

**Exacerbated hepatic inflammatory response to diet-induced non-alcoholic fatty liver disease in male 11 $\beta$ -HSD1 knockout mice**

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## ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) defines a spectrum of diseases from simple steatosis to non-alcoholic steatohepatitis (NASH) and cirrhosis, and can be regarded as the hepatic manifestation of the metabolic syndrome (MetS). The initial stages of NAFLD are characterized by intra-hepatocyte lipid accumulation. Glucocorticoid (GC) excess can promote steatosis by stimulating lipolysis within adipose tissue, FFA delivery to the liver and hepatic *de novo* lipogenesis. GCs can be reactivated in the liver through 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ -HSD1) enzyme activity. Inhibition of 11 $\beta$ -HSD1 has been suggested as a potential treatment for NAFLD. To test this, male mice with global (11 $\beta$ -HSD1KO) and liver-specific (LKO) 11 $\beta$ -HSD1 loss-of-function were fed the American Lifestyle Induced Obesity Syndrome (ALIOS) diet, known to recapitulate the spectrum of NAFLD, and metabolic and liver phenotypes assessed. Body weight, muscle and adipose tissue mass, and parameters of glucose homeostasis showed that 11 $\beta$ -HSD1KO and LKO mice were not protected from systemic metabolic disease. Evaluation of hepatic histology, triglyceride content and blinded NAS assessment indicted that levels of steatosis were similar between 11 $\beta$ -HSD1KO, LKO and control mice. Unexpectedly, histological analysis did reveal significantly increased levels of immune foci present in livers of 11 $\beta$ -HSD1KO but not LKO or control mice, suggestive of a transition to NASH. This was endorsed by elevated hepatic expression of key immune cell and inflammatory markers. These data indicate that 11 $\beta$ -HSD1 deficient mice are not protected from metabolic disease or hepatosteatosis in the face of a NAFLD inducing diet. However, global deficiency of 11 $\beta$ -HSD1 did increase markers of hepatic inflammation and suggests a critical role for 11 $\beta$ -HSD1 in restraining NASH.

## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) defines a spectrum of diseases ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, progressing in rare cases to cirrhosis and hepatocellular carcinoma (HCC) [1-4]. Around 30% of the USA and 25% of the European adult populations have NAFLD with 3-5% diagnosed with NASH [5, 6], leading to increased morbidity and mortality [7].

Accumulating evidence supports an association between NAFLD and metabolic syndrome (MetS). Around 75% of obese people have NAFLD, with insulin resistance a key mechanistic factor between both conditions [8]. As such, NAFLD can be regarded as the hepatic manifestation of the metabolic syndrome (MetS), which together increase the risk of developing cardiovascular disease [8, 9].

Patients affected by glucocorticoid (GC) excess (Cushing's syndrome) present with many of the disorders associated with MetS and can develop NAFLD [10]. GCs promote steatosis through multiple mechanisms including stimulation of lipolysis within adipose tissue resulting in increased free fatty acid (FFA) delivery for utilization in the liver to produce lipids through enhanced hepatic *de novo* lipogenesis [11-13]. In most patients with NAFLD and MetS, circulating GC concentrations are not elevated [14]. However, GCs can be activated in a tissue-specific manner through the pre-receptor activity of the 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ -HSD1) enzyme, with the site of greatest activity being the liver [15]. Thus, hepatic metabolism affected by GCs is a balance between circulating delivery and 11 $\beta$ -HSD1 mediated intracellular activation.

Pre-clinical studies using 11 $\beta$ -HSD1 knockout (KO) and transgenic mice have exemplified the role 11 $\beta$ -HSD1 can play in determining hepatic metabolic phenotype. Global deletion of 11 $\beta$ -HSD1 protects against high fat diet induced obesity and glucose intolerance whereas liver-specific 11 $\beta$ -HSD1 deletion does not [16-18]. 11 $\beta$ -HSD1KO mice are protected from hepatosteatosis in the face of circulating GC excess and reveal the importance of adipose tissue in determining hepatic phenotype [16]. Furthermore, mice with transgenic overexpression of 11 $\beta$ -HSD1, specifically in adipose and liver, develop hepatosteatosis in the context of a high fat diet [19, 20].

Data from studies in humans support the idea that with steatosis there is decreased hepatic 11 $\beta$ -HSD1 mediated GC reactivation, possibly due to decrease local GC availability and preservation of

metabolic phenotype [21]. 11 $\beta$ -HSD1 inhibitors have been the subject of interest regarding their use in the treatment of conditions associated with MetS, with in-excess of 170 compounds having been developed for this purpose [22-25]. Human clinical studies evaluating NAFLD patients showed that pharmacological inhibition of 11 $\beta$ -HSD1 was able to modestly reduce liver fat content over a 12-week treatment period, though whether this was a direct or peripheral effect was unclear [26]. Given the important role that 11 $\beta$ -HSD1 mediated GC metabolism plays in determining systemic and liver metabolic phenotype, we hypothesized that global deletion (11 $\beta$ -HSD1KO) and hepatocyte specific deletion (LKO) mice would be protected from MetS and hepatosteatosis when subjected to a potent steatogenic diet. To this end we fed 11 $\beta$ -HSD1KO mice the American Lifestyle Induced Obesity Syndrome (ALIOS) diet which, unlike a standard high fat diet, is known to more faithfully recapitulate the spectrum of NAFLD, from steatosis to NASH [27]. Our data suggest that 11 $\beta$ -HSD1 loss of function affords no protection from ALIOS induced obesity, glucose intolerance, insulin resistance or hepatosteatosis. Unexpectedly, we show that in the context of hepatosteatosis, 11 $\beta$ -HSD1KO mice have hastened progression to NASH as revealed by an accumulation of hepatic immune foci and increased expression of immune and inflammatory markers. These results suggest a role for 11 $\beta$ -HSD1 in restraining hepatic inflammation in NAFLD.

## MATERIALS AND METHODS

### *Animal husbandry*

Male mice aged 7-8 weeks, with global (11 $\beta$ -HSD1KO) and hepatocyte-specific (LKO) deletions of 11 $\beta$ -HSD1 [18, 28]; along with age-matched C57BL/6 control mice (Charles River) were used. Mice were housed 2-3 per cage and maintained at the Biomedical Services Unit at the University of Birmingham, UK and all procedures conducted in accordance with the Animals (Scientific Procedures) Act 1986; regulated by the UK Home Office. Mice were maintained on a 12:12 hour light-dark cycle at 21-22 °C and were fed *ad libitum*, a diet consisting of 45% of calories from fat, of which 11.6% were derived from trans-fats (Research Diets, Inc.; D13022701). Mice were also given a high fructose corn syrup equivalent drinking water replacement (55% fructose: 45% glucose (w/v) deionised H<sub>2</sub>O at a concentration of 42 g/L). This feeding regimen, referred to as the ALIOS (American Lifestyle Induced Obesity Syndrome) diet [27], was maintained for 16 weeks, following which mice were subjected to exsanguination via cardiac puncture under general anaesthetic (isoflurane), followed by cervical dislocation; upon which tissues were excised and weighed. Once weighed, tissues were dissected then promptly snap-frozen in liquid nitrogen (LN<sub>2</sub>) or fixed in 4% formaldehyde (10% formalin (v/v) PBS).

