Dietary Macronutrient Composition Determines the Contribution of DGAT1 to Alcoholic Steatosis

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Background: The first stage of alcoholic liver disease is hepatic steatosis. While alcohol is known to profoundly impact hepatic lipid metabolism, gaps in our knowledge remain regarding the mechanisms leading to alcohol-induced hepatic triglyceride (TG) accumulation. As the sole enzymes catalyzing the final step in TG synthesis, diacylglycerol O-acyltransferase (DGAT) 1 and 2 are potentially important contributors to alcoholic steatosis. Our goal was to study the effects of dietary fat content on alcohol-induced hepatic TG accumulation, and the relative contribution of DGAT1 and DGAT2 to alcoholic steatosis.

Methods: These studies were carried out in wild-type (WT) mice fed alcohol-containing high-fat or low-fat formulations of Lieber-DeCarli liquid diets, as well as follow-up studies in *Dgat1*$^{-/-}$ mice.

Results: A direct comparison of the low-fat and high-fat liquid diet in WT mice revealed surprisingly similar levels of alcoholic steatosis, although there were underlying differences in the pattern of hepatic lipid accumulation and expression of genes involved in hepatic lipid metabolism. Follow-up studies in *Dgat1*$^{-/-}$ mice revealed that these animals are protected from alcoholic steatosis when consumed as part of a high-fat diet, but not a low-fat diet.

Conclusions: Dietary macronutrient composition influences the relative contribution of DGAT1 and DGAT2 to alcoholic steatosis, such that in the context of alcohol and a high-fat diet, DGAT1 predominates.

Key Words: Alcohol, DGAT, Liver, Steatosis, Triglyceride.

ALCOHOLIC LIVER DISEASE (ALD) is one of the many adverse effects caused by chronic alcohol consumption (Rehm et al., 2009). Hepatic steatosis, or fatty liver, represents the first stage of ALD and is characterized by the accumulation of triglyceride (TG) in the liver. With continued alcohol consumption, fatty liver can progress into steatohepatitis, cirrhosis, hepatocellular carcinoma, and liver failure (Mann et al., 2003; Purohit et al., 2009). The precise mechanisms underlying alcoholic fatty liver are complex and have been the subject of much research in both human and animal models (Purohit et al., 2009; Sozio et al., 2010). Alcohol has been postulated to have multiple effects on hepatic lipid metabolism including increased fatty acid uptake and de novo lipogenesis, as well as decreased fatty acid oxidation and very low density lipoprotein (VLDL) secretion, although the relative contribution of these different factors is unknown (Baraona and Lieber, 1979; Orman et al., 2013). Regarding TG synthesis, chronic alcohol consumption increases the hepatic expression of genes involved in TG synthesis, suggesting a direct role of this pathway in the development of alcoholic steatosis (Clugston et al., 2011, 2014; Wang et al., 2010; Yang et al., 2017).

One of the leading experimental approaches to study the effects of chronic alcohol consumption in rodents is the use of Lieber-DeCarli liquid diets (Bertola et al., 2013; De La Motte Hall et al., 2001). First described in 1967, the use of nutritionally complete liquid diets containing alcohol is a well-established approach to study ALD (Bertola et al., 2013; DeCarli and Lieber, 1967; De La Motte Hall et al., 2001). The primary formulation of these diets provides 36% of calories from fat and therefore can be considered a "high-fat diet." On the other hand, with 12.5% of calories from fat, a "low-fat" version of this diet has been described and is commercially available (Lieber et al., 1989). We recently established that *Cd36*$^{-/-}$ mice are resistant to alcoholic steatosis when fed this low-fat formulation of the Lieber-DeCarli liquid diet (Clugston et al., 2014). Follow-up studies using both high- and low-fat liquid diets led us to focus on

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Received for publication May 15, 2018; accepted September 2, 2018.

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DOI: 10.1111/acer.13881

potential mechanistic differences in the induction of alcoholic steatosis in mice consuming alcohol as part of diets with different fat content. While it is known that diets with different macronutrient composition can contribute differently to nonalcoholic fatty liver disease (NAFLD; Li et al., 2015b; Pierce et al., 2016), to our knowledge this has not been previously explored at the molecular level in the context of liquid diet consumption and alcoholic hepatic steatosis.

