REV-ERB Agonism Improves Liver Pathology in a Mouse Model of NASH

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Abstract

Non-alcoholic fatty liver disease (NAFLD) affects a significant number of people worldwide and currently there are no pharmacological treatments. NAFLD often presents with obesity, insulin resistance, and in some cases cardiovascular diseases. There is a clear need for treatment options to alleviate this disease since it often progresses to much more the much more severe non-alcoholic steatohepatitis (NASH). The REV-ERB nuclear receptor is a transcriptional repressor that regulates physiological processes involved in the development of NAFLD including lipogenesis and inflammation. We hypothesized that pharmacologically activating REV-ERB would suppress the progression of fatty liver in a mouse model of NASH. Using REV-ERB agonist SR9009 in a mouse NASH model, we demonstrate the beneficial effects of REV-ERB activation that led to an overall improvement of hepatic health by suppressing hepatic lipogenesis and inflammation.

Introduction

Among the metabolic disorders, non-alcoholic fatty liver disease (NAFLD) is considered a hepatic manifestation of metabolic syndrome (MetS) and it is one of the prominent health challenges of the twenty-first century as NAFLD is the most prevalent liver disease worldwide affecting 25-30% of the general population and its prevalence could reach 70-90% in specific populations with comorbidities such as morbid obesity or type 2 diabetes mellitus. NAFLD can often progress to non-alcoholic steatohepatitis (NASH), which is associated with progressive liver disease [1]. NASH has been mainly associated with higher morbidity and mortality than other diseases in the NAFLD spectrum and, although there are pharmacological therapies under clinical investigation for treatment of NASH [2], no drugs are approved by the Federal Drug Administration (FDA) or the European Medicines Agency (EMA) for the NASH treatment [3].

Nuclear receptors (NRs) are transcription factors generally activated by ligands and involved in diverse biological processes such as cell growth and differentiation, apoptosis, gene expression during tumor formation and metabolism. They bind to specific sequences of DNA allowing them to regulate the expression of adjacent genes. Many diseases including NASH are directly or indirectly related to nuclear receptor signaling and many NRs have become favored targets for drug discovery

[4]. NRs play an important role in liver diseases and they are key modulators in the onset and progression NAFLD, including the peroxisome proliferator-activated receptors (PPAR) α/β/γ; liver X receptors (LXR) α/β; farnesoid X receptors (FXR); constitutive androstane receptor (CAR); and pregnane X receptor (PXR). All of these NRs form obligate heterodimers with retinoid X receptor (RXR) α/β/γ in order to modulate corresponding target genes in the nucleus. [5,6]

REV-ERB nuclear receptors (REV-ERB□ and REV-ERB□) are transcriptional repressors that regulate a variety of physiological processes including lipogenesis, inflammation, circadian regulation, and muscle regeneration and are expressed in all tissues but has significantly higher expression in liver, skeletal muscle, adipose tissue, and brain [7]. Although REV-ERBs play a regulatory role in hepatic metabolism, inflammation and lipogenesis, these NRs have yet to be validated as a potential therapeutic target for liver disease [8–11]. Here, we show that REV-ERB agonist SR9009 treatment in *ob/ob* mice fed a high-fat, high-fructose (NASH) diet has beneficial effects and may provide some translational groundwork for further developing REV-ERB agonists for metabolic diseases, specifically NAFLD.

Materials and Methods

Animals

Animal studies were performed as previously described [12–14]. Briefly, six-week old B6 V-Lep^{ob}/J (*ob/ob*) male mice were purchased from Jackson Labs (Bar Harbor, ME). Upon receipt, mice were housed individually in standard cages with huts and immediately placed on NASH diet (D09100301; Research Diets) [15]. Mice were maintained on this diet throughout the experiment. Mice were handled and weighed weekly while acclimating to the diet. At 12-weeks of age, mice were assigned into weight-matched groups (n = 6) and dosing began. Mice were weighed daily and food-intake was monitored daily. At the termination of the study, mice were euthanized by CO_2 and blood was collected by cardiac puncture for clinical chemistry (Roche COBAS) and ELISA analysis (EMD Millipore). Tissues were collected and flash-frozen in liquid nitrogen for gene expression, or placed in 4% Paraformaldehyde (PFA) in PBS for paraffin-embedding or 10% Neutral-Buffered Formalin

(NBF) for cryo-sectioning. All animal work was performed in accordance with the Institutional Animal Care and Use Committee (IACUC) at Washington University in St. Louis (Protocol #20180062).

Compounds and Dosing

SR9009 was formulated as 100mg/kg at 10mg/ml in 5% DMSO, 15% Cremophore EL (Sigma), 80% PBS as previously described [16]. Both vehicle (5% DMSO, 15% CremophoreEL (Sigma), 80% PBS) and SR9009 were filter sterilized (Millipore Steriflip) prior to dosing. Mice were given once daily i.p. injections within an hour of "lights on" (ZT0-ZT1). Dosing was performed for 30 days by the same researcher.