### *Metabolic analyses and plasma analytes*

Following 13 weeks of ALIOS treatment, blood from each mouse was collected into Microvette EDTA lined hematological tubes, from lateral tail veins and blood-glucose concentrations measured using an Accu-Chek® monitor. The following week, mice were fasted overnight and glucose tolerance tests (GTT) performed; whereby intraperitoneal (IP) injections of 20% glucose (v/v) 0.9% sterile saline solutions at a dose of 2 g/kg were administered. Blood-glucose concentrations were measured at times (T) 0, 15, 30, 60, 90 and 120 minutes post-glucose injections. Blood samples were also collected at T0 and T30 post-glucose injection and plasma insulin concentrations measured using the Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem). Insulin tolerance testing (ITT) was performed during week 15 of the study. Mice were fasted for 4 hours and blood-glucose concentrations measured (T0), followed by the administration of insulin (Actrapid) via IP injection at

a concentration of 0.1 IU/mL (0.1% 100 IU/mL (v/v) 0.9% sterile saline) at a dose of 0.75 IU/kg. Blood-glucose concentrations were subsequently measured at 15, 30, 60, 90 and 120 minutes post-insulin injections. Plasma free fatty acid (FFA), triglyceride (TAG) and high density/low density lipoprotein (HDL/LDL) cholesterol concentrations were measured from blood taken via terminal cardiac bleeds using protocols outlined according to the manufacturer's instructions (BioVision).

#### ***Hepatic triglyceride quantification***

Hepatic triglyceride content was ascertained using Biovision's colorimetric assay (Cambridge Bioscience, Cambridge, UK). Briefly, 100 mg of frozen liver tissue was homogenized in 5% Nonidet P-40 before being heated for 5 minutes at 95 °C in a water bath to solubilize triglycerides, samples were left to cool to room temperature and the process repeated before centrifugation at 13000 rpm. The resultant supernatants were diluted in distilled H<sub>2</sub>O and analysed on a 96 well Wallac plate reader at  $\lambda$ 570 nm.

#### ***Histological analyses***

Freshly excised livers were dissected, processed and embedded in paraffin wax, from which 5  $\mu$ m sections were cut for analyses via histological staining. Hematoxylin and Eosin (H&E) staining was used to score hepatosteatosis and inflammation using the NAFLD activity score (NAS) [29] and Van Gieson staining to access fibrosis. All images pertaining to histological analyses were viewed via light microscopy and photomicrographs taken using a Leica imaging system.

#### ***Real time quantitative polymerase chain reaction***

Fragments of frozen liver tissue were homogenized using TRI Reagent (Sigma-Aldrich; Dorset, UK) for total RNA isolation. 1  $\mu$ g of total RNA was reversed transcribed to cDNA using Applied Biosystems' High Capacity cDNA Reverse Transcription Kit (Life Technologies, Paisley, UK) following the manufacturer's instructions. Reaction mixes consisting of Applied Biosystems' TaqMan Universal PCR Master Mix and primer/probes (assays on demand), along with 1  $\mu$ L cDNA, were made to 20  $\mu$ L with nuclease free water. All primer/probes targeted to genes of interest were labelled

with FAM, whereas the reference gene, invariably 18s, was labelled with VIC; all reactions were singleplex and performed using Applied Biosystems' ABI 7500 sequence detection system. Gene expression data was graphically represented as fold changes against controls. Reference genes' (18s) Cycle threshold (Ct) values from each sample were subtracted from Ct values of corresponding samples genes' of interest for delta Ct values ( $\Delta Ct$ ). These values were then averaged and controls averages taken from those of KOs' to give  $\Delta\Delta Ct$  values which were then incorporated into the equation  $2^{-\Delta\Delta Ct}$ ; to normalize cohort controls values to 1 and KO cohort as fold changes compared to controls [29].

### ***Statistical analyses***

All data is derived from n=13-controls, 11-11 $\beta$ -HSD1KO and 9-LKO and 9-LKO controls male mice with data presented as the mean values  $\pm$  SEM. Statistical analyses were performed using Mann-Whitney and Student's *t*-tests and statistical significance assigned where P values were <0.05. Although graphical representation of RTqPCR data is presented as fold change verses controls, statistical analyses were performed using  $\Delta Ct$  values.

## **RESULTS**

### **Effect of ALIOS diet feeding on metabolic parameters**

NAFLD is considered the hepatic manifestation of the MetS. Thus we used the ALISO diet as a means to induce MetS and fatty liver disease in 11 $\beta$ -HSD1KO and control mice. After 16 weeks of ALIOS diet, controls and 11 $\beta$ -HSD1KO mice showed similar rates of body weight gain (Fig. 1A). Body weight normalized subcutaneous fat (SF), gonadal fat (GF) and peri-renal fat (PRF) pad weights showed no differences, with evidence for moderately increased accumulation of mesenteric fat (MF) in 11 $\beta$ -HSD1KOs compared to controls ( $p < 0.01$ , Fig. 1B). No differences were seen in lean mass (quadriceps, tibialis anterior and soleus muscles) between 11 $\beta$ -HSD1KO and control mice (Fig. 1C). 11 $\beta$ -HSD1KO mice showed no difference in glucose or insulin tolerance when compared to control mice (Fig. 1D, E). Furthermore, neither fed nor fasted state blood glucose concentrations were different between 11 $\beta$ -HSD1KO and control mice (Fig. 1F). Blood insulin concentrations in the fasted state and 30 minutes after glucose bolus injection showed no differences between 11 $\beta$ -HSD1KO and control mice (Fig. 1G). We also assessed plasma lipid profiles and show HDL and LDL cholesterol, free fatty acids (FFA) and triglycerides (TAG) were no different between 11 $\beta$ -HSD1KO and control mice (Fig. 1H, I, J). Taken together, these data suggest that global deletion of 11 $\beta$ -HSD1 does not protect from the metabolic dysregulation associated with 16 weeks of ALIOS diet feeding, a time frame validated to cause obesity, insulin resistance and dyslipidemia [27].

### **Hepatic histology and steatosis scoring**

ALIOS diet is also an established means to induce the classical features of hepatosteatosis and fatty liver disease [27], from which 11 $\beta$ -HSD1KO mice should be protected. However, there were no differences in the macroscopic appearance of the liver, or body weight normalized liver weight between 11 $\beta$ -HSD1KO and control mice (Fig. 2A). Hepatic TAG content endorsed the liver weight data, with no differences observed (Fig. 2B). Steatosis analysis, using blind scoring of H&E stained livers of 11 $\beta$ -HSD1KO and control mice, showed microvesicular and macrovesicular steatosis in both 11 $\beta$ -HSD1KO and control mice. Although the severity varied across the cohorts, no differences in overall distribution in lipid accumulation were observed (Fig. 2C). Indeed, qualitative assessment using NAS showed there was no statistically significant difference between hepatosteatosis in 11 $\beta$ -HSD1KO and control mice (Fig. 2D). Overall, we find that deletion of 11 $\beta$ -HSD1 in mice treated

with the ALIOS diet affords no protection from hepatic TAG accumulation or classical histological profile associated with hepatosteatosis and an overall MetS.

#### **Metabolic and hepatic analysis of hepatocyte-specific 11 $\beta$ -HSD1 deletion**

We also wished to delineate the contribution of hepatocyte specific-11 $\beta$ -HSD1 deletion to the development of a MetS and fatty liver in ALIOS fed mice given the high level of enzyme expression in this cell type and its prominent role in GC regulated lipid metabolism. To do this, we subjected hepatocyte-specific KO mice (LKO) to ALIOS diet for 16 weeks. As in 11 $\beta$ -HSD1KO, LKO mice gained weight at a similar rate to controls over the 16 weeks, with no discernible differences in endpoint weight observed (Control  $34.73 \pm 1.47$  g vs. LKO  $36.56 \pm 0.70$  g). No differences in body weight normalized adipose or lean tissue weights were seen. In terms of glucose metabolism, Area under the curve analysis of GTTs and ITTs again showed no differences between LKO and control mice (GTT AUC, control  $17.23 \pm 2.26$  mM vs. LKO  $20.32 \pm 2.22$  mM; ITT AUC, control  $7.92 \pm 0.33$  mM vs. LKO  $7.35 \pm 0.37$  mM). Furthermore, there were no differences between normalized liver weight and hepatic TAG content between control and LKO mice, being equivalent to the 11 $\beta$ -HSD1KO (Fig. 3A,B). Hepatosteatosis analysis was conducted and showed equally distributed microvesicular and macrovesicular steatosis in both LKO and control mice (Fig. 3C). NAS assessed LKO and control livers showed similar levels of hepatosteatosis (Fig. 3D). These data confirmed the overall view that ALIOS diet and its associated induction of MetS and NALFD is unaffected by the 11 $\beta$ -HSD1 loss of function.