As part of our research into macronutrient composition and alcoholic steatosis, our attention became focused on hepatic TG synthesis. It is known that the final step of TG synthesis is mediated by either diacylglycerol O-acyltransferase (DGAT) 1 or 2, which catalyze the addition of a fatty acyl-CoA to diacylglycerol (Liu et al., 2012; Yen et al., 2008). These 2 enzymes are encoded by 2 distinct genes, which do not share sequence homology (Cases et al., 1998, 2001; Lardizabal et al., 2001; Oelkers et al., 1998). Both genes are ubiquitously expressed but with the highest expression in tissues where TG synthesis is active such as adipose tissue, mammary glands, small intestine, and liver (Liu et al., 2012). *DGAT1* expression is up-regulated in the livers of humans with NAFLD (Kohjima et al., 2007), and in agreement with this, overexpression of *Dgat1* in animal models causes excess accumulation of hepatic TGs (Liang et al., 1998; Millar et al., 2006; Monetti et al., 2007; Yamazaki et al., 2005). Conversely, restricting DGAT1 activity through genetic ablation of *Dgat1* or pharmacological inhibition decreases hepatic TG content and protects against high-fat diet-induced steatosis (Sachdev et al., 2016; Smith et al., 2000; Villanueva et al., 2009; Yamaguchi et al., 2008). Investigations of DGAT2 in vivo have been limited as mice with genetic *Dgat2* ablation die shortly after birth due to impaired skin barrier formation and severe lipopenia (Stone et al., 2004). Overexpression of *Dgat2* has parallels to *Dgat1*, such that there is increased hepatic steatosis and associated hepatic insulin resistance (Choi et al., 2007; Liu et al., 2008; Smith et al., 2000; Yu et al., 2005). Similarly, reducing DGAT2 activity using antisense nucleotides reduces high-fat diet-induced obesity and impairs the formation of hepatic cytoplasmic lipid droplets (Choi et al., 2007; Yu et al., 2005).

There are clearly important contributions of both DGAT1 and DGAT2 to hepatic TG synthesis, with more recent studies attempting to dissect distinct roles for each enzyme. For example, in vitro studies have suggested that DGAT2 preferentially uses endogenous fatty acids, that is those generated by de novo lipogenesis to synthesize TG (Qi et al., 2012; Wurie et al., 2012). This contrasts with DGAT1, which preferentially uses exogenous fatty acids as substrate (Qi et al., 2012; Villanueva et al., 2009). In terms of the metabolic fate of TGs synthesized by DGAT1 or DGAT2, it appears that TG synthesized by DGAT1 is primarily channeled toward oxidation, whereas TG synthesized by DGAT2 is primarily channeled toward lipidation of VLDL (Li et al., 2015a). These different roles for DGAT1 and DGAT2 are reflected in the paradigm that they contribute to different lipid droplet populations in hepatocytes. It is thought that DGAT1 preferentially contributes to small lipid droplets (<1 μm), referred to as initial lipid droplets. On the other hand, DGAT2 preferentially contributes to larger lipid droplets (2 to 3 μm or greater), referred to as expanding lipid droplets (Wilfling et al., 2013; Xu et al., 2012).

While there has been considerable focus on the role of DGAT1 and DGAT2 in the context of NAFLD, there have been no mechanistic studies exploring their role in ALD, with the extant literature primarily focused on the effect of alcohol on the expression level of these enzymes. For example, expression of *Dgat1* and *Dgat2* has both been reported to be increased in response to alcohol feeding in mice, rats, and the human HepG2 cells (Clugston et al., 2011, 2014; Wang et al., 2010; Yang et al., 2017). Wang and colleagues (2010) further suggested a direct role of DGAT2 in the pathogenesis of ALD by contributing to hepatic steatosis in association with increased de novo lipogenesis.