Gene Expression Analysis

Total RNA was isolated from liver using the trizol (Invitrogen) method [12]. Samples were analyzed by QPCR using Fatty Liver and Fibrosis QPCR array plates (Bio-Rad; 384-well format) and Bio-Rad supplied SYBR reagents (per manufacturer's protocol). Each sample was run in duplicate and analyzed on the PrimePCR software supplied by Bio-Rad. Multiple reference genes were utilized (including Gapdh, ActinB, and Cyclophillin) for analysis [14]. Results were plotted in GraphPad prism software as Gene Regulation using mean +/- SEM.

Tissue Lipid Analysis and Histology

Livers for cryosectioning were incubated in 10% NBF at 4^{o}C for 24-hours, then placed in 30% Sucrose in PBS at 4^{o}C for cry-protection until the livers sank (approximately 2-6 days). Livers were then embedded in OCT freezing media in an ethanol-dry ice bath. Livers were cryo-sectioned at 10 um thickness and floated in 12-well dishes in 1X PBS for Bodipy staining (10 sections per mouse). Sections were washed three times in cold 1X PBS and stained with Bodipy 493/503 per manufacturer's protocol [12,14,17]. Livers that were placed in 4% PFA were paraffin-embedded and sectioned at 10 μm onto slides at the Saint Louis University Histology Core Facility. H&E and Masson's Trichrome staining was performed at the core as a fee-for-service. Ten-sections per mouse

were slide scanned at the core facility and images were analyzed using ImageJ software (staining:total area) and plotted in GraphPad Prism [12,14,17].

Statistics

All data are expressed as mean +/- SEM (n = 4 or greater). All statistical analysis was performed using unpaired Student's t-test with Tukey's post-hoc analysis in GraphPad prism software.

Results and Discussion

Given that REV-ERBs have been demonstrated to play a regulatory role in hepatic lipid metabolism [18] as well as inflammation [16,18,19], we sought to examine the effects of pharmacologically activating REV-ERB in a mouse model of NASH and determine whether REV-ERB may be a therapeutically relevant target. We opted to utilize a diet-induced NASH model using *ob/ob* mice as previously described [14,15] as the time period for development of NAFLD with fibrosis (NASH) is relatively short as compared to other models. During the acclimation and NASH development period, mice were fed a diet that contains Primex as a trans-fat source, fructose, and cholesterol *ad libitum* and monitored for weight gain and food intake. These parameters were also monitored daily throughout the dosing period to validate that any weight-loss was not due to loss of appetite. As shown in Figure 1A, both groups (Vehicle and SR9009) gained weight throughout the experimental period, however the SR9009-treated group gained weight at a consistently slower rate. The slower weight gain was not due to lower food intake in the SR9009-treated mice since this group consistently consumed the same amount of food as the vehicle group (Figure 1B). After 30-days of dosing was completed, mice were euthanized, and we performed a variety of tissue and plasma analyses to determine whether SR9009 treatment had a beneficial effect in this model. While we did not see a significant effect in overall liver weight (Figure 1C), we did observe a significant decrease in blood-glucose levels in the SR9009-treated mice. The *ob/ob* mouse model is typically hyperglycemic and addition of the high fat/high fructose diet hyperglycemia can be particularly prominent. Thus, our observation that of lowered hyperglycemia was promising and potentially

relevant to human NASH patients who often present with co-morbidities such as obesity and diabetes [20–24].

Figure 1: REV-ERB agonist treatment of ob/ob NASH diet-fed mice.
Mouse weight (A) and food intake (B) were recorded daily and averaged weekly for each group. At the termination of the experiment, mice were euthanized and blood and tissues were collected for analysis. Panel C shows the average liver weight as a percentage of the total body weight for each group. Blood glucose (D), ALT (E), AST (F), circulating triglycerides (G), and total protein (H) were also analyzed from blood plasma.

We were interested in determining whether SR9009 had any utility in improving the hepatic health of these mice. In order to make this determination, we performed clinical chemistry analysis on blood plasma samples to examine liver enzyme levels (ALT and AST) (Figures 1E and 1F respectively), as well as circulating triglyceride levels (Figure 1G) and total protein levels (Figure 1H) in both mouse groups. Plasma levels of liver enzymes are typically elevated in NASH due to liver damage and we observed that ALT levels were significantly reduced in the SR9009-treated mice (Figure 1E). These data clearly suggest that that the amount of liver damage due to the diet may be suppressed by treatment with REV-ERB agonist SR9009. While not statistically significant, AST levels in the SR9009 group were also trending lower than the vehicle group consistent with a benefit due to SR9009 treatment. We also observed significantly reduced circulating triglycerides as well as total protein in the plasma of SR9009 mice as compared to vehicle-treated mice. Our overall impression from the clinical chemistry data and mouse observations is that SR9009 treatment may have beneficial effects in a NASH model.

As the SR9009 group maintained a lower body weight throughout the dosing period and had significantly decreased circulating lipid levels in blood plasma, we investigated whether the SR9009-treated mice had reduced hepatosteatosis by staining for neutral lipids with Bodipy 493/503 [12,14,17]. Figure 2A shows representative results of the Bodipy staining (green) in vehicle and SR9009-treated liver sections, which suggests that there are fewer lipid droplets in the SR9009 group. The Bodipy staining was quantified using ImageJ software and normalized to total area, plotted as relative fluorescence units (RFU) in Figure 2B. There was a significant decrease in lipid staining in

the SR9009-treated group, suggesting that SR9009 treatment suppressed circulating lipids (Figure 1G), it was also suppressed the storage/accumulation of triglycerides in the livers of the treated animals.