#### **Hepatic inflammation analysis in 11 $\beta$ -HSD1KO**

It became apparent during the process of blind assessing histological NAS scores that certain sections displayed accumulations of hepatic immune foci in the context of steatosis. To better characterize this, blinded NAS system quantification of inflammatory and immune foci was conducted and revealed an average of 80% of fields of view from 11 $\beta$ -HSD1KO livers had 2 or more inflammatory foci compared to 30% in controls ( $P < 0.001$ , Fig. 4A and B). Importantly, scoring of LKO immune foci showed no discernible differences with their controls (Fig. 4C). These data suggest that despite no

differences in systemic or hepatic metabolic parameters being observed, 11 $\beta$ -HSD1KO livers are potentially engaged in the early transition to inflammatory disease, and that extra hepatocyte 11 $\beta$ -HSD1 activity is important to this process.

### **Markers of inflammation, immune cell infiltration and fibrosis in 11 $\beta$ -HSD1KO mice**

Following the observation of increased immune foci and early stage inflammatory disease in 11 $\beta$ -HSD1KO mice, we conducted gene expression analysis for markers of cytokines, macrophages, lymphocyte and fibrosis to further characterize this finding. The pro-inflammatory genes *Tnf* and *Ccl2* were significantly increased in livers of 11 $\beta$ -HSD1KO compared to controls (Fig. 5A). Further to this, macrophage markers *Lgals3*, *Fsp1* and *Cd11b*, known to be involved in the early modulation of NASH, were significantly increased, as were lymphocyte specific markers *Cd8* and *B220* in 11 $\beta$ -HSD1KO compared to controls (Fig. 5B, C). These suggest that 11 $\beta$ -HSD1 expression in non-hepatocyte cells is important to restraining the immune response to ectopic liver fat accumulation.

As the pathogenesis of NASH involves the deposition of collagen and the formation of fibrotic lesions as hallmark features of advancing disease, we assessed the expression of fibrosis markers and show that *Colla1*, encoding the major protein component of type 1 collagen is significantly increased (P=0.004) in 11 $\beta$ -HSD1KO but not in control liver. Reelin, (*Reln*) expressed by hepatic stellate cells can act as a marker of hepatocyte damage, and was shown not to be altered (Fig. 6A). However, Van Gieson staining showed no ectopic collagen deposition evident in either 11 $\beta$ -HSD1KO or control liver, with only collagen of the tunica externa of blood vessels stained (Fig. 6B); suggesting increased *Colla1* expression was a marker of the very earliest progression to NASH only seen in 11 $\beta$ -HSD1KO mice.

## **DISCUSSION**

Mice deficient in 11 $\beta$ -HSD1, or treated with 11 $\beta$ -HSD1 inhibitors, resist obesity and a MetS phenotype when fed conventional high fat diets [17, 23]. Based on observations from such models and recent human clinical studies we postulated that 11 $\beta$ -HSD1KO and hepatocyte-specific KO (LKO) mice would be protected against the development of MetS and resist hepatosteatosis when challenged with the ALIOS diet. Following 16 weeks of ALIOS, we show that male 11 $\beta$ -HSD1KO and LKO mice are indistinguishable from control mice displaying obesity, glucose intolerance, dyslipidemia or indeed hepatosteatosis. An increase in mesenteric adipose tissue mass was measured in 11 $\beta$ -HSD1KO mice. This runs contrary to that found for 11 $\beta$ -HSD1KO mice fed a high fat diet, in which there was preferential adipose deposition away from the mesenteric depot [17]. None the less, positive correlations have been shown between mesenteric fat mass and hepatosteatosis in human subjects [30-32].

Unlike conventional high fat diets, ALIOS diet includes trans-fats and fructose, both of which can drive hepatic lipid metabolism and accumulation, and have been shown to exacerbate the development of MetS beyond that achieved by conventional high fat diets [27, 33-35]. Indeed ALIOS is also validated to induce a more vigorous progression from simple steatosis to NASH, something that high fat diets rarely achieve [27, 36].

We had further hypothesized that KO mice would be protected from developing overt fatty liver and show a decrement in lipid accumulation. However, hepatic lipid accumulation and quantitative NAS analysis demonstrated no differences between 11 $\beta$ -HSD1KO, LKO or thier control mice. A number of preclinical animal models have demonstrated the potential for 11 $\beta$ -HSD1 to modulate lipid accumulation and the NAFLD phenotype. Mice with transgenic over expression of 11 $\beta$ -HSD1, specifically in adipose and liver develop steatosis in the context of a HFD [19, 20]. 11 $\beta$ -HSD1KO and adipose-specific 11 $\beta$ -HSD1KO mice resist hepatosteatosis when GCs are in circulatory excess, achieved through the lack of adipose 11 $\beta$ -HSD1 limiting lipolysis and hepatic FFA delivery, whereas LKO maintained florid steatosis as in WT mice [16, 18]. Furthermore, a recent phase 1B clinical trial using the 11 $\beta$ -HSD1 inhibitor RO5093151 effectively reduced liver-fat content of those treated; however, the tissues mediating this effect were not identified. [26].

On this basis we conclude that in the context of the ALIOS diet, neither global nor hepatocyte-specific 11 $\beta$ -HSD1 loss-of-function in male mice has any protective effect against the development of hepatosteatosis.

Simple and benign steatosis is a reversible condition which precedes more destructive NASH, fibrosis and cirrhosis [37]. Our unanticipated observations of inflammatory disease only, in 11 $\beta$ -HSD1KO mice, implied that we had collected liver transitioning to the early stage of NASH. Inflammatory scoring was not a primary end-point measure in terms of our initial hypothesis regarding metabolic disease. However, there is accumulating evidence for a prominent role for 11 $\beta$ -HSD1 in modulating local tissue inflammatory responses, regulating early restraint of the acute inflammatory process. 11 $\beta$ -HSD1 deficiency has been shown to worsen acute inflammation with greater infiltration of inflammatory cells into target sites in experimental models of rheumatoid arthritis and myocardial infarction [38, 39]. However, this is not a universal observation. Reduced inflammation in adipose tissue has been observed in 11 $\beta$ -HSD1KO mice, suggesting tissue-specific inflammatory phenotypes are important [40].