The initial goal of this study was to explore the effects of dietary macronutrient composition on the development of alcoholic steatosis, by comparing hepatic TG accumulation in mice consuming the low-fat and high-fat formulations of the Lieber-DeCarli liquid diet. While our results indicated that the extent of hepatic steatosis was comparable between diets, there was evidence for differential contributions of DGAT1 and DGAT2 to this process. Specifically, it appeared that DGAT1 mediated alcohol-induced hepatic TG accumulation in the context of a high-fat diet, whereas DGAT2 was more important in the context of a low-fat diet. Based on these observations, we tested the hypothesis that *Dgat1*$^{-/-}$ mice would be resistant to alcoholic steatosis when fed alcohol as part of a high-fat diet, but not as part of a low-fat diet. The results described below provide evidence that dietary macronutrient composition determines the relative contribution of DGAT1 to the pathogenesis of alcoholic steatosis.

MATERIALS AND METHODS

Animals and Alcohol Feeding Protocol

All animal experiments were approved by the Institutional Animal Care and Use Committee of Columbia University. Age-matched (~6 months old) male mice were used for all experiments and housed in a barrier facility with a 12 hour light:dark cycle. The generation of *Dgat1*$^{-/-}$ mice has previously been described (Smith et al., 2000). *Dgat1*$^{-/-}$ mice on a C57BL/6 background and wild-type (WT) C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

As specified in the Results section below, mice were fed either a high-fat or low-fat formulation of the Lieber-DeCarli liquid diet, the macronutrient composition of which is presented in Fig. 1*A*. We used nondenatured 200 proof ethanol (EtOH) for all our alcohol feeding studies (Decon Labs, King of Prussia, PA). Alcohol groups were adapted to EtOH consumption by introducing 0, 2.1, and 4.2% alcohol by volume at days 1, 3, and 7, respectively, in a 10-day adaptation period. Control mice were pair-fed their respective diet with maltodextrin substituted for EtOH to maintain isocaloric intake relative to alcohol-consuming mice. Feeding was conducted for 6 weeks following the conclusion of the alcohol adaptation period at a final alcohol concentration of 5.1% alcohol by volume. The blood alcohol concentration (BAC) of alcohol-fed mice was assayed

using blood samples collected at midnight, 1 week before the end of the feeding protocol, using a NAD-alcohol dehydrogenase reagent (Sigma-Aldrich, St. Louis, MO).

The 4 experimental groups in our initial comparison of the low-fat and high-fat liquid diets were as follows: (i) control, low-fat diet (LF-C; $n = 5$), (ii) alcohol-containing low-fat diet (LF-A; $n = 5$), (iii) control, high-fat diet (HF-C; $n = 5$), and (iv) alcohol-containing high-fat diet (HF-A; $n = 5$). The 4 experimental groups in our study of alcohol and a low-fat diet in *Dgat1*$^{-/-}$ mice were as follows: (i) WT control ($n = 6$), (ii) WT alcohol ($n = 7$), (iii) *Dgat1*$^{-/-}$ control ($n = 6$), and (iv) *Dgat1*$^{-/-}$ alcohol ($n = 5$). The 4 experimental groups in our study of alcohol and a high-fat diet in *Dgat1*$^{-/-}$ mice were as follows: (i) WT control ($n = 6$), (ii) WT alcohol ($n = 9$), (iii) *Dgat1*$^{-/-}$ control ($n = 6$), and (iv) *Dgat1*$^{-/-}$ alcohol ($n = 4$). All liquid diets were purchased from Bio-Serv (Flemington, NJ): LF-C (F6506SP); LF-A (F6507SP); HF-C (F5937SP); and HF-A (F5938SP).

Tissue Collection and Liver Histology

At the end of each alcohol feeding study, mice were fasted for 4 hours. Next, blood and the following tissues were collected: liver, white adipose tissue (WAT; epigonadal depot), and brown adipose tissue (BAT; intrascapular depot). All tissues were weighed at the time of collection, snap frozen in liquid nitrogen, and stored at −80°C prior to further analysis. At the time of tissue collection, 2 pieces of the liver were also set aside for histological processing by the Pathology Core Facility at the Columbia University Medical Center. Cryopreserved tissue was processed, sectioned with a cryostat, and stained with Oil Red O. Formalin-fixed tissue was embedded in paraffin, sectioned with a microtome, and stained with hematoxylin and eosin (H&E). All images of stained liver sections were captured using an FSX100 microscope (Olympus, Center Valley, PA).

