that shift fat distribution towards subcutaneous fat storage rather than visceral fat storage.³³ As a result, there is a reduction in circulating FFA along with a decrease in hepatic fat deposition, hepatic insulin resistance, and hepatic gluconeogenesis. Furthermore, TZDs suppress the transcription of inflammatory genes and upregulate the production of adiponectin which has AMPK mediated effects that promote hepatic fat oxidation, glucose uptake, and insulin sensitivity.^{26,34,35} Studies investigating the treatment of NAFLD with TZDs have shown promising results on liver histology, improving steatosis and inflammation. In a two year randomized controlled trial that compared Pioglitazone, a commonly prescribed TZD, to placebo in patients without diabetes, Pioglitazone showed an improvement in all histological features except fibrosis and a resolution of NASH compared to the placebo (47% vs 21%).³⁶ Similarly, another study found that Pioglitazone administered for 18 months showed improvements in the NAFLD Activity Score (which includes hepatic steatosis, lobular inflammation,

and ballooning) in NASH patients with T2DM (58% vs 17% placebo), and these improvements were maintained for 36 months post treatment. Moreover, 51% of patients were found to have a resolution of NASH compared to 19% of patients receiving placebo.³⁷ Based on these studies, the AASLD Practice Guideline for NAFLD, allows for the use of Pioglitazone to treat steatohepatitis for NASH patients. Unfortunately, TZDs have also exhibited some concerning side effects. Body weight gain, fluid retention, an increased fracture risk, cardiac failure, and a possible increased risk of bladder cancer, make it less appealing and necessary for clinicians to weigh the benefits and risks during long term use.²⁶

Problem with TZDs/Development of CHS-131

Pioglitazone and Rosiglitazone are the two TZDs (PPAR- γ agonists), that are approved to treat T2DM, and have been clinically validated to increase insulin sensitivity, albeit with the negative side effects mentioned earlier. Other PPAR- γ full agonists that are structurally unrelated have also shown a similar set of side effects, leading to the conclusion that the negative effects are associated with full receptor agonism.³⁸ The dose response curves for both the therapeutic effects of TZDs and the negative side effects overlap, which means that greater doses exhibit both increased benefits and negative effects. This also means that the full therapeutic potential of PPAR- γ activation by TZDs

may not be realized since patients are not able to tolerate the drawbacks present at maximally beneficial doses.³⁹

CHS-131, a selective PPAR- γ modulator was specifically designed to have the positive insulin sensitizing and glucose lowering effects of TZDs, without the undesirable side effects.³⁸ The molecule binds to PPAR- γ at the same binding pocket as TZDs, but differs in its interaction with the activation helix of the nuclear receptor, leading to a different pattern of gene transcription. In vitro studies showed that CHS-131, compared to other TZDs, caused a reduction in the induction of adipogenic genes resulting in less adipocyte differentiation of preadipocytes along with less lipid accumulation.³⁸ In animal models, CHS-131 causes markedly less weight gain and volume expansion than TZDs. In a study comparing CHS-131 to a TZD in a rodent model of T2DM, CHS-131 was more potent in reducing serum glucose, insulin, triglyceride, and non-esterified fatty acid (NEFA) concentrations. Moreover, CHS-131 was also more potent in improving glucose tolerance and increasing levels of adiponectin in the mice. Unlike TZDs, CHS-131 did not cause fluid retention, or exhibit the adverse cardiac effects that are caused by an increased plasma volume expansion.³⁸

Toxicology studies have found that CHS-131 is well tolerated at high doses and does not exhibit the toxicities associated with TZDs. In rats given doses two to three orders of magnitude greater than efficacious clinical doses for six months, CHS-131 did not show signs of fluid accumulation or increased heart weight, typical of PPAR- γ agonists. Safety

testing in monkeys given doses more than 70-fold greater than the highest expected clinical dose also confirmed these results.³⁹

The aforementioned preclinical studies have led to Phase I and Phase II trials with CHS-131 for the treatment of T2DM that have shown promising results. A multicenter, double blinded, placebo controlled study in patients with T2DM found that CHS-131 lowered plasma glucose levels in patients, and stimulated adiponectin levels, with a marked separation of the negative side effects of edema and weight gain present in PPAR- γ full agonists.⁴⁰

SPECIFIC AIMS⁴¹

This project aims to investigate the effects of CHS-131 on metabolic parameters and liver pathology in a diet-induced and biopsy-confirmed mouse model of NASH. This will allow us to assess and further understand the potential for CHS-131 to be a novel therapeutic with less adverse side effects for the treatment of NAFLD.

MATERIALS AND METHODS⁴¹

Study Design

NASH was induced in 5-week old male C57BL/6JRj mice that were supplied by JanVier (France). The mice were fed a high fat diet consisting of 40% fat with trans-fat, 20% fructose, and 2% cholesterol (AMLN diet or D09100301, Research Diets Inc., USA) for 36 weeks. A control group of mice were fed a regular chow diet. To confirm the presence

of NASH, a biopsy was performed three weeks prior to the start of the study. Using a validated scoring system for NAFLD⁴², the study only included NASH-affected mice based on a steatosis score ≥ 2 and a fibrosis score ≥ 1 . Before the initiation of treatment, the mice were randomized into four treatment groups of at least 12 mice each. The four groups were:

- a) mice fed a chow diet treated with a vehicle
- b) mice fed an AMLN diet treated with a vehicle
- c) mice fed an AMLN diet treated with low dose CHS-131 (10mg/kg)
- d) mice fed an AMLN diet treated with high dose CHS-131 (30mg/kg)

The doses of CHS-131 or vehicle were administered orally via an oral gavage and were performed once a day for the study's 12 week duration.

Legal and Permissions

All animal experiments were conducted with accordance to Gubra bioethical guidelines, and were fully compliant with internationally accepted guidelines for the care and use of laboratory animals.

Animal Welfare

Animals were checked once a day for signs of abnormal behavior, abnormal locomotor activity, ataxia, or clinical signs of disease. A clinical veterinarian or technician judged health status in case additional examination was necessary. If abnormal behavior or

clinical signs of disease presented during the study period, animals were terminated from the study.

Animal Identification

Unique microchips implanted subcutaneously under light CO₂ anesthesia were used to identify animals in Gubra's animal facility. Animals were identified using a WS-1 weigh station (MBrose, Faaborg, Denmark) connected to a laptop running an HM02Lab software (Ellegaard Systems, Faaborg, Denmark) which was used to match body weight with ID and calculate dosing volume.

Animal Housing

Animals were housed in a controlled environment (temperature 22±1°C, relative humidity 50±10%), and under a 12:12 hours light:dark cycle. During the acclimatization and diet induction period, the mice were group housed with ten animals per cage in custom-made cabinets, while animals were single-housed during post-operative recovery and throughout the study treatment period. Animals always had access to bedding material, shelters, and chewing sticks. During the diet-induction phase and the study period, animals had access to an AMLN diet and tap water.

Pre-biopsy procedure and randomization

A pre-biopsy was performed to confirm NASH and to include NASH affected mice only. This took place 3 weeks prior to the start of the study. Mice were anesthetized with

isoflurane (2-3%) in oxygen. A small abdominal incision was made in the midline and a cone shaped wedge of liver tissue was extracted from the left lateral lobe of the liver. The tissue was fixated in 10% neutral buffered formalin (4% formaldehyde) for histopathological analyses. The cut surface of the liver was electrically coagulated, the liver was returned to the abdominal cavity, and wound sutured and closed. Mice were given carprofen (5mg/mL – 0.01mL/10g) subcutaneously on the day of operation and on post-operation day 1 and day 2. Animals were continuously evaluated for general health and body weight. The pre-biopsy was analyzed to evaluate liver steatosis score and fibrosis stage for study inclusion based on previously outlined methods (Kleiner et al.).⁴² Additionally, liver Collagen 1a1 (Col1a1) was quantified via morphometry and used to stratify NASH-affected animals into study groups.