Figure 2: SR9009 reduces hepatosteatosis in ob/ob mice maintained on a NASH diet.
Bodipy 493/503 staining (green) for neutral lipids in 10 μm liver cryosections counterstained with DAPI (blue) in vehicle- and SR9009-treated mice. Quantification of Bodipy staining was performed using ImageJ and demonstrates that the amount of lipids is significantly decreased in SR9009 mice as compared to vehicle.

To validate the clinical chemistry data suggesting that the SR9009 reduced liver damage and the Bodipy data suggesting that SR9009 treatment decreased hepatosteatosis, we performed hematoxylin and eosin (H&E) staining on paraffin-embedded liver tissue and examined liver morphology (Figure 3). The top panels are 10X imaging of representative vehicle (left) and SR9009-treated (right) liver sections. While both groups display some level of steatosis, it is clear that the SR9009 group displayed reduced macrosteatosis and improved tissue morphology. The 20X (bottom) panels clearly illustrate that both macrosteatosis and microsteatosis are reduced in SR9009 tissue. H&E staining also indicated severe disease in vehicle sections, with an increase in the number of inflammatory foci and hepatocellular ballooning, in addition to increased steatosis, which is indicative of a NASH phenotype while the SR9009 group did not appear to progress further than a simple fatty liver phenotype.

Figure 3: H&E stained paraffin-embedded liver sections illustrating improved tissue morphology in SR9009-treated mice.
Top panels (10X); Bottom panels (20X). Blue arrows indicate macrosteatosis; Yellow arrows indicate microsteatosis; orange arrows indicate inflammatory foci; and green arrows indicate hepatic ballooning.

To continue our investigation into whether pharmacological activation of REV-ERB is beneficial in an NASH model, we analyzed gene expression of inflammatory markers by QPCR from frozen mouse liver tissues. For this analysis, we focused on expression changes related to inflammatory markers that indicate progression of NAFLD towards a NASH pathology. Previous work from our lab [16] and others have shown that REV-ERBs regulate a variety of genes involved

in the pathogenesis of metabolic diseases including those associated with inflammation. Specifically, we were interested to see whether we were suppressing the progression of NAFLD towards NASH with SR9009 by not only alleviating liver damage as assessed in Figure 1E-H, but by also suppressing hepatic inflammation in these animals, which was suggested in the H&E staining (Figure 3). Indeed, when compared to the vehicle-treated group, the SR9009 mice display significantly lower levels of expression of inflammatory cytokines including *IL-1a*, *IL-1b*, *TNFa*, and *Interferon gamma* (*IFNg*), all of which have been implicated as biomarkers in NASH (Figure 4A).

Figure 4: Expression of inflammatory markers are downregulated by SR9009 treatment in a mouse model of NASH.
(A) Gene expression of inflammatory cytokines in mouse liver tissues that are involved in the pathogenesis of NAFLD-NASH. (B) Masson-Trichrome staining of Vehicle-treated and SR9009-treated mouse liver paraffin-embedded sections (10um). Blue staining indicates collagen, suggesting that hepatic fibrosis is present. (C) Collagen staining was quantified using ImageJ software and suggests that SR9009 treatment suppressed the development of fibrosis in these samples. (D) An ELISA for mouse TNFα was performed using plasma samples from each mouse in triplicate and shows that SR9009 mice had significantly reduced circulating TNFα. (E) Gene expression was performed for *Col3A1* and *Stat1* genes, both of which are involved in the development of fibrosis in NAFLD/NASH. As shown in the graphs, both genes are significantly downregulated in SR9009 mice.

Progression of NAFLD to NASH is associated with hepatic fibrosis, and in order to determine the level of fibrosis in this model we stained paraffin-embedded liver sections with Masson's trichrome (Figure 4B). Similar to the H&E stained liver sections, hepatic tissue from SR9009-treated mice display a more "normal" appearance with less macrosteatosis. The hallmark of this staining technique is the bright blue stain indicative of collagen fibers, which we quantitated as shown in Figure 4B. The livers from vehicle-treated mice appeared to have significantly increased blue staining, suggesting the progression of the disease towards a NASH phenotype with collagen deposits and fibrosis. While there is some indication of collagen deposits in the 20X image of the SR9009 group, when quantified, there is a significant decrease in collagen staining suggesting that SR9009 treatment inhibited fibrosis in these mice (Figure 4C). To further characterize the effects of SR9009 on inflammation, we also assessed plasma TNFα levels. High levels of TNFα are associated with increased hepatic inflammation and poorer prognosis in NASH patients. As shown in Figure 4D, the SR9009-treated group had significantly lower plasma TNFα levels, which is in agreement with the

hepatic gene expression data (Figure 4A). Additionally, we assessed the level of expression of additional genes specifically involved in the development of hepatic fibrosis. *Collagen 3A1* gene (*COL3A1*) and *STAT1* are both considered biomarkers of NASH development and progression [25]. In our experiments, both of these genes were significantly down regulated in SR9009-treated mice (Figure 4E), indicating that pharmacological activation of REV-ERB may dampen the progression of NAFLD towards NASH by not only suppressing hepatic lipid storage/accumulation, but also suppressing the activation of pro-inflammatory cytokines that are involved in the development of hepatic fibrosis.