We endorsed our histological observations by showing elevated expression of markers of inflammation and immune cell infiltration/activation in livers of 11 $\beta$ -HSD1KO mice when compared to controls. Fibroblast-specific protein-1 (*Fsp1/S100a4*), a marker for a sub-population of macrophages specific to liver damage was increased yet *F4/80* and *Cd68*, pan macrophage markers, and *Clec4f*, expressed in Kupffer and migrant liver macrophages were not. Although no firm conclusions can be drawn, they do suggest differential regulation of FSP1<sup>+ve</sup> macrophages in the context of 11 $\beta$ -HSD1 and diet-induced hepatic inflammation. Importantly, FSP1<sup>+ve</sup> macrophages also express *Cd11b*, *Ccl2* and *Tnf*, all of which were also significantly increased in 11 $\beta$ -HSD1KO mice. As several liver resident immune cells types express these markers, elucidation of the sub-populations involved could not be ascertained [41-44]. In healthy livers, resident immune cells are found in portal tracts and migrate into the parenchyma upon initiation of an inflammatory response [45-47]. It has been shown that they auto-modulate their physiology via intracrine 11 $\beta$ -HSD1 mediated re-activation of GC regulates processes such as mast cell degranulation, dendritic cell differentiation, and

neutrophil and dendritic cell susceptibility to apoptosis [48-52]. This evidence suggests the intrinsic ability of immune cells to auto-regulate availability of GC is crucial to appropriate immune function. We postulate that ALIOS induced hepatic inflammation is initially restrained by extra-hepatocyte 11 $\beta$ -HSD1 activity in resident hepatic immune cells or/and by peripheral extravasated immune cells such as macrophages [53, 54]. This is supported by data showing macrophages deficient in 11 $\beta$ -HSD1 fail to phagocytose apoptotic neutrophils when treated *in vitro* with 11 $\beta$ -HSD1 substrate; with 11 $\beta$ -HSD1KO mice having delayed macrophage phagocytic competence during induced peritonitis [51]. The enhanced inflammatory phenotype exhibited by 11 $\beta$ -HSD1KO mice also endorses clinical data, whereby steatohepatitis was accompanied with an increase in 11 $\beta$ -HSD1, suggesting a requirement for increased GC activity in inflamed livers [21].

While we show significantly increased hepatic expression of *Colla1*, an early marker of liver collagen deposition [55], we could not demonstrate histological evidence of fibrosis, and may be due to 16 weeks of ALIOS being insufficient to evoke fibrotic lesions. ALIOS diet fed for 12 months has been shown to recapitulate the development of NASH, as 80% of animals developed fibrosis and 60% HCC [36].

These data indicate ALIOS as a useful means to examine the relationship between hepatic 11 $\beta$ -HSD1 mediated GC metabolism, MetS and inflammatory disease. Importantly, we highlight the need to further explore 11 $\beta$ -HSD1 in the context of hepatic inflammation and its role in the mechanisms that gateway the development of NASH. Further work is required to elucidate the cell types and mechanisms critical to resolving hepatic inflammation in the setting of ALIOS induced NAFLD and MetS. Ultimately, the clinical application of 11 $\beta$ -HSD1 inhibitors as treatments for many of the conditions associated with MetS may not be advisable when NAFLD is also present.

## **DECLARATION OF INTEREST, FUNDING AND ACKNOWLEDGMENTS**

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**Figure 1. Loss of 11 $\beta$ -HSD1 does not protect mice from the adverse metabolic effects from being fed the ALIOS diet for 16 weeks.**

(A) Body weight. (B) Adipose depot weights; 11 $\beta$ -HSD1KO mice showed statistically significant differences only in mesenteric depot weights. (C) Muscle bed weights. (D) Glucose tolerance. (E) Insulin tolerance. (F) Fed and fasted blood glucose concentrations. (G) Plasma insulin concentrations at T0 and T30 following glucose bolus injection. (H) Total, HDL and LDL plasma cholesterol concentrations. (I) Plasma FFA. (J) Plasma TAG. SCF-subcutaneous fat, GF-gonadal fat, MF-mesenteric fat, PRF- peri-renal fat, TA-tibialis anterior, HDL-high-density lipoprotein, LDL-low-density lipoprotein, FFA-free fatty acids, TAG-triglyceride. Mean  $\pm$  SEM; \*\*P<0.01 using student's *t*-test; n = 13 (controls), 11 (11 $\beta$ -HSD1KO).

**Figure 2. Loss of 11 $\beta$ -HSD1 does not reduce or delay hepatic lipid accumulation in mice fed the ALIOS diet for 16 weeks.**

(A) Liver/body weight ratios in controls and 11 $\beta$ -HSD1KO. (B) Hepatic triglyceride content in controls and 11 $\beta$ -HSD1KO mice. (C) H&E stained sections cut at 5  $\mu$ m of controls and 11 $\beta$ -HSD1KO liver demonstrating levels of macro- and micro- vesicular steatosis. (D) Dot plot representing steatosis in livers of control and 11 $\beta$ -HSD1KO mice was assessed using the NAS grading system. Mean  $\pm$  SEM (A,B), interquartile range (D); n = 13 (control), 11 (11 $\beta$ -HSD1KO); scale bars 200  $\mu$ m.

**Figure 3. Mice with hepatocyte-specific deletion of 11 $\beta$ -HSD1 have steatosis comparable to global KO mice.**

(A) Liver/body weight ratios in controls and LKO. (B) Hepatic triglyceride content in controls and LKO mice. (C) H&E stained sections cut at 5  $\mu$ m of control and LKO liver demonstrating levels of macro- and micro- vesicular steatosis. (D) Dot plot representing steatosis in livers of control and LKO mice, assessed using the NAS grading system. Mean  $\pm$  SEM (A,B), interquartile range (D); n = 9 (control), 9 (LKO); scale bars 200  $\mu$ m.

**Figure 4. Loss of 11 $\beta$ -HSD1 results in an increased frequency of hepatic inflammatory foci compared to control after 16 weeks of ALIOS diet.**

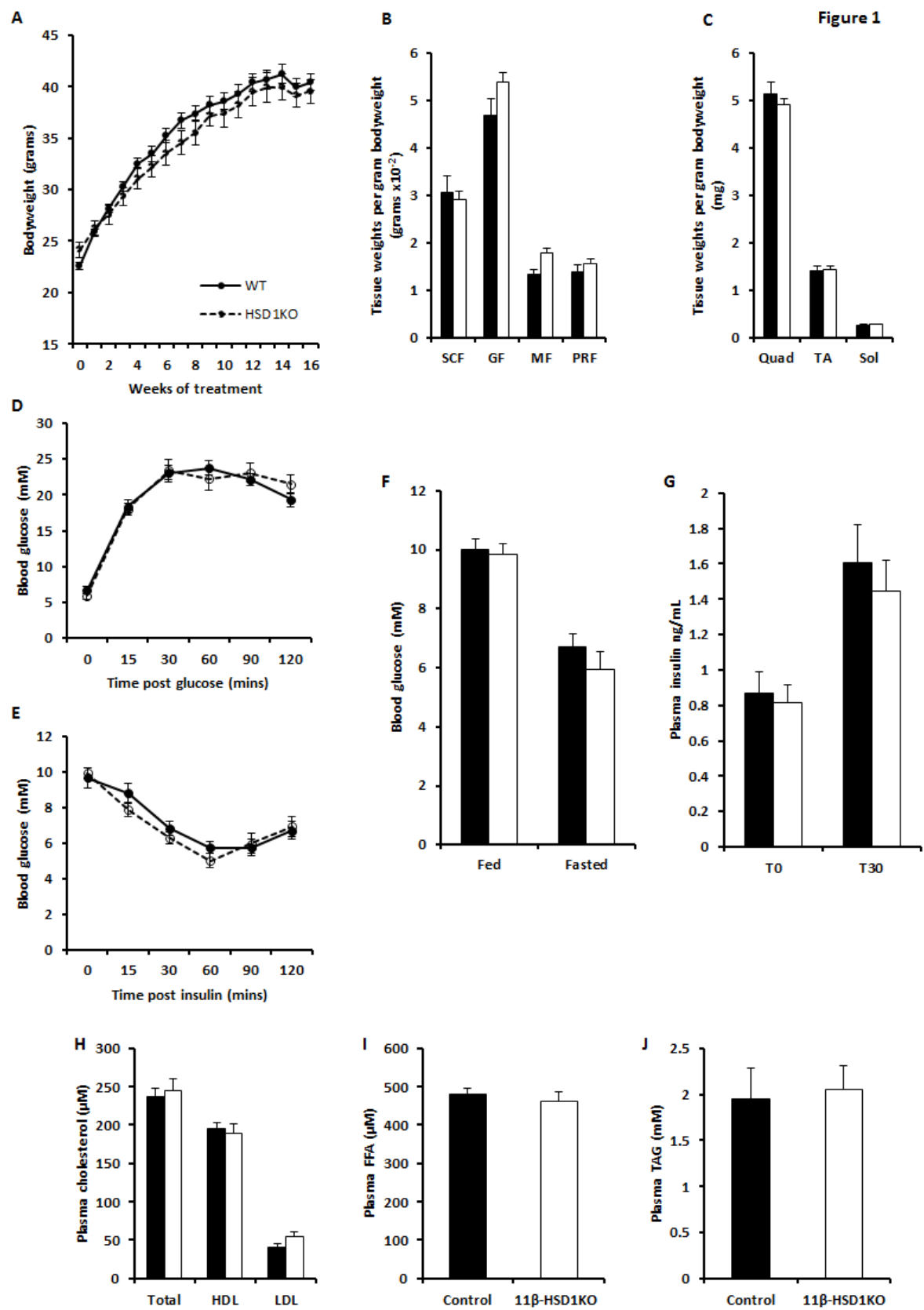
(A) H&E stained sections cut at 5  $\mu$ m show inflammatory foci (arrows) in livers of controls and 11 $\beta$ -HSD1KO mice, areas inside boxes highlight inflammatory foci (magnified an additional 2X). (B) Dot plot showing % fields of view with 2 or more inflammatory foci; using the Mann-Whitney test there was a significant increase in 11 $\beta$ -HSD1KO mice compared to controls, which was not seen in livers of LKO mice when compared to their controls (C). \*\*\*P<0.001, interquartile range; n = 13 (control), 11 (11 $\beta$ -HSD1KO), 9 (LKO control), 9 (LKO); Foci per field of view at X200 magnification; scale bars 200  $\mu$ m.