Tolerance Tests

On the 7th and 10th week of treatment respectively, an oral glucose tolerance test (OGTT) and intraperitoneal insulin tolerance test (IPITT) were performed. The tests were performed after a 6 hour fast. For the IPITT, .5 U/kg of rapid acting insulin (Novorapid) was administered and for the OGTT 2g/kg of glucose PO. Blood was collected sixty minutes before and up to one hundred and eighty minutes after administration of glucose or insulin. Upon collection, blood was suspended into a glucose/lactate solution buffer and blood glucose was measured using a BIOSEN c-Line glucose meter. After the last blood sample was taken, the animals returned to a normal feeding schedule, and were dosed with compounds just after the 60 minute blood sample.

Blood Sampling and Sample Collection

Tail blood was drawn through the capillary of a Microvette/Vacurette and mixed with anticoagulant via inversion. Blood was placed in a 4 degree Celsius refrigerator until centrifugation and centrifuged at 3000xg for 10 minutes at 4 degrees Celsius. Plasma supernatants were transferred to new tubes and immediately frozen and stored at -80 degrees Celsius.

Biochemical Measurements

Plasma alanine transaminase (ALT) from Roche Diagnostics, aspartate transaminase (AST) from Roche Diagnostics, triglycerides (TG) from Roche Diagnostics, total cholesterol (TC) from Roche Diagnostics, creatinine from Roche Diagnostics, and urea from Roche Diagnostics, were measured via commercial kits using the Cobas c 501 autoanalyzer. The manufacturer's instructions were followed on all kits. The Meso Scale Diagnostics platform was used to measure mouse insulin in single determinations. Liver hydroxyproline, a protein marker for liver fibrosis was quantified. First, liver samples were homogenized and hydrolyzed in 6M HCl in order to degrade collagen. Then the samples were centrifuged and hydroxyproline levels were measured in the supernatant using Quickzyme Biosciences colorimetric assay. All biochemical measurements were performed during the last (12th) week of treatment. For liver triglycerides (TG) and total cholesterol (TC) quantification, TG and TC were extracted from homogenized liver samples using 5% NP-40 and heating twice to 90 degrees

Celsius. The samples were centrifuged and commercial kits (Roche Diagnostics) were used to measure TG and TC content in the supernatant using the Cobas c 501 autoanalyzer using the manufacturer's instructions.

Termination

Mice were terminated after 12 weeks of treatment in a non-fasting state. The mice were put under anesthesia using isoflurane, the abdominal cavity was opened, and cardiac blood was drawn. Blood was processed and stored similarly to sample collection methods. Upon necropsy, the whole liver was collected, weighed, and sampled for histological and biochemical analyses.

Liver Sampling

Mice livers were biopsied, fixated in formalin, and embedded in paraffin. Liver biopsies for liver triglycerides and cholesterol were dissected from the medial lobe and frozen in liquid nitrogen. Liver biopsies for hydroxyproline measurement were dissected from the caudal lobe and frozen in liquid nitrogen. A liver sample for RNA isolation and gene expression was dissected from the left lateral lobe and frozen in liquid nitrogen.

Histological Tissue Preparation and IHC Staining

Liver samples fixated in formalin and embedded in paraffin were cut into 3 μ M thin sections on a Microm HM340E microtome. Slides were deparaffinated in xylene and rehydrated in a series of graded ethanol prior to staining. Slides were then stained with

HE and Sirius Red. According to the validated scoring system for NASH⁴², a histopathology specialist scored the samples. Immunohistochemistry was used to further detect a protein marker of fibrosis, Colla1 (antibody provided by Southern Biotech). Furthermore, fibrogenesis and hepatic stellate cell activation was assessed via alpha-smooth muscle actin (antibody provided by Abcam), and inflammation-macrophage activation was assessed via Galectin-3 (antibody provided by Biolegend) immunohistochemistry.

Quantitative Assessment of Immunoreactivity and Steatosis

Immunoreactivity and steatosis were quantitatively assessed by calculation of area fraction. Visiomorph software was used to analyze the virtual slides. First, the crude detection of tissue was recorded at a low magnification (1x objective). Second, detection of IHC positive staining or steatosis and tissue staining was recorded. The areas were quantitatively assessed by calculation of area fraction using the following equations:

IHC Positive staining:

$$AF_{IHC-pos.} = \text{Area}_{IHC-pos} / (\text{Area}_{fat} + \text{Area}_{tissue} + \text{Area}_{IHC-pos})$$

Steatosis:

$$\text{Area fraction}_{Steatosis} = \text{Area}_{Steatosis} / (\text{Area}_{Tissue} + \text{Area}_{Steatosis})$$

EchoMRI Body Composition

An EchoMRI 3-1 body composition analyzer (EcoMRI, US) was used to assess the body composition of the mice. Mice were placed inside the MRI scanner for 80 seconds, and the analyzer expressed body composition as fat mass, fat free mas (lean mass), and water.

Gene Expression*

A Trizol/chloroform extraction process and RNeasy spin columns (RNeasy Plus Universal Mini Kit, Qiagen, Valencia, Canada) was used to isolate RNA from liver and adipose samples. The RNA was reverse transcribed to cDNA using SSIV VIIo Mastermix with EzDNase (Life Technologies, New York, USA). The QuantStudio 3 real-time PCR system with Taqman custom array plates was used to analyze gene expression (see Table 1 and 2 for primers)

Table 1. Taqman primers used for liver gene expression analysis

Assay ID	Gene	
	Symbol(s)	Gene name(s)

Hs9999901_s1	18s rRNA	-
Mm02619580_g1	Actb	actin, beta
Mm04260181_s1	Rnr2	16S ribosomal RNA
Mm01247058_m1	Pck1	phosphoenolpyruvate carboxykinase 1, cytosolic
Mm00839363_m1	G6pc	glucose-6-phosphatase, catalytic
Mm00662319_m1	Fasn	fatty acid synthase
Mm02342723_m1	Mlxip1	MLX interacting protein-like
Mm01304257_m1	Acaca	acetyl-Coenzyme A carboxylase alpha
Mm00550338_m1	Srebf1	sterol regulatory element binding transcription factor 1
Mm01306292_m1	Srebf2	sterol regulatory element binding factor 2
Mm00440940_m1	Pparg	peroxisome proliferator activated receptor gamma
Mm00445878_m1	Fabp4	fatty acid binding protein 4, adipocyte
Mm01278327_m1	Irs1	insulin receptor substrate 1
Mm01231183_m1	Cpt1a	carnitine palmitoyltransferase 1a, liver
Mm00802529_m1	Adgre1	adhesion G protein-coupled receptor E1 (also known as F4/80)
Mm00444293_m1	Acadvl	acyl-Coenzyme A dehydrogenase, very long chain
Mm01246834_m1	Acox1	acyl-Coenzyme A oxidase 1, palmitoyl
Mm01323360_g1	Acadm	acyl-Coenzyme A dehydrogenase, medium chain
Mm01166879_m1	Pdk4	pyruvate dehydrogenase kinase, isoenzyme 4
Mm00443258_m1	Tnf	tumor necrosis factor
Mm00441259_g1	Ccl3	chemokine (C-C motif) ligand 3
Mm00445235_m1	Cxcl10	chemokine (C-X-C motif) ligand 10
Mm00434228_m1	Il1b	interleukin 1 beta
Mm00803184_m1	Ppard	peroxisome proliferator activator receptor delta
Mm00441242_m1	Ccl2	chemokine (C-C motif) ligand 2
Mm00434455_m1	Itgam	integrin alpha M (also known as CD11B)
Mm00498701_m1	Itgax	integrin alpha X (also known as CD11C)
Mm00440939_m1	Ppara	peroxisome proliferator activated receptor alpha
Mm00801477_m1	Chil1	chitinase-like 1