Over the last several years, many studies by our lab and others have demonstrated that the REV-ERBs regulate a variety of genes involved in lipogenesis, metabolism, and inflammation. Several studies have investigated potential of REV-ERB agonists as potential therapeutics for cardio-metabolic diseases including atherosclerosis which is a common comorbidity with NAFLD or NASH [16,19]. Understanding the critical role that the REV-ERBs play in these pathways, we hypothesized that pharmacological activation of REV-ERB with the tool compound SR9009 in a mouse model of NASH would provide beneficial metabolic effects in the liver leading to reduced NASH pathology. Our data suggests that not only did SR9009 improve hepatic morphology by reducing hepatosteatosis, it also suppressed the activation of pro-inflammatory cytokines that are essential for the progression of NAFLD to NASH. While the SR9009 group did not gain weight at the same rate as the vehicle group and we cannot validate whether this was a direct effect of the compound, although feeding and other behaviors were observed to be the same for both groups throughout the study. In summary, our data suggests that REV-ERB is a potential therapeutic target to treat NASH. The REV-ERB agonist SR9009 displayed efficacy in reduction of hepatic pathology associated with NASH. SR9009 is effective in suppressing clinical markers of liver damage, circulating lipids, hepatic fibrosis and markers of inflammation. Our data suggests that REV-ERB agonists may offer novel therapies for NAFLD or NASH.

Acknowledgments

Barb Nagel at Saint Louis University School of Medicine (St. Louis, MO) Histology Core for paraffin embedding and performing histological staining of liver sections. Melissa Kazantzis at The Scripps Research Institute in Jupiter, FL for performing clinical chemistry analysis on samples. Sherry Burris for performing the cryopreservation and cryo-sectioning of liver samples for Bodipy analysis.

References

1. Fazel Y, B. Koenig A, Sayiner M, D. Goodman Z, M. Younossi Z. Epidemiology and natural history of non-alcoholic fatty liver disease [Internet]. Metabolism. 2016. pp. 1017 – 1025. doi:https://doi.org/10.1016/j.metabol.2016.01.012

2. Polyzos SA, Kang ES, Boutari C, Rhee E-J, Mantzoros CS. Current and emerging pharmacological options for the treatment of nonalcoholic steatohepatitis. Metabolism. Elsevier BV; 2020; 154203. doi:10.1016/j.metabol.2020.154203

3. Konerman MA, Jones JC, Harrison SA. Pharmacotherapy for NASH: Current and emerging. Journal of Hepatology. Elsevier BV; 2018;68: 362–375. doi:10.1016/j.jhep.2017.10.015

4. Yang X, Gonzalez FJ, Huang M, Bi H. Nuclear receptors and non-alcoholic fatty liver disease: An update. Liver Research. Elsevier BV; 2020; doi:10.1016/j.livres.2020.03.001

5. Tanaka N, Aoyama T, Kimura S, Gonzalez FJ. Targeting nuclear receptors for the treatment of fatty liver disease. Pharmacology & Therapeutics. Elsevier BV; 2017;179: 142–157. doi:10.1016/j.pharmthera.2017.05.011

6. López-Velázquez JA, Carrillo-Córdova LD, Chávez-Tapia NC, Uribe M, Méndez-Sánchez N. Nuclear Receptors in Nonalcoholic Fatty Liver Disease. Journal of Lipids. Hindawi Limited; 2012;2012: 1–10. doi:10.1155/2012/139875

7. Preitner N, Damiola F, Luis-Lopez-Molina, Zakany J, Duboule D, Albrecht U, et al. The Orphan Nuclear Receptor REV-ERBα Controls Circadian Transcription within the Positive Limb of the Mammalian Circadian Oscillator. Cell. Elsevier BV; 2002;110: 251–260. doi:10.1016/s0092-8674(02)00825-5

8. Li T, Eheim AL, Klein S, Uschner FE, Smith AC, Brandon-Warner E, et al. Novel role of nuclear receptor rev-erbα in hepatic stellate cell activation: Potential therapeutic target for liver injury. Hepatology. Wiley; 2014;59: 2383–2396. doi:10.1002/hep.27049

9. Stujanna EN, Murakoshi N, Tajiri K, Xu D, Kimura T, Qin R, et al. Rev-erb agonist improves adverse cardiac remodeling and survival in myocardial infarction through an anti-inflammatory mechanism. Fan G-C, editor. PLOS ONE. Public Library of Science (PLoS); 2017;12: e0189330. doi:10.1371/journal.pone.0189330