**Figure 5. Increased hepatic expression of pro-inflammatory cytokines and macrophage markers in 11 $\beta$ -HSD1KO mice.**

(A) Increased expression of pro-inflammatory cytokines *Tnf* and *Ccl2* in the livers of 11 $\beta$ -HSD1KO mice. (B) Increased expression of macrophage specific markers, *Lgals3*, *Fsp1* and *Cd11b* in 11 $\beta$ -HSD1KO compared to controls. (C) Significant increases were seen in the expression of lymphocyte specific markers *Cd8* and *B220*. Mean  $\pm$  SEM; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 using student's t-test; n = 13 (control), 11 (11 $\beta$ -HSD1KO).

**Figure 6. Increased expression of fibrosis-associated gene does not correlate with histological phenotype.**

(A) Significant increases in *Coll1a1* mRNA, a marker of fibrosis, in 11 $\beta$ -HSD1KO mice. Hepatic stellate cell marker Reelin (*Reln*), was not increased. (B) Assessment of fibrosis using Van Gieson stained sections cut at 5  $\mu$ m showed no ectopic collagen in either control or 11 $\beta$ -HSD1KO mice. Mean  $\pm$  SEM; \*\*P<0.01 using student's t-test; n = 13 (control), 11 (11 $\beta$ -HSD1KO); scale bars 100  $\mu$ m.