Mm01178820_m1	Tgfb1	transforming growth factor, beta 1
Mm00440181_m1	Lepr	leptin receptor
Mm01291334_mH	Adipor1	adiponectin receptor 1
Mm01184032_m1	Adipor2	adiponectin receptor 2
Mm01211875_m1	Insr	insulin receptor

Table 2. Taqman primers used for adipose tissue gene expression analysis

Assay ID	Gene Symbol(s)	Gene Name(s)
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Mm00437762_m1	B2m	beta-2 microglobulin
Mm04260181_s1	Rnr2	16S ribosomal RNA
Mm01247058_m1	Pck1	phosphoenolpyruvate carboxykinase 1, cytosolic
Mm00662319_m1	Fasn	fatty acid synthase
Mm02342723_m1	Mlxip1	MLX interacting protein-like
Mm01304257_m1	Acaca	acetyl-Coenzyme A carboxylase alpha
Mm00550338_m1	Srebf1	sterol regulatory element binding transcription factor 1
Mm01306292_m1	Srebf2	sterol regulatory element binding factor 2
Mm00445878_m1	Fabp4	fatty acid binding protein 4, adipocyte
Mm01211875_m1	Insr	insulin receptor
Mm01278327_m1	Irs1	insulin receptor substrate 1
Mm00802529_m1	Adgre1	adhesion G protein-coupled receptor E1 (also known as F4/80)
Mm01246834_m1	Acox1	acyl-Coenzyme A oxidase 1, palmitoyl
Mm00432554_m1	Cidea	cell death-inducing DNA fragmentation factor, alpha subunit-like effector A
Mm01244861_m1	Ucp1	uncoupling protein 1 (mitochondrial, proton carrier)
Mm00468164_m1	Elov13	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3
Mm00851223_s1	Elov16	ELOVL family member 6, elongation of long chain fatty acids (yeast)
Mm00712556_m1	Prdm16	PR domain containing 16
Mm01208835_m1	Ppargc1a	peroxisome proliferative activated receptor, gamma, coactivator 1 alpha
Mm00495359_m1	Lipe	lipase, hormone sensitive
Mm00503040_m1	Pnpla2	patatin-like phospholipase domain containing 2
Mm00447485_m1	Tfam	transcription factor A, mitochondrial
Mm00468869_m1	Hif1a	hypoxia inducible factor 1, alpha subunit
Mm00434764_m1	Lpl	lipoprotein lipase
Mm00443258_m1	Tnf	tumor necrosis factor
Mm00445235_m1	Cxcl10	chemokine (C-X-C motif) ligand 10
Mm00441242_m1	Ccl2	chemokine (C-C motif) ligand 2

Mm00434455_m1	Itgam	integrin alpha M (also known as CD11B)
Mm00440940_m1	Pparg	peroxisome proliferator activated receptor gamma
Mm00440939_m1	Ppara	peroxisome proliferator activated receptor alpha
Mm00803184_m1	Ppard	peroxisome proliferator activator receptor delta
Mm00456425_m1	Adipoq	adiponectin, C1Q and collagen domain containing
Mm00434759_m1	Lep	leptin
Mm00440181_m1	Lepr	leptin receptor
Mm01291334_mH	Adipor1	adiponectin receptor 1
Mm01184032_m1	Adipor2	adiponectin receptor 2

Metabolomics-Lipidomics

Samples were weighed and soaked in a 1:1 dichloromethane:methanol mixture overnight at 4 degrees Celsius. The supernatants were extracted using a modified Bligh-Dyer extraction using methanol, water, and dichloromethane in the presence of deuterated internal standards. Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy was performed by Metabolon Inc (Morrisville, NC) using libraries based on the authenticated standards. Peaks were quantified using area under the curve. Individual lipid species were quantified by taking the ratio of signal intensity of each target compound to that of its assigned standard, then multiplying by the concentration of the internal standard added to the sample. Lipid class concentrations were calculated by taking the sum of all molecular species within a class and fatty acid compositions were calculated by taking the proportion of individual fatty acids within each class.

Statistics

A one-way ANOVA was performed for single timepoint continuous data and by $p < 0.05$ Fisher's least significant difference (LSD) was used to compare treatments between the NASH and vehicle group. For multiple timepoint continuous data, a two-way ANOVA with the factors time and treatment was performed and by $p < 0.05$ for the interaction of time with treatment, post-hoc LSD comparing the different treatments with NASH + vehicle at each individual timepoint was performed. Data from categorical endpoints (histopathological scoring values) were partitioned into a 2x2 contingency table consisting of mice with lower vs same/higher scores. These were analyzed using Fisher's exact test with the hypothesis that CHS-131 treatment will have positive effects and lower the score on liver histology. Graphpad Prism 8 was used to perform the analyses. Relative expression (mean \pm SEM) compared to NASH mice treated with vehicle was reported. A one way ANOVA with post-hoc LSD test for parametric values or Kruskal-Wallis test followed by Dunn's test for non-parametric values was performed. For metabolomic analysis, values were normalized as raw area counts, while for lipidomic analysis, values were normalized as concentrations (nmol/mg). Each value was rescaled to a set median of 1. Missing values were replaced with the minimum value. A sparse partial least squares discriminant analysis was performed to visualize data. A pathway analysis with metabolites identified with HMDB ID with an enrichment analysis following a global t-test, as well as a topology analysis following the relative-

betweenness Centrality was used. The mus musculus pathway library of KEGG (October 2019) was used. The Welch's two-sample t-test was used to compare individual lipid or metabolic species or groups. The MetaboAnalystR was used for the analysis.

**Aditya Joshi contributed to gene expression experiments. All other experiments were conducted by fellow lab members. The experiments in this thesis have been adapted from:
Perakakis N, Joshi A, Peradze N, et al. The selective PPAR_γ modulator CHS-131 improves liver histology in a diet-induced and biopsy-confirmed mouse model of NASH. Submitted for Publication.

RESULTS

CHS-131 has no effect on body weight, body composition and energy intake and significantly improves insulin sensitivity

Mice that were fed an AMLN diet for 36 weeks had a higher body weight and total energy intake (measured on Day 11) compared to the mice fed a chow diet (Figure 1b-c). There was not a significant change in body weight, energy intake, fat mass, lean mass, and water mass in response to treatment with CHS-131 at both doses compared to the NASH vehicle treated group (Figure 1b-d). NASH induced mice were found to have elevated levels of fasting blood glucose compared to mice fed a chow diet (Figure 1e-f). NASH induced mice were found to have much greater fasting insulin levels compared to mice fed a chow diet (Figure 1e-f). NASH induced mice were mildly insulin resistant but not clearly diabetic based on findings from the OGTT and ipITT. The tests found that NASH mice had similar glucose levels during the OGTT and slightly elevated glucose levels during the ipITT compared to chow mice (Figure 1e-f), indicating insulin resistance. Treatment with CHS-131 at both low and high doses resulted in lower fasting insulin levels and a lower area under the curve (AUC) in ipITT (Figure 1f) without having an impact on glucose levels during fasting or OGTT (Figure 1e), indicating an improved insulin sensitivity.