10. Kojetin D.J. and Burris T.P. A Role for Rev-erbα Ligands in the Regulation of Adipogenesis. Current Pharmaceutical Design. Bentham Science Publishers Ltd.; 2011;17: 320–324. doi:10.2174/138161211795164211

11. Pariollaud M, Gibbs JE, Hopwood TW, Brown S, Begley N, Vonslow R, et al. Circadian clock component REV-ERBα controls homeostatic regulation of pulmonary inflammation. Journal of Clinical Investigation. American Society for Clinical Investigation; 2018;128: 2281–2296. doi:10.1172/jci93910

12. Griffett K, Solt LA, El-Gendy BE-DM, Kamenecka TM, Burris TP. A Liver-Selective LXR Inverse Agonist That Suppresses Hepatic Steatosis. ACS Chemical Biology. American Chemical Society (ACS); 2012;8: 559–567. doi:10.1021/cb300541g

13. Griffett K, Burris TP. Promiscuous activity of the LXR antagonist GSK2033 in a mouse model of fatty liver disease. Biochemical and Biophysical Research Communications. Elsevier BV; 2016;479: 424–428. doi:10.1016/j.bbrc.2016.09.036

14. Griffett K, Welch RD, Flaveny CA, Kolar GR, Neuschwander-Tetri BA, Burris TP. The LXR inverse agonist SR9238 suppresses fibrosis in a model of non-alcoholic steatohepatitis. Molecular Metabolism. Elsevier BV; 2015;4: 353–357. doi:10.1016/j.molmet.2015.01.009

15. Trevaskis JL, Griffin PS, Wittmer C, Neuschwander-Tetri BA, Brunt EM, Dolman CS, et al. Glucagon-like peptide-1 receptor agonism improves metabolic, biochemical, and histopathological indices of nonalcoholic steatohepatitis in mice [Internet]. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2012. pp. G762–G772. doi:10.1152/ajpgi.00476.2011

16. Sitaula S, Billon C, Kamenecka TM, Solt LA, Burris TP. Suppression of atherosclerosis by synthetic REV-ERB agonist. Biochemical and Biophysical Research Communications. Elsevier BV; 2015;460: 566–571. doi:10.1016/j.bbrc.2015.03.070

17. Sengupta M, Griffett K, Flaveny CA, Burris TP. Inhibition of Hepatotoxicity by a LXR Inverse Agonist in a Model of Alcoholic Liver Disease. ACS Pharmacology & Translational Science. American Chemical Society (ACS); 2018;1: 50–60. doi:10.1021/acsptsci.8b00003

18. Solt LA, Wang Y, Banerjee S, Hughes T, Kojetin DJ, Lundasen T, et al. Regulation of circadian behaviour and metabolism by synthetic REV-ERB agonists. Nature. Springer Science and Business Media LLC; 2012;485: 62–68. doi:10.1038/nature11030

19. Pourcet B, Zecchin M, Ferri L, Beauchamp J, Sitaula S, Billon C, et al. Nuclear Receptor Subfamily 1 Group D Member 1 Regulates Circadian Activity of NLRP3 Inflammasome to Reduce the Severity of Fulminant Hepatitis in Mice. Gastroenterology. Elsevier BV; 2018;154: 1449–1464.e20. doi:10.1053/j.gastro.2017.12.019

20. Petit J-M, Vergès B. GLP-1 receptor agonists in NAFLD. Diabetes & Metabolism. Elsevier BV; 2017;43: 2S28–2S33. doi:10.1016/s1262-3636(17)30070-8

21. Scorletti E, Byrne CD. Extrahepatic Diseases and NAFLD: The Triangular Relationship between NAFLD, Type 2-Diabetes and Dysbiosis. Digestive Diseases. S. Karger AG; 2016;34: 11–18. doi:10.1159/000447276

22. Obesity, Type 2 diabetes and NAFLD. Diabetic Medicine. Wiley; 2014;31: 1–3. doi:10.1111/dme.12377_1

23. Portillo-Sanchez P, Cusi K. Treatment of Nonalcoholic Fatty Liver Disease (NAFLD) in patients with Type 2 Diabetes Mellitus. Clinical Diabetes and Endocrinology. Springer Science and Business Media LLC; 2016;2. doi:10.1186/s40842-016-0027-7

24. Bonora E. Novel predictors of diabetes - NAFLD, diabetes and CVD. Endocrine Abstracts. Bioscientifica; 2017; doi:10.1530/endoabs.49.s29.3

25. Feng G, Li X-P, Niu C-Y, Liu M-L, Yan Q, Fan L-P, et al. Bioinformatics analysis reveals novel core genes associated with nonalcoholic fatty liver disease and nonalcoholic steatohepatitis [Internet]. Gene. 2020. p. 144549. doi:https://doi.org/10.1016/j.gene.2020.144549