Analysis of Plasma and Hepatic Lipids

The concentration of circulating free fatty acids (FFAs) and TGs was directly measured in plasma as described below. The concentration of hepatic FFAs and TGs was measured in lipid extracts obtained using a Folch solution (chloroform and methanol; 2:1 ratio; Folch et al., 1957). Lipid extracts were solubilized in a 2% Triton X-100 solution prior to analysis. The concentration of FFAs in samples was determined using a HR Series NEFA-HR(2) kit according to the manufacturer's instructions (Wako Diagnostics, Richmond, VA). The concentration of TG in samples was determined using an Infinity TG reagent according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). All protocols were adapted to a 96-well plate format while preserving sample-to-reagent ratios. Plates were read using a SpectraMax 250 Microplate Reader (Molecular Devices, Sunnyvale, CA). The individual acyl composition of plasma and liver FFA was obtained by liquid chromatography-mass spectrometry (LC/MS) using a Waters Xevo TQ MS ACQUITY UPLC system (Waters, Milford, MA), according to our previously published method (Clugston et al., 2011).

Analysis of Hepatic Gene Expression by Quantitative Polymerase Chain Reaction

Total mRNA was extracted from liver tissues homogenized in TRIzol Reagent (Ambion, Carlsbad, CA), according to the manufacturer's protocol. Crude mRNA extracts were purified of