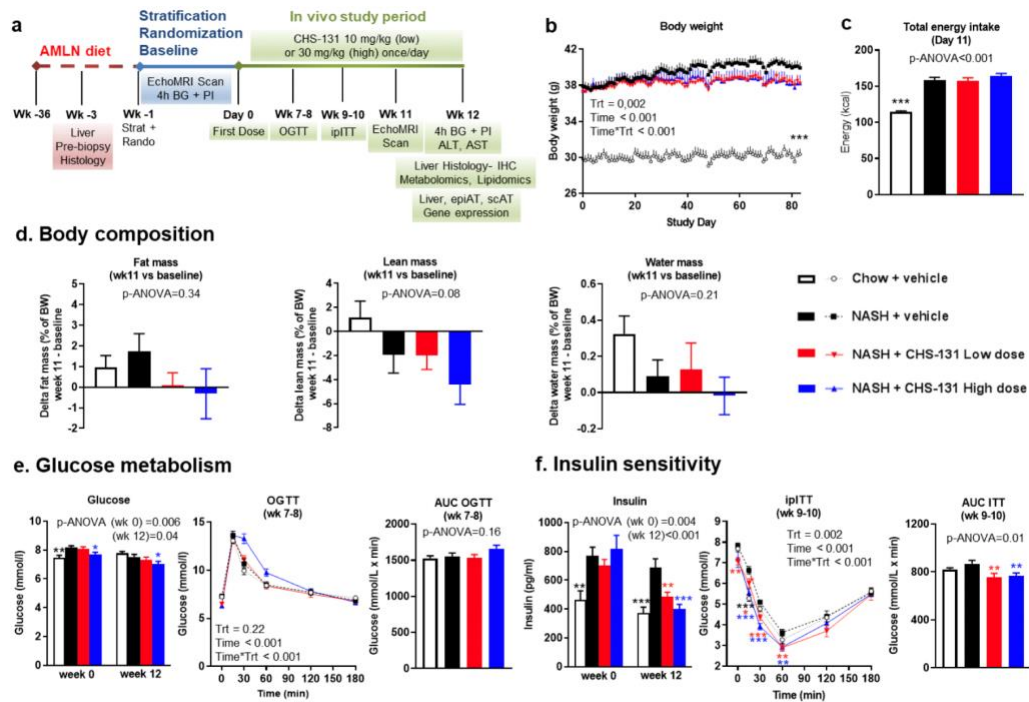


Figure 1: CHS-131 improves insulin sensitivity without affecting body weight, body composition, energy intake and glucose levels (prepared by Dr. Nikolaos Perakakis)⁴¹

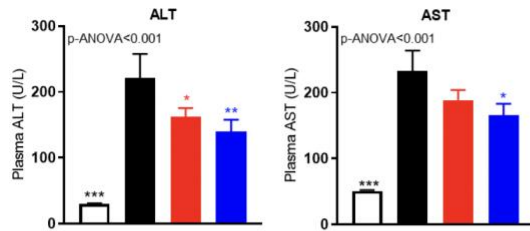
Perakakis)⁴¹

a. Schematic representation of study design, b. body weight over the course of 12 weeks of treatment, c. total energy intake at 11 days of treatment, d. changes in body composition as a percentage of body weight from the start of treatment up to week 11, e. Glucose levels after a four hour fast at week 0 and week 12 of treatment and during an OGTT during weeks 7-8. AUC of OGTT is also displayed, f. Insulin levels after a four hour fast at week 0 and week 12 of treatment and during an ipITT during weeks 9-10. AUC of ipITT is also displayed. A one-way ANOVA was performed for all single-time points and two-way ANOVA was performed for parameters with multiple time points.

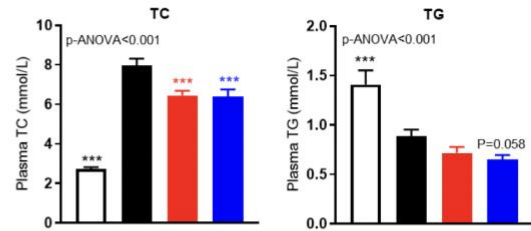
CHS-131 improves plasma levels of liver transaminases, total cholesterol, and adiponectin

Diet induced NASH mice had much higher plasma levels of ALT, AST and total cholesterol compared to the mice fed a chow diet (Figure 2a-b). Treatment with the high dose of CHS-131 resulted in 37% lower levels of ALT ($p < 0.01$), 29% of AST ($p < 0.01$), and 20% of total cholesterol ($p < 0.001$) (Figures 2a-b). While diet induced NASH mice had lower plasma levels of triglycerides, treatment with CHS-131 still resulted in a decrease in plasma triglycerides ($p = 0.058$) (Figure 1b). The decreased triglyceride level in NASH mice could possibly be explained by an increase in uptake and accumulation of triglycerides by the liver. Plasma adiponectin levels were significantly increased with treatment of CHS-131 (114% with low dose, 137% with high dose) ($p < 0.001$) and both doses did not influence the elevated leptin levels of NASH induced mice (Figure 1c). Urea levels were used as a marker for hydration, and were not affected by treatment with CHS-131 (Figure 2d).

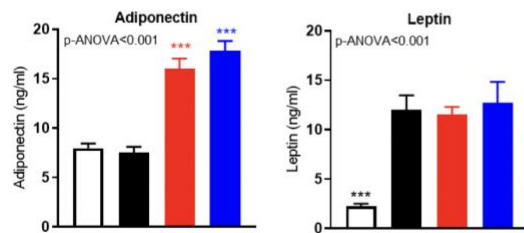
a. Liver transaminases



b. Lipids



c. Adipokines



d. Marker of hydration

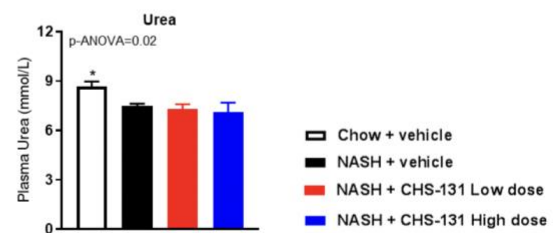


Figure 2: CHS-131 reduces transaminases and total cholesterol and increases adiponectin levels (Prepared by Dr. Nikolaos Perakakis)⁴¹

a. Plasma levels of liver transaminases (ALT and AST), b. Plasma levels of lipids (total cholesterol and triglycerides), c. Plasma levels of adipokines (adiponectin and leptin), and d. Plasma levels of urea (a marker of hydration) after 12 weeks of treatment.

CHS-131 improves NAFLD activity score mainly by affecting lobular inflammation and hepatocellular ballooning

After 33 weeks on the AMLN or normal chow diet, a liver biopsy was performed on the mice before they were randomized into the four treatment groups. The biopsy found that diet induced NASH mice (on the AMLN diet) had a relatively high NAFLD activity score (NAS) of approximately 5 points compared to the score mice given a chow diet received, of approximately 0 points (11 out of 12 mice scored zero). Furthermore, upon treatment with a high dose of CHS-131, diet induced NASH mice experienced a significant reduction in NAS score (from baseline, delta change in NAS = -0.77 points; 7 mice with a lower score, 5 mice had the same score, and 1 mouse had a higher score after treatment) compared to diet induced NASH mice treated with the vehicle (from baseline delta change in NAS = $+0.15$; 2 mice with lower scores, 7 mice with the same score, and 4 with a higher NAS score after treatment) (Figure 3a-b). The change in the NAFLD activity score was due to a result of improvements in lobular inflammation and hepatocellular ballooning (Figure 3e-f). Steatosis score and fibrosis stages were also measured. Markers of steatosis (liver weight, lipid content, total triglycerides, total cholesterol) along with steatosis scores were not significantly affected by the treatment (Figure 6a-b, Figure 3d). While the fibrosis stage did not show a significant improvement (figure 3c), there was a significant reduction in hydroxyproline, a marker of liver fibrosis (28% reduction ($p < 0.01$), and a nonsignificant reduction ($p = 0.05 - 0.1$) in Colla1 (24%

reduction), a-SMA (21% reduction), and Galectin-3 (18% reduction), also markers of liver fibrosis (Figure 6c).