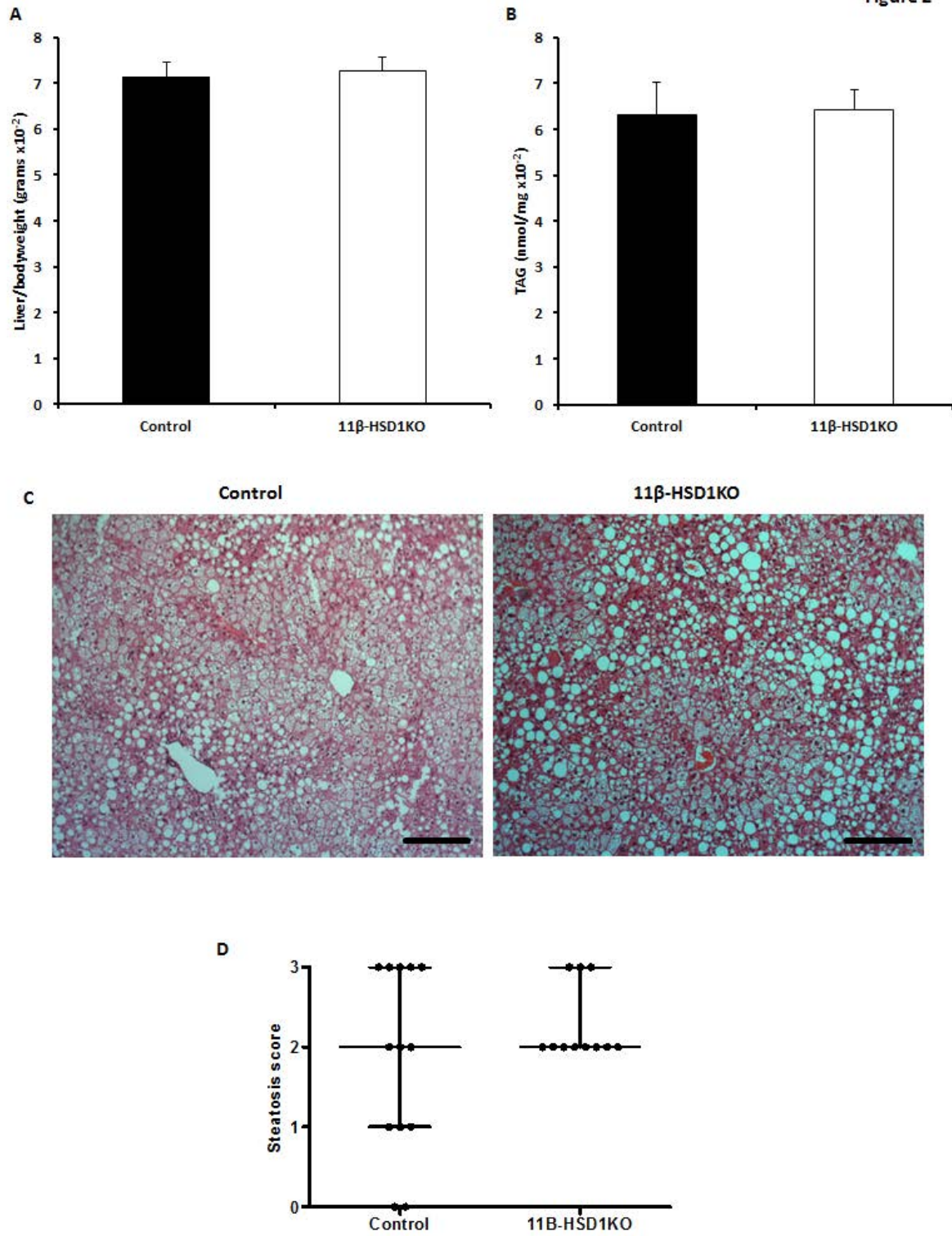


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Figure 2



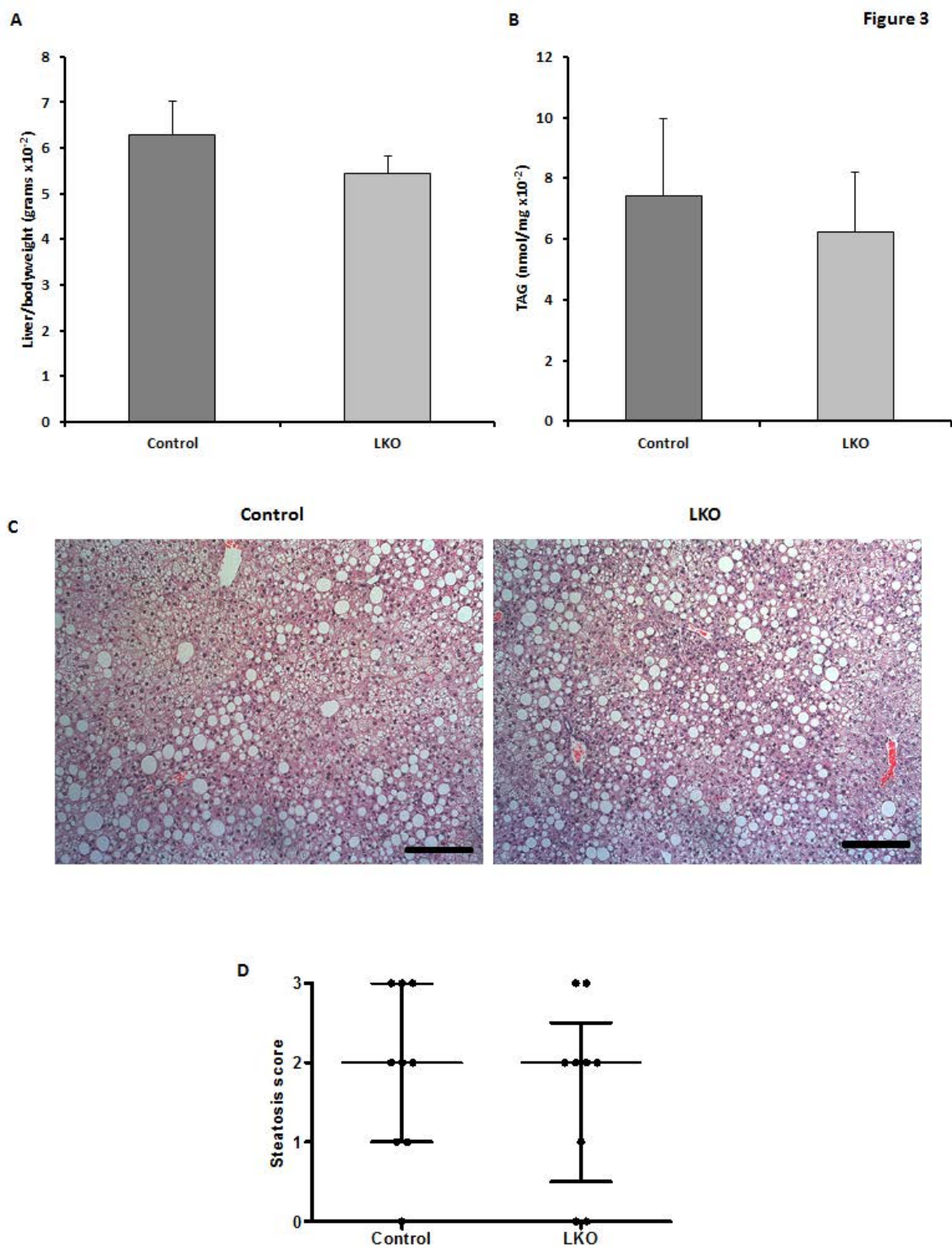
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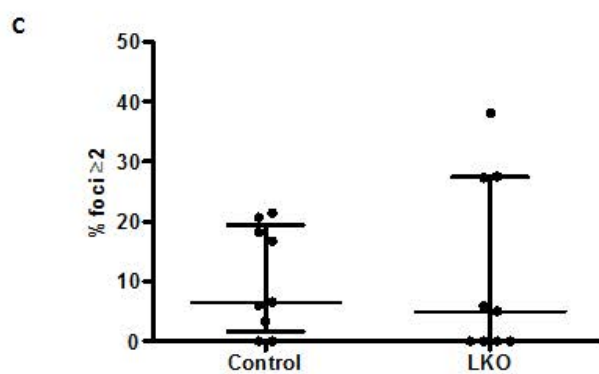
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**A**

11 $\beta$ -HSD1KO

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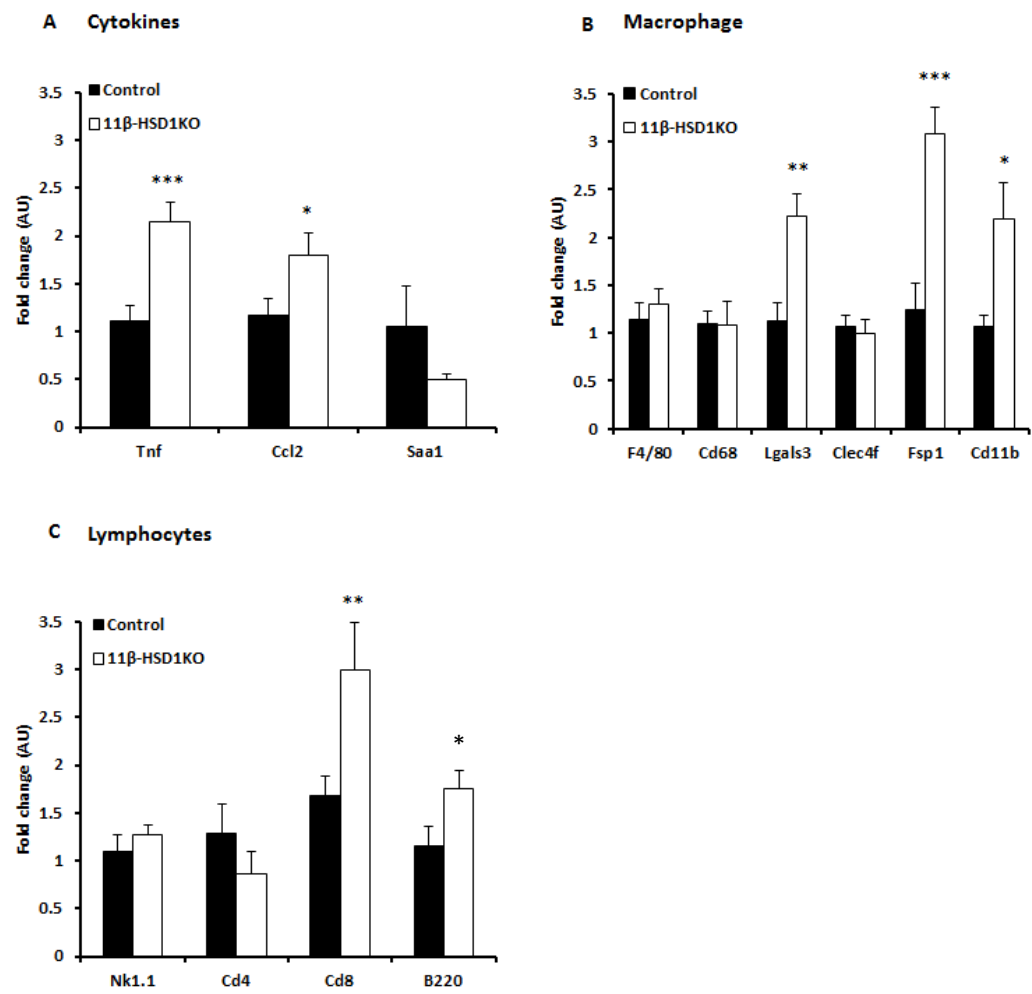
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Figure 5