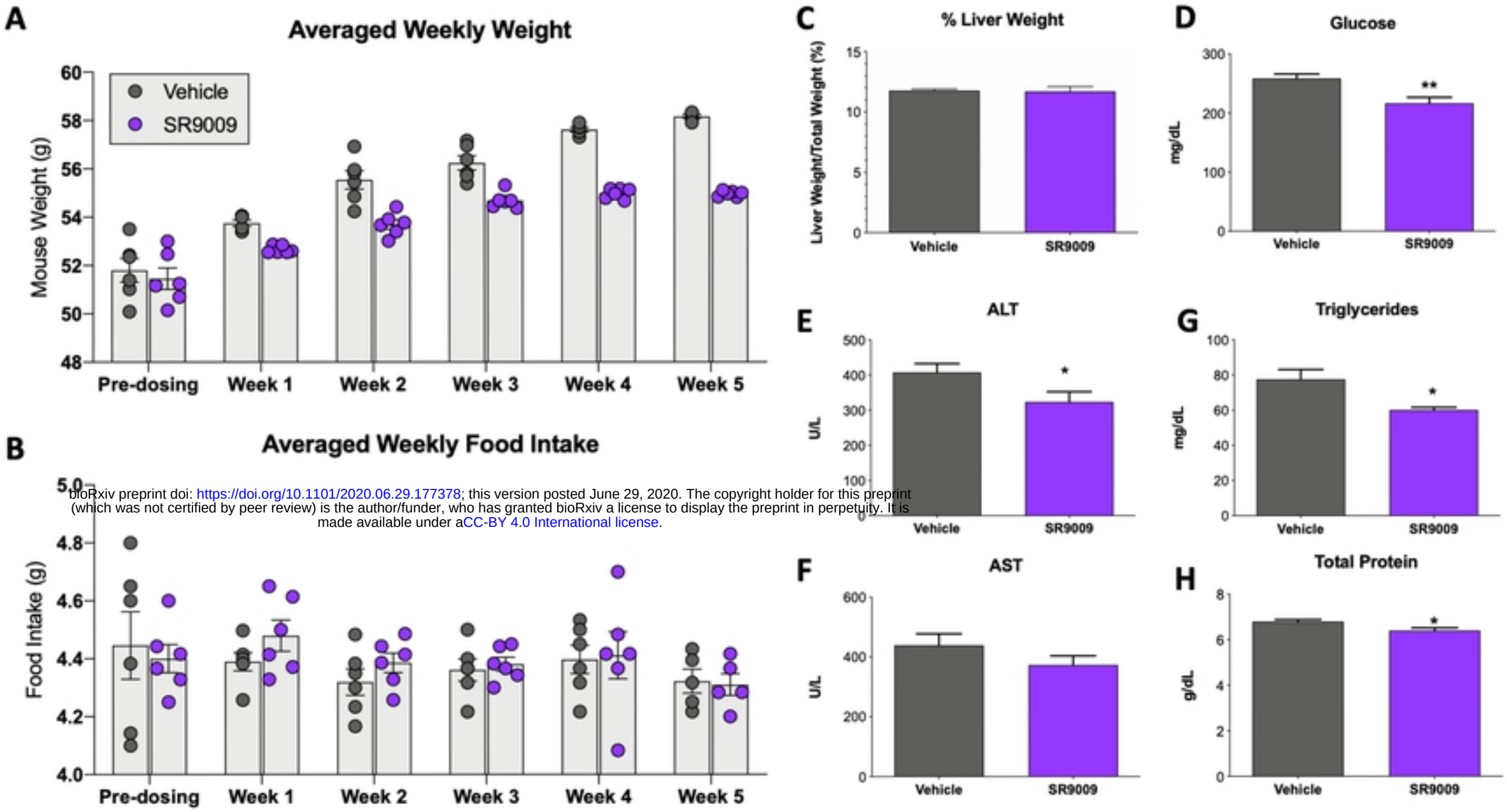
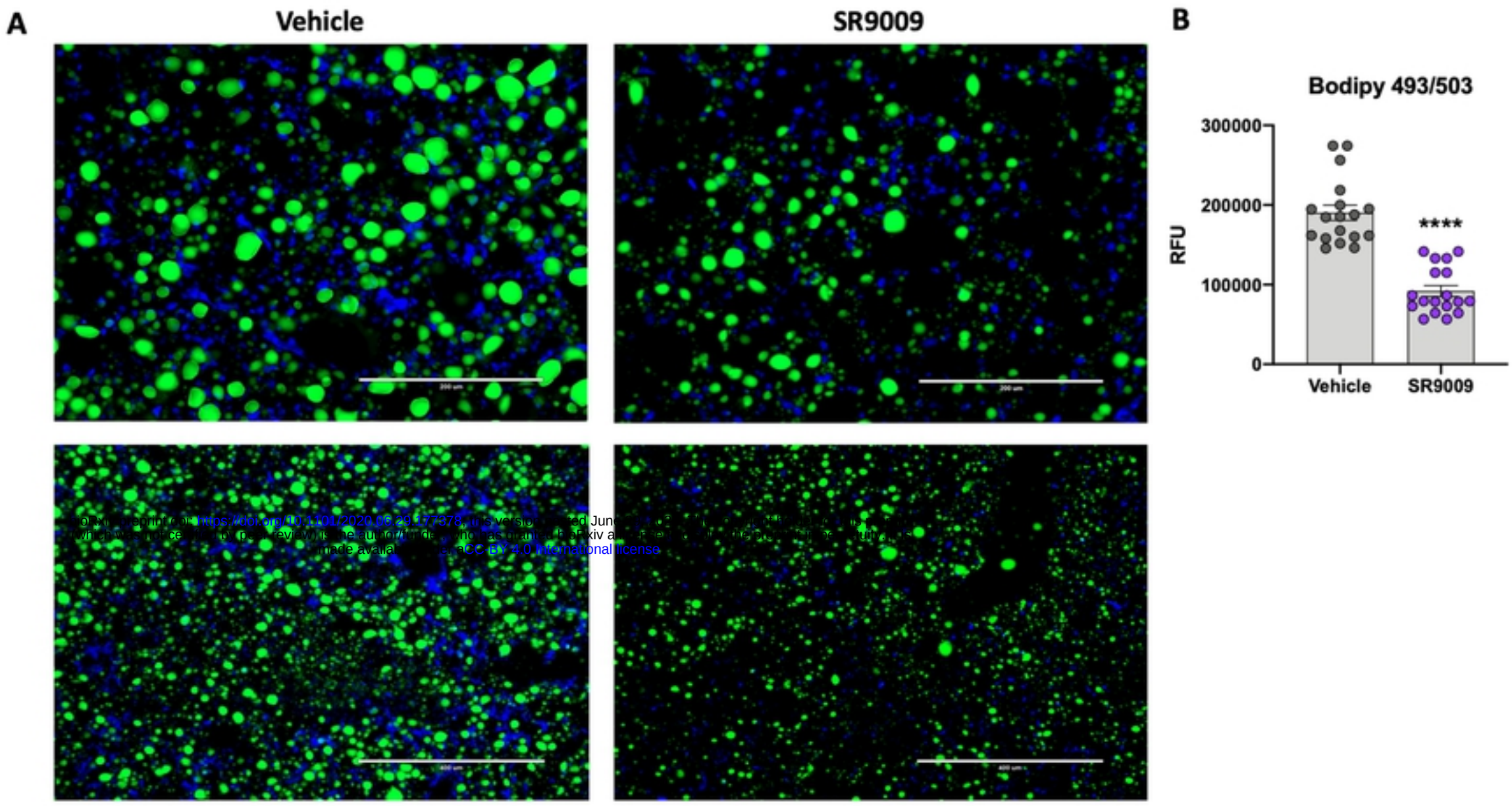