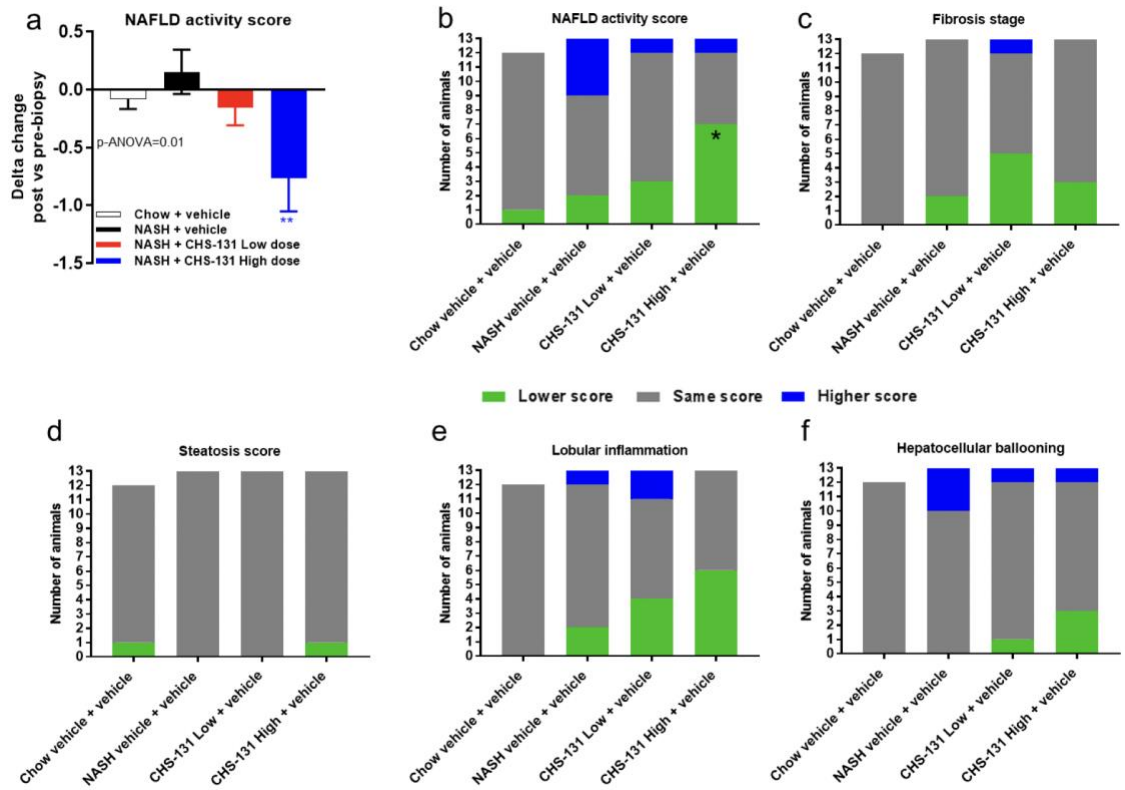


Figure 3: CHS-131 reduces NAFLD activity score mainly by improving lobular inflammation and hepatocellular ballooning (Prepared by Dr. Nikolaos Perakakis)⁴¹

Comparisons of the histologic outcomes in post vs. pre-treatment biopsy in a. Delta change of the NAFLD activity score; Number of animals with lower, same or higher scores in b. NAFLD activity score, c. fibrosis stage, d. steatosis, e. lobular inflammation, f. hepatocellular ballooning.

CHS-131 reverses the changes in lipid and metabolite composition in the liver of NASH induced mice

We conducted a lipidomic analysis of liver tissues and identified 987 different lipid species present in the mice. Using a sparse partial squares discriminant analysis (sPLS-DA) with two main components consisting of 10 lipid species each, we separated the mice based on their different lipid profiles. Diet induced NASH mice with CHS-131 treatment (blue dots in upper left), diet induced NASH mice with vehicle treatment (green dots in lower left), and mice fed a normal chow diet (red dots in middle right) converged into three different clusters (Figure 4a). Component 1 is a vertical separation of chow mice from diet induced NASH mice (consisting of both vehicle and CHS-131 treated) and consisted of phosphatidylethanolamines (PEs) and phosphatidylcholines (PCs). Component 2 is a horizontal separation of the vehicle and CHS-131 treated diet induced NASH mice and consisted mainly of long-chain triacylglycerols (TAGs) and diacylglycerols (DAGs) as well as lesser amounts of PCs and PEs (Figure 4a). Although there was not a significant change in total TGs and TCs between vehicle and CHS-131 treated mice, (Figure 6b), CHS-131 treated mice have lower concentrations of many TAGs (237 out of 518), DAGs (24 out of 54), and cholesterol ester (9 out of 26) species and higher concentrations of many PC esters, PE esters, and PE plasmalogens (Figure 4b). Of the lower concentrations of TAGs and DAGs seen with CHS-131 treatment, the majority of cases were seen in species with a large number of carbons (very long-chain

lipids) in contrast to species with a smaller number of carbons, suggesting that there was an increased breakdown of more complex lipids.

A metabolomic analysis identified 592 known metabolites, and a pathway analysis found that the greatest differences between the three groups was observed in amino acid synthesis and metabolism (Figure 4d). We found that diet induced NASH mice treated with a vehicle had approximately 50% lower concentration of 15 amino acids compared to mice fed a chow diet. While NASH-mice treated with CHS-131 also exhibited lower amino acid levels compared to chow mice, they had significantly increased levels compared to the NASH-mice treated with vehicle (Figure 4e).