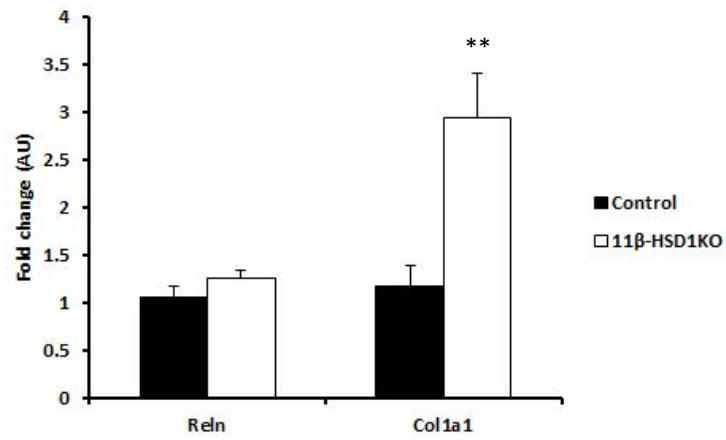
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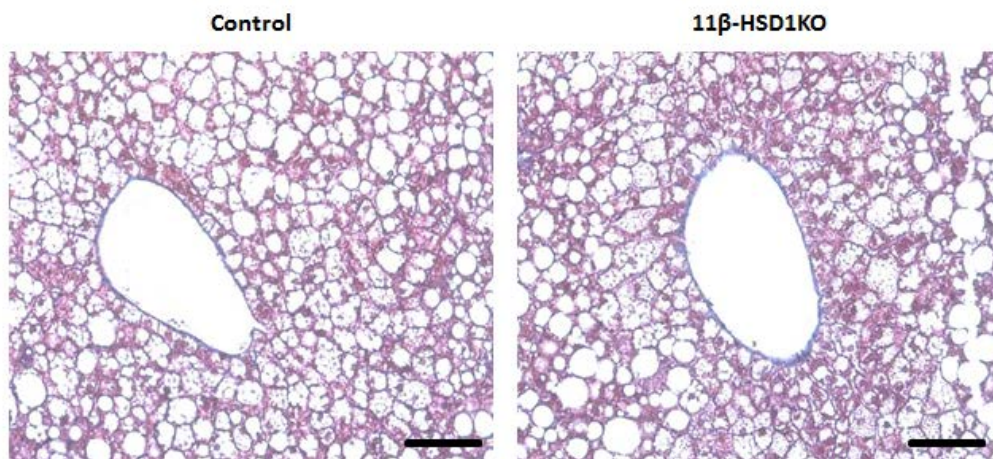
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Figure 6

**A Fibrosis**



**B**



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## 449 REFERENCES

- 450 1. Ludwig, J., et al., *Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto*  
451 *unnamed disease*. Mayo Clin Proc, 1980. **55**(7): p. 434-8.
- 452 2. Ascha, M.S., et al., *The incidence and risk factors of hepatocellular carcinoma in patients with*  
453 *nonalcoholic steatohepatitis*. Hepatology, 2010. **51**(6): p. 1972-8.
- 454 3. Starley, B.Q., C.J. Calcagno, and S.A. Harrison, *Nonalcoholic fatty liver disease and*  
455 *hepatocellular carcinoma: A weighty connection*. Hepatology, 2010. **51**(5): p. 1820-1832.
- 456 4. Ludwig, J., et al., *Nonalcoholic steatohepatitis: Mayo clinic experiences with a hitherto*  
457 *unnamed disease*. Mayo Clin Proc, 1980. **55**: p. 434-438.
- 458 5. Vernon, G., A. Baranova, and Z.M. Younossi, *Systematic review: the epidemiology and*  
459 *natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults*.  
460 *Aliment Pharmacol Ther*, 2011. **34**(3): p. 274-85.
- 461 6. WGO, *Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis*. World  
462 *Gastroenterology Organisation Global Guidelines*, 2012.
- 463 7. Ekstedt, M., et al., *Fibrosis stage is the strongest predictor for disease-specific mortality in*  
464 *NAFLD after up to 33 years of follow-up*. Hepatology, 2015. **61**: p. 1547-1554.
- 465 8. Fabbrini, E., S. Sullivan, and S. Klein, *Obesity and nonalcoholic fatty liver disease:*  
466 *biochemical, metabolic, and clinical implications*. Hepatology, 2010. **51**(2): p. 679-89.
- 467 9. Marchesini, G., et al., *Nonalcoholic fatty liver disease: a feature of the metabolic syndrome*.  
468 *Diabetes*, 2001. **50**(8): p. 1844-50.
- 469 10. Rockall, A., et al., *Hepatic steatosis in Cushing's syndrome: a radiological assessment using*  
470 *computed tomography*. European Journal of Endocrinology, 2003. **149**(6): p. 543-548.
- 471 11. Baxter, J.D. and P.H. Forsham, *Tissue effects of glucocorticoids*. Am J Med, 1972. **53**(5): p.  
472 573-89.
- 473 12. Hellerstein, M.K., *De novo lipogenesis in humans: metabolic and regulatory aspects*.  
474 *European Journal of Clinical Nutrition*, 1999. **53**: p. S53-S65.
- 475 13. Dolinsky, V.W., et al., *Regulation of the enzymes of hepatic microsomal triacylglycerol*  
476 *lipolysis and re-esterification by the glucocorticoid dexamethasone*. Biochemical Journal,  
477 2004. **378**: p. 967-974.
- 478 14. Fraser, R., et al., *Cortisol effects on body mass, blood pressure, and cholesterol in the general*  
479 *population*. Hypertension, 1999. **33**: p. 1364 - 1368.
- 480 15. Gathercole, L.L., et al., *11beta-Hydroxysteroid dehydrogenase 1: translational and*  
481 *therapeutic aspects*. Endocr Rev, 2013. **34**(4): p. 525-55.
- 482 16. Morgan, S.A., et al., *11beta-HSD1 is the major regulator of the tissue-specific effects of*  
483 *circulating glucocorticoid excess*. Proc Natl Acad Sci U S A, 2014.
- 484 17. Morton, N.M., et al., *Novel adipose tissue-mediated resistance to diet-induced visceral*  
485 *obesity in 11 beta-hydroxysteroid dehydrogenase type 1-deficient mice*. Diabetes, 2004.  
486 **53**(4): p. 931-8.
- 487 18. Lavery, G.G., et al., *Lack of significant metabolic abnormalities in mice with liver-specific*  
488 *disruption of 11B-hydroxysteroid dehydrogenase type 1*. Endocrinology, 2012. **153**: p. 3236-  
489 3248.
- 490 19. Livingstone, D.E. and B.R. Walker, *Is 11beta-hydroxysteroid dehydrogenase type 1 a*  
491 *therapeutic target? Effects of carbenoxolone in lean and obese Zucker rats*. J Pharmacol Exp  
492 *Ther*, 2003. **305**(1): p. 167-72.
- 493 20. Masuzaki, H., et al., *A transgenic model of visceral obesity and the metabolic syndrome*.  
494 *Science*, 2001. **294**(5549): p. 2166-70.
- 495 21. Ahmed, A., et al., *A switch in hepatic cortisol metabolism across the spectrum of non*  
496 *alcoholic fatty liver disease*. PLoS One, 2012. **7**(2): p. e29531.
- 497 22. Masuzaki, H. and J.S. Flier, *Tissue-specific glucocorticoid reactivating enzyme, 11B-*  
498 *hydroxysteroid dehydrogenase type 1 (11B-HSD1) - a promising drug target for the*