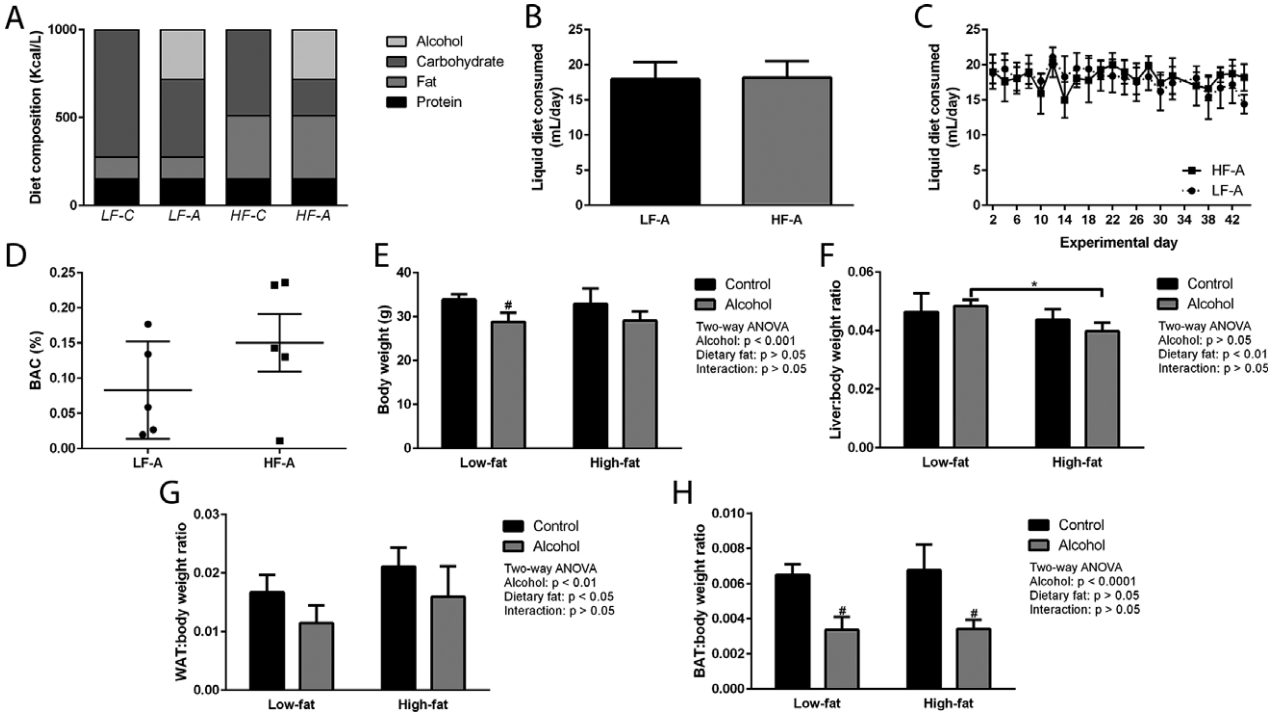

Fig. 1. Comparison of liquid diet macronutrient composition and consumption, and physical characteristics of mice consuming alcohol as part of a high-fat or low-fat liquid diet. The macronutrient composition of the low-fat control (LF-C), low-fat alcohol (LF-A), high-fat control (HF-C), and high-fat alcohol (HF-A) liquid diets is shown (**A**). The overall average (**B**) and daily average (**C**) volume of liquid diet consumed by experimental mice in the LF-A and HF-A groups throughout the experimental time course is presented. A scatterplot showing the average blood alcohol concentration (BAC) in mice from the LF-A and HF-A groups is shown (**D**). The body weight of experimental mice is presented (**E**) alongside the liver (**F**), white adipose tissue (WAT; **G**), and brown adipose tissue (BAT; **H**) weight, normalized to body weight for each experimental group. All data are shown as mean ± SD. (**E**–**H**) Data analyzed by 2-way ANOVA; * represents a significant posttest between animals with the same dietary alcohol content; # represents a significant posttest between animals consuming the same amount of dietary fat.

potential DNA contamination using a RNeasy Mini kit with an on-column DNase digestion step (Qiagen, Valencia, CA). RNA concentration was determined before and after purification using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Reverse transcription of purified RNA was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA), according to the manufacturer's protocol. A LightCycler 480 (Roche Applied Science, Indianapolis, IN) was used to perform quantitative polymerase chain reaction (qPCR) on diluted cDNA, using a SYBR Green I Master Mix (Roche Applied Science), as previously described (Clugston et al., 2011). The full gene name and oligonucleotide sequence of gene-specific primers are provided in Table S1. The expression level of target genes was calculated relative to the reference gene cyclophilin A (*CypA*) using the delta–delta Ct method adapted from Pfaffl (2001). All gene expression data are presented as the calculated expression ratio of the target gene relative to reference gene (*CypA*) expression.

Statistical Analyses

All data are presented as mean ± standard deviation (SD). Comparisons between 2 groups were performed using a Student's *t*-test. Comparisons amongst 4 groups were determined using a 2-way analysis of variance (ANOVA) followed by a Bonferroni posttest between groups using Prism 6 (GraphPad Software, La Jolla, CA). A p-value <0.05 was considered statistically significant.

RESULTS

Alcohol-Consuming WT Mice Have an Equal Preference for Low-Fat and High-Fat Liquid Diets

The primary goal of our first alcohol feeding study was to compare the effect of chronic alcohol consumption as part of a low-fat or high-fat liquid diet on hepatic lipid accumulation. The macronutrient composition of these diets is summarized in Fig. 1*A*. To ensure a fair comparison between alcohol-consuming mice, we compared the volume of liquid diet consumed by LF-A and HF-A mice throughout the alcohol feeding study. The average daily liquid diet consumption between mice in the LF-A and HF-A groups was not significantly different (Fig. 1*B*); similarly, we observed no obvious difference in daily liquid diet consumption at any point throughout the alcohol feeding protocol (Fig. 1*C*). Importantly, there was no significant difference in the BAC of mice consuming either the LF-A or HF-A diet (Fig. 1*D*). As such, the mice consuming alcohol as part of a low-fat or high-fat diet had comparable levels of caloric and alcohol intake.