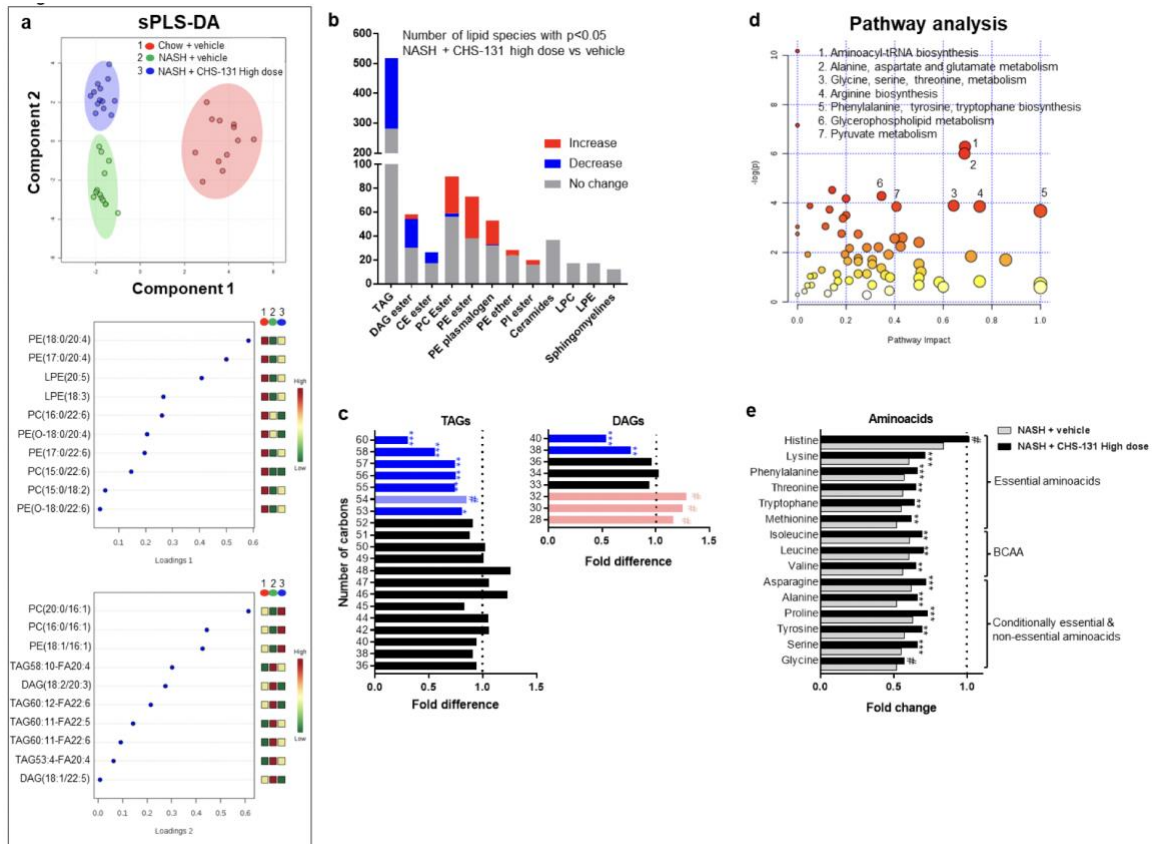


Figure 4: Effects of CHS-131 treatment on hepatic lipidomic and metabolomic profile (Prepared by Dr. Nikolaos Perakakis)⁴¹

a. Score-plot and loadings of the two main components in sPLS-DA analysis, b. Number of lipid species with significant increase, decrease or no change in their hepatic concentrations after treatment with high dose CHS-131 compared to vehicle. c, Mean fold changes in hepatic concentrations of triglycerides (TAGs) and diacylglycerides (DAGs) according to their number of carbons after treatment with a high dose of CHS-131 (columns) compared to the vehicle (dotted line) in mice with NASH. d. Score-plot of

pathway analysis indicating the pathway impact (x axis) in relation to p-value (y axis) in metabolites in CHS-131 vs. vehicle treated mice with NASH. e. Mean fold changes in amino acid concentrations in CHS-131 treated (black columns) or vehicle treated (gray columns) NASH induced mice vs non-NASH chow-fed mice (dotted line).

CHS-131 increases the expression of genes related to mitochondrial function and decreases the expression of genes related to inflammation in adipose tissue

We investigated whether CHS-131 affects the expression of key genes involved in cellular metabolic processes in both the liver and adipose tissues. Mice that were treated with CHS-131 showed a profound increase in gene expression of key regulators of mitochondrial function. Specifically, UCP1 and Elovl3, which are genes involved in mitochondrial browning and thermogenesis were increased up to ~33 fold (Figure 5a) in epididymal adipose tissue and up to ~80 fold in subcutaneous adipose tissue in mice treated with CHS-131 compared to mice treated with a vehicle. This was true for both low and high doses. Furthermore, in epididymal adipose tissue, we observed a two-fold increase in the gene expression of PGC-1 α , a major regulator of mitochondrial biogenesis as well as in Acox1, a gene that encodes the first enzyme in the fatty acid beta oxidation pathway (Figure 5b). Genes that encode proteins that stimulate the synthesis of long chain saturated fatty acid and triglycerides (Fasn, Mlxipl) had reduced expression in both epididymal and subcutaneous adipose tissue (Figure 5d). These changes point towards an increase in browning and fatty acid oxidation as well as a decrease in long chain fatty acid synthesis and triglyceride synthesis. Furthermore, the expression of CIDEA was also increased in both epididymal and subcutaneous adipose tissue, suggesting a reduction in lipolysis and enhanced lipid accumulation in adipose tissues.

Regarding expression of PPARs, there were no changes in the expression of PPAR- γ in epididymal adipose, subcutaneous adipose, or in the liver (Figure 5c, Figure 7). The

expression of PPAR- δ was reduced in both epididymal and subcutaneous adipose tissue whereas the expression of PPAR- α was increased in epididymal adipose tissue but not in the liver (where it is mainly expressed) (Figure 5c, Figure 7). Lepr in epididymal adipose tissue as well as Insr and Irs1 in subcutaneous adipose tissue were reduced by ~50% (Figure 5e). Lastly, the expression of the macrophage marker F4/80 and of the proinflammatory cytokine Ccl2, which recruits monocytes, T cells and dendritic cells, were reduced with low doses of CHS-131 in epididymal adipose tissue (not in subcutaneous adipose tissue or in the liver) (Figure 5f, Figure 7).

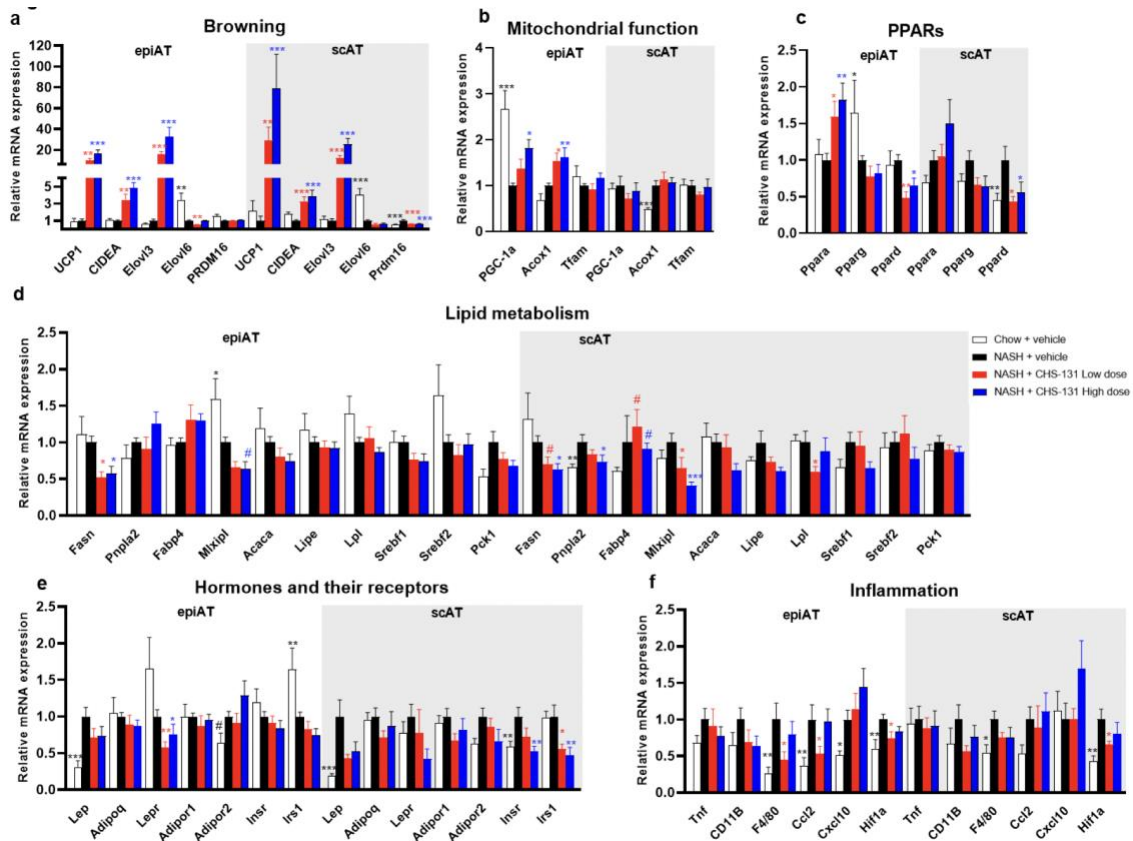


Figure 5: Effects of CHS-131 treatment on gene expression profile in epididymal adipose tissue and subcutaneous adipose tissue (Prepared by Dr. Nikolaos Perakakis)⁴¹

Relative mRNA expression levels of genes involved in a. browning, b. mitochondrial function, c. PPARs, d. lipid metabolism, e. hormones and their receptors, f. inflammation.