Figure 1



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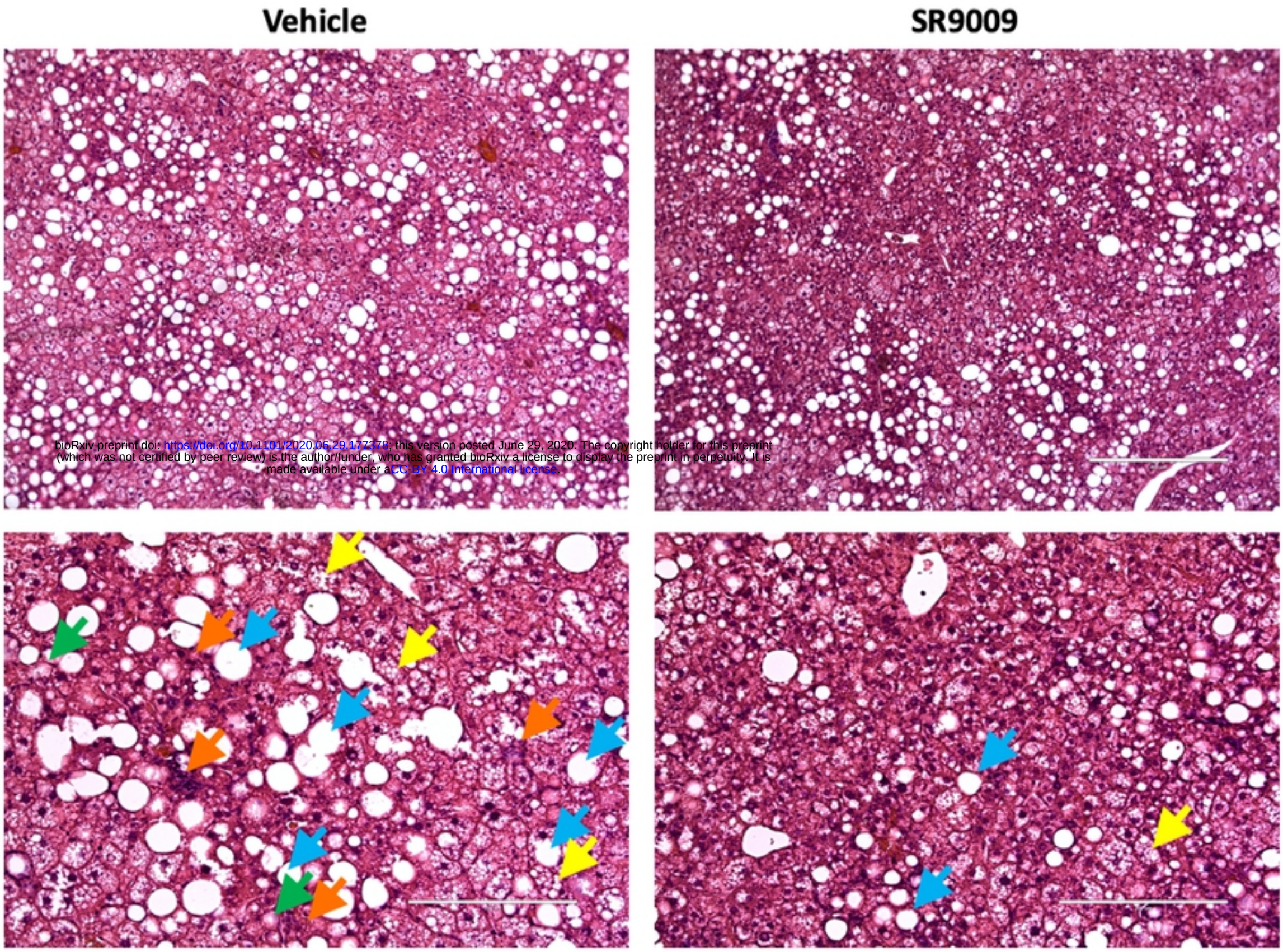