Physical Characteristics of WT Mice Consuming Alcohol as Part of a Low-Fat or High-Fat Liquid Diet

The effect of dietary fat content and alcohol on body, liver, WAT, and BAT weight was assessed (Table 1; Fig. 1*E–H*). Our 2-way ANOVA revealed that there was no effect of dietary fat content on body weight in control or alcohol-consuming mice (Fig. 1*E*). Alcohol-fed mice were significantly lighter than control mice, reflecting a 4 to 5-g difference in body weight, although the posttest analysis was only significant in mice consuming the low-fat diet (Fig. 1*E*). Dietary fat content was associated with significantly smaller livers in mice consuming a high-fat liquid diet, with no observed effect of alcohol (Fig. 1*F*). In terms of adiposity, dietary fat content was associated with significantly more WAT in mice consuming a high-fat diet, and our 2-way ANOVA revealed a significant effect of alcohol on the WAT:body weight ratio, reflecting an alcohol-associated drop in WAT weight (Table 1; Fig. 1*G*). Consistent with our recent publication (Blaner et al., 2017), we observed a significant effect of alcohol on BAT, such that LF-A and HF-A mice had significantly less BAT compared to their respective controls (Table 1; Fig. 1*H*). Despite this strong alcohol effect, there was no significant effect of dietary fat content on BAT.

Effect of Consuming Alcohol as Part of a Low-Fat or High-Fat Liquid Diet on Circulating and Hepatic TGs

We assessed the effect of dietary fat content on alcohol-induced hepatic lipid by biochemical analysis of hepatic TG content and histological staining of hepatic lipids using Oil Red O (Fig. 2). Our 2-way ANOVA revealed a significant effect of alcohol on hepatic TG concentration, such that alcohol-consuming mice had higher hepatic TG levels than control mice (Fig. 2*A*). This analysis also revealed a significant effect of dietary fat content. Surprisingly, animals consuming a high-fat diet had lower hepatic TG concentration (Fig. 2*A*). This difference was confirmed by Oil Red O staining in the liver of experimental mice (Fig. 2*C–F*). In general, alcohol-fed mice had more Oil Red O staining than control mice, which is consistent with our previous data and data reported by others (Fig. 2*B,D*; Clugston et al., 2014; Mehlem et al., 2013). Interestingly, this effect was most pronounced in the LF-A group, which appeared to have much larger lipid droplets than the HF-A group (Fig. 2*D*). In terms of plasma lipids, alcohol increased the concentration of circulating TGs, which was most pronounced in the HF-A group (Fig. 2*B*). There was also a significant diet effect on circulating TGs, such that animals on a high-fat diet had higher levels of TG in their circulation.

Effect of Consuming Alcohol as Part of a Low-Fat or High-Fat Liquid Diet on Hepatic FFAs

In addition to TG concentration, we also measured the effect of alcohol and dietary fat content on hepatic FFAs (Fig. 3). Our 2-way ANOVA revealed that alcohol significantly increased total hepatic FFA concentration, which is driven by a significant increase in hepatic FFAs in the HF-A group (Fig. 3*A*). Reflecting this, dietary fat consumption was also associated with a significantly higher FFA level in the liver. A complete breakdown of the concentration of the 17 hepatic FFAs obtained by LC/MS is provided in Table S2. To better understand what was driving the changes in total hepatic FFA concentration, we conducted a

Table 1. Physical Characteristics of WT Mice Consuming Alcohol as Part of a High-Fat or Low-Fat Diet

	Low-fat control	Low-fat alcohol	High-fat control	High-fat alcohol	*p*-Value (2-way ANOVA) Alcohol	Diet
Body weight (g)	33.8 ± 1.25	28.8 ± 2.06#	32.86 ± 3.52	29.1 ± 2.10	<0.001	n.s.
Liver weight (g)	1.56 ± 0.17	1.40 ± 0.14	1.43 ± 0.13	1.16 ± 0.09#	<0.01	<0.01
WAT weight (g)	0.57 ± 0.11	0.33 ± 0.10	0.70 ± 0.18	0.47 ± 0.18	<0.005	n.s.
BAT weight (g)	0.22 ± 0.01	0.10 ± 0.03#	0.22 ± 0.07	0.10 ± 0.01#	<0.0001	n.s.
Liver:body ratio	0.05 ± 0.01	0.05 ± 0.002	0.04 ± 0.003	0.04 ± 0.003*	n.s.	<0.01
WAT:body ratio	0.02 ± 0.003	0.01 ± 0.003	0.02 ± 0.003	0.02 ± 0.005	<0.01	<0.05
BAT:body ratio	0.006 ± 0.0006	0.003 ± 0.0007#	0.006 ± 0