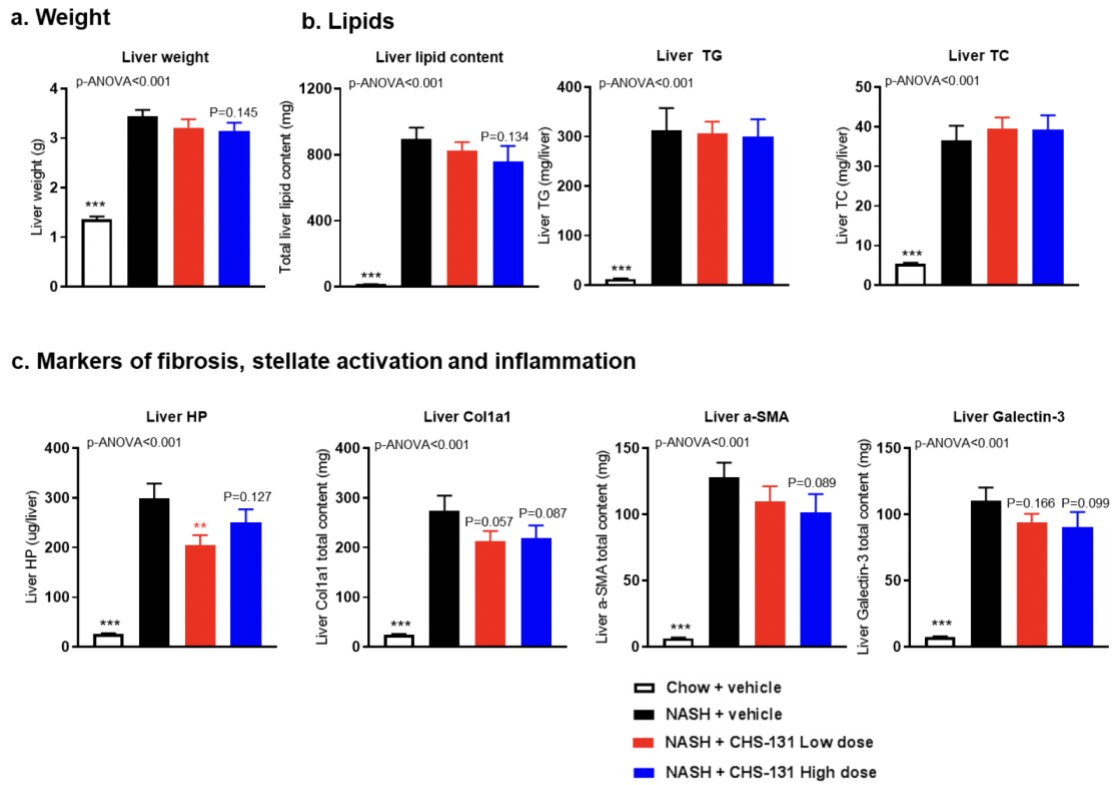


Figure 6: Effect of treatment with CHS-131 on liver parameters (Prepared by Dr. Nikolaos Perakakis)⁴¹

a. Liver weight, b. Liver lipids expressed as liver lipid content, liver triglycerides, liver total cholesterol, c. Markers of hepatic fibrosis, stellate activation, and inflammation (specific markers used are hydroxyproline, Col1a1, a-SMA and Galectin-3).

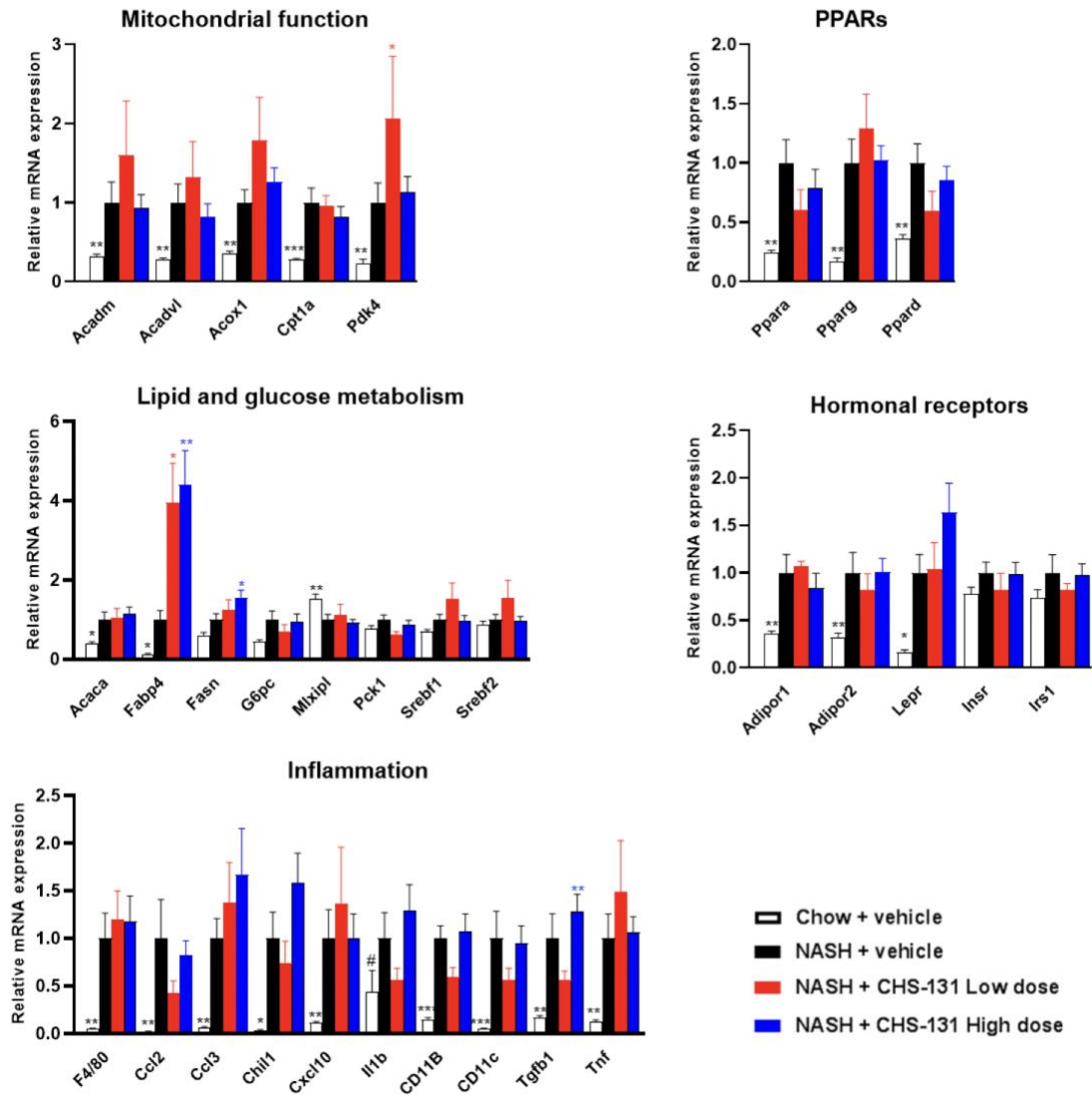


Figure 7: Effects of CHS-131 treatment on gene expression profile in the liver

(Prepared by Dr. Nikolaos Perakakis)⁴¹

Relative mRNA expression levels of genes involved in mitochondrial function, PPARs, lipid and glucose metabolism, hormonal receptors, and inflammation.

DISCUSSION

The use of CHS-131 on a diet induced and biopsy confirmed mouse model of NASH was demonstrated to improve liver histology by several parameters. We used a previously established mouse model of NASH induced via a diet high in trans-fat, fructose, and cholesterol, that resembled the metabolic human phenotype through elevated plasma liver enzymes as well as clinical manifestations of NASH (liver inflammation, steatosis, fibrosis, and ballooning).⁴³ We saw that CHS-131 reduced hepatic lobular inflammation and hepatocyte ballooning in addition to shifting hepatic TAG and DAG composition from very-long chained to long and medium chained TAGs and DAGs. We also saw an increase in hepatic amino acid levels. Based on the findings from our study, these beneficial effects were likely a result of changes in adipose tissue function, increased adiponectin levels, and a systemic improvement in insulin sensitivity.

The NAS score is a scoring system of histological features of NAFLD/NASH that quantifies levels of steatosis, inflammation, ballooning.⁴² In mice treated with a high dose of CHS-131 (equivalent to 10mg of rosiglitazone ⁴⁰) compared to placebo, we saw a lower NAS score due to improvements in lobular inflammation and ballooning. There was no change in steatosis score, but we saw a significant shift in intrahepatic lipid composition of TAGs and DAGs to shorter chain fatty acids. It has been shown that a higher ratio of shorter chain fatty acids are associated with suppressed autophagy and reduced hepatic lipotoxicity.⁴⁴ Furthermore, we saw an upregulation of the *Fabp4* and

Fasn genes in the liver. *Fabp4* is a carrier protein for fatty acids and *Fasn* catalyzes fatty acid synthesis, indicating that the shift in lipid composition could be due to an increase in hepatic fatty acid uptake, transport, and synthesis. We did not see any changes in genes related to fatty acid oxidation in the liver. However, in adipose tissue, we saw a reduction in the expression of *Fasn* and *Mlxipl* (involved in fatty acid/triglyceride synthesis) and an increase in the expression of *PGC-1a* and *Acox1* (stimulate fatty acid oxidation). This leads us to conclude that the shift in hepatic lipid composition due to CHS-131 treatment was most likely due to reduced fatty acid synthesis and increased beta oxidation in adipose tissues.

Although we saw a reduction in histological inflammation with CHS-131 treatment, we did not see any significant changes in the expression of genes regulating liver inflammatory processes (TNF, CD11B, Cxcl10). However, we did see a reduction (50%) in the inflammatory markers F4/80 and CCL2 in epididymal adipose tissue. It has been demonstrated previously that CHS-131 downregulates proinflammatory molecules in both adipose tissue and the brain, but not the liver.⁴⁰ This supports our findings and suggests that the reduction in hepatic inflammation is due to the downregulation of inflammation in adipose tissue.

Our study also saw a significant reduction in various markers of liver fibrosis (Hydroxyproline, Col1a1, α -SMA and Gal-3), despite the nonsignificant changes in NAS fibrosis scores. Based on previous studies examining the mechanistic effects of full

PPAR- γ agonists, CHS-131 reduces levels of hepatic stellate cells, either through increased apoptosis, lower activation, reduced proliferation.⁴⁵

Moreover, treatment with CHS-131 increased circulating levels of adiponectin by approximately 2.4-fold. Adiponectin has been shown previously to promote hepatic fat oxidation, glucose uptake, and insulin sensitivity as well as to reduce inflammation.^{30,31,26} Treatment with TZDs has shown similar increases in adiponectin levels in humans⁴⁶ as a result of PPAR- γ activation, and increased levels of adiponectin via CHS-131 are crucial to its metabolic and hepatic effects.

Our study also showed that the AMLN diet unexpectedly reduced hepatic concentrations of amino acids. Although previous studies have shown that the concentration of hepatic branched chain amino acids (BCAAs) increase as NAFLD progresses to NASH, during liver cirrhosis the decreased capacity for urea synthesis results in a buildup of ammonia, and BCAAs are depleted as they are used for ammonia detoxification.^{47,48} In our study, CHS-131 treatment resulted in an increase in amino acid concentrations, which could possibly contribute to its beneficial hepatic effects. BCAA supplementation has been shown to improve hepatic steatosis, liver injury, glucose tolerance, and insulin sensitivity, indicating possible mechanisms for the effects of CHS-131 on amino acid levels.^{49,50,51} As previously discussed, TZDs present a variety of negative side effects that limit their use as treatments for NAFLD/NASH. Particularly, an increase in body weight due to water retention which can lead to cardiac failure, and an increased fracture risk have

made it less appealing for use by clinicians.³⁹ Moreover, in patients that are co-administered insulin, water retention has been shown to be greater.⁵² Our study found that CHS-131 did not increase body weight, or lead to increased water retention in mice, in agreement with previous safety trials done on humans.⁵³ Overall, these results show that CHS-131 could be an effective treatment for the metabolic and hepatic complications in NASH that avoids the negative side effects of weight gain and water retention. Further studies are necessary however, to evaluate the efficacy and safety of CHS-131 in humans with NAFLD/NASH.

List of Abbreviated Journal Titles:

Aliment Pharmacol Ther	Alimentary Pharmacology & Therapeutics
Ann Intern Med	Annals Internal Medicine
Cancer Prev Res	Cancer Prevention Research
Cell Metab	Cell Metabolism
Clin Exp Med	Clinical and Experimental Medicine
Clin Gastroenterol Hepatol	Clinical Gastroenterology and Hepatology
Clin Liver Dis	Clinical Liver Disease
Clin Liver Dis	Clinical Liver Disease
Diabetes Metab	Diabetes Metabolism
Drug Saf	Drug Safety
Eur J Gastroenterol Hepatol	European Journal of Gastroenterology & Hepatology
FASEB J	The Federation of American Societies for Experimental Biology Journal
Front Pharmacol	Frontiers of Pharmacology
J Clin Endocrinol Metab	Journal of Clinical Endocrinology and Metabolism
J Clin Endocrinol Metab	Journal of Clinical Endocrinology and Metabolism
J Clin Invest.	Journal of Clinical Investigation
J Mol Biol	Journal of Molecular Biology
JAMA - J Am Med Assoc.	JAMA – Journal of the American Medical Association

Liver Int.	Liver International
Mol Med	Molecular Medicine
N Engl J Med	New England Journal of Medicine
Nat Med	Nature Medicine
Nat Rev Gastroenterol Hepatol	Nature Reviews Gastroenterology & Hepatology
Nutr Metab	Nutrition Metabolism
PLoS One.	Public Library of Science One
PPAR Res	PPAR Research
Sci Rep	Scientific Reports
Trends Pharmacol Sci	Trends Pharmacological Sciences
World J Hepatol	World Journal of Hepatology

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CURRICULUM VITAE