Figure 3

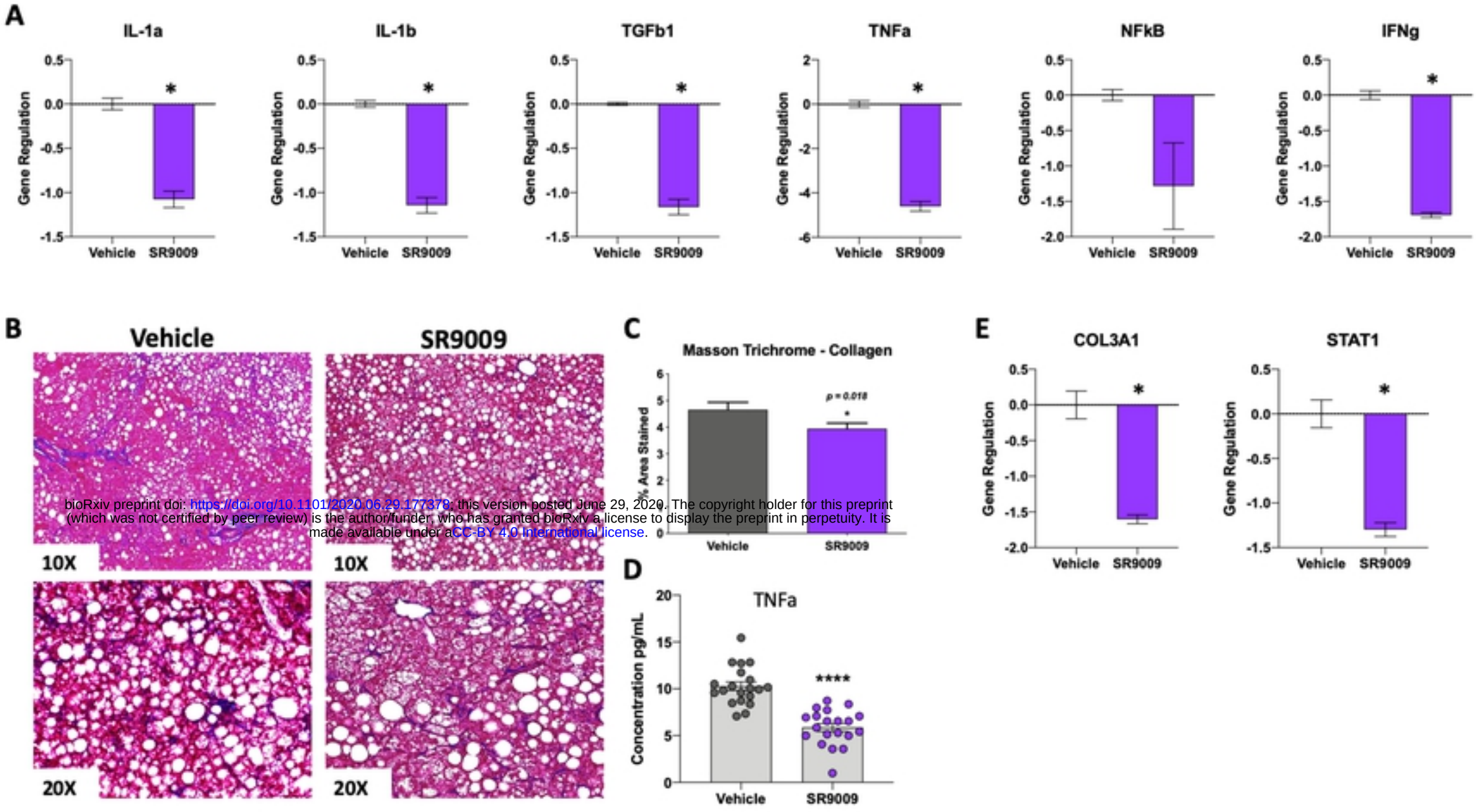

Figure 4