- treatment of metabolic syndrome. *Current Drug Targets- Immune, Endocrine & Metabolic Disorders*, 2003. **3**: p. 255-262.
23. Hermanowski-Vosatka, A., et al., *11beta-HSD1 inhibition ameliorates metabolic syndrome and prevents progression of atherosclerosis in mice*. *J Exp Med*, 2005. **202**(4): p. 517-27.
  24. Tomlinson, J.W. and P.M. Stewart, *Mechanisms of disease: selective inhibition of 11B-hydroxysteroid dehydrogenase type 1 as a novel treatment for the metabolic syndrome*. *Nature Reviews Endocrinology*, 2005. **1**: p. 92-99.
  25. Anderson, A. and B.R. Walker, *11beta-HSD1 inhibitors for the treatment of type 2 diabetes and cardiovascular disease*. *Drugs*, 2013. **73**(13): p. 1385-93.
  26. Stefan, N., et al., *Inhibition of 11beta-HSD1 with RO5093151 for non-alcoholic fatty liver disease: a multicentre, randomised, double-blind, placebo-controlled trial*. *Lancet Diabetes Endocrinol*, 2014. **2**(5): p. 406-16.
  27. Tetri, L.H., et al., *Severe NAFLD with hepatic necroinflammatory changes in mice fed trans fats and a high-fructose corn syrup equivalent*. *Am J Physiol Gastrointest Liver Physiol*, 2008. **295**(5): p. G987-95.
  28. Semjonous, N.M., et al., *Hexose-6-Phosphate Dehydrogenase Contributes to Skeletal Muscle Homeostasis Independent of 11B-Hydroxysteroid Dehydrogenase Type 1*. *Endocrinology*, 2011. **152**: p. 93-102.
  29. Kleiner, D.E., et al., *Design and validation of a histological scoring system for nonalcoholic fatty liver disease*. *Hepatology*, 2005. **41**(6): p. 1313-21.
  30. Jakobsen, M.U., et al., *Abdominal obesity and fatty liver*. *Epidemiologic Reviews*, 2007. **29**: p. 77-87.
  31. Liu, K.H., et al., *Mesenteric fat thickness as an independent determinant of fatty liver*. *International Journal of Obesity*, 2006. **30**: p. 787-793.
  32. Ma, R.C.W., et al., *Sonographic measurement of mesenteric fat predicts presence of fatty liver among subjects with polycystic ovary syndrome*. *J Clin Endocrinol Metab*, 2011. **96**: p. 799-807.
  33. Basciano, H., L. Federico, and K. Adeli, *Fructose, insulin resistance, and metabolic dyslipidemia*. *Nutr Metab (Lond)*, 2005. **2**(1): p. 5.
  34. Kohli, R., et al., *High-fructose, medium chain trans fat diet induces liver fibrosis and elevates plasma coenzyme Q9 in a novel murine model of obesity and nonalcoholic steatohepatitis*. *Hepatology*, 2010. **52**(3): p. 934-44.
  35. Dhibi, M., et al., *The intake of high fat diet with different trans fatty acid levels differentially induces oxidative stress and non alcoholic fatty liver disease (NAFLD) in rats*. *Nutr Metab (Lond)*, 2011. **8**(1): p. 65.
  36. Dowman, J.K., et al., *Development of Hepatocellular Carcinoma in a Murine Model of Nonalcoholic Steatohepatitis Induced by Use of a High-Fat/Fructose Diet and Sedentary Lifestyle*. *The American Journal of Pathology*, 2014. **184**(5): p. 1550-1561.
  37. Wong, V.W.-S., et al., *Disease progression of non-alcoholic fatty liver disease: a prospective study with paired liver biopsies at 3 years*. *Gut*, 2010. **59**(7): p. 969-974.
  38. Coutinho, A.E., et al., *11beta-Hydroxysteroid dehydrogenase type 1, but not type 2, deficiency worsens acute inflammation and experimental arthritis in mice*. *Endocrinology*, 2012. **153**(1): p. 234-40.
  39. McSweeney, S.J., et al., *Improved heart function follows enhanced inflammatory cell recruitment and angiogenesis in 11betaHSD1-deficient mice post-MI*. *Cardiovasc Res*, 2010. **88**(1): p. 159-67.
  40. Wamil, M., et al., *Novel fat depot-specific mechanisms underlie resistance to visceral obesity and inflammation in 11 beta-hydroxysteroid dehydrogenase type 1-deficient mice*. *Diabetes*, 2011. **60**(4): p. 1158-67.
  41. Christensen, J.E., et al., *CD11b expression as a marker to distinguish between recently activated effector CD8(+) T cells and memory cells*. *Int Immunol*, 2001. **13**(4): p. 593-600.

42. Van Coillie, E., J. Van Damme, and G. Opdenakker, *The MCP/eotaxin subfamily of CC chemokines*. Cytokine Growth Factor Rev, 1999. **10**(1): p. 61-86.
43. Caron, G., et al., *Human NK cells constitutively express membrane TNF-alpha (mTNFalpha) and present mTNFalpha-dependent cytotoxic activity*. Eur J Immunol, 1999. **29**(11): p. 3588-95.
44. Mueller, L., et al., *TNF-alpha similarly induces IL-6 and MCP-1 in fibroblasts from colorectal liver metastases and normal liver fibroblasts*. Biochem Biophys Res Commun, 2010. **397**(3): p. 586-91.
45. Norris, S., et al., *Resident human hepatic lymphocytes are phenotypically different from circulating lymphocytes*. J Hepatol, 1998. **28**(1): p. 84-90.
46. Doherty, D.G., et al., *The human liver contains multiple populations of NK cells, T cells, and CD3+CD56+ natural T cells with distinct cytotoxic activities and Th1, Th2, and Th0 cytokine secretion patterns*. J Immunol, 1999. **163**(4): p. 2314-21.
47. Jomantaite, I., et al., *Hepatic dendritic cell subsets in the mouse*. Eur J Immunol, 2004. **34**(2): p. 355-65.
48. Zhang, T.Y., X. Ding, and R.A. Daynes, *The expression of 11 beta-hydroxysteroid dehydrogenase type I by lymphocytes provides a novel means for intracrine regulation of glucocorticoid activities*. J Immunol, 2005. **174**(2): p. 879-89.
49. Coutinho, A.E., et al., *Mast cells express 11beta-hydroxysteroid dehydrogenase type 1: a role in restraining mast cell degranulation*. PLoS One, 2013. **8**(1): p. e54640.
50. Freeman, L., et al., *Expression of 11beta-hydroxysteroid dehydrogenase type 1 permits regulation of glucocorticoid bioavailability by human dendritic cells*. Blood, 2005. **106**(6): p. 2042-9.
51. Gilmour, J.S., et al., *Local amplification of glucocorticoids by 11 beta-hydroxysteroid dehydrogenase type 1 promotes macrophage phagocytosis of apoptotic leukocytes*. J Immunol, 2006. **176**(12): p. 7605-11.
52. Soulier, A., et al., *Cell-intrinsic regulation of murine dendritic cell function and survival by prereceptor amplification of glucocorticoid*. Blood, 2013. **122**(19): p. 3288-97.
53. Henning, J.R., et al., *Dendritic cells limit fibroinflammatory injury in nonalcoholic steatohepatitis in mice*. Hepatology, 2013. **58**(2): p. 589-602.
54. Thieringer, R., et al., *11 Beta-hydroxysteroid dehydrogenase type 1 is induced in human monocytes upon differentiation to macrophages*. J Immunol, 2001. **167**(1): p. 30-5.
55. Ala-Kokko, L., et al., *Gene expression of type I, III and IV collagens in hepatic fibrosis induced by dimethylnitrosamine in the rat*. Biochem J, 1987. **244**(1): p. 75-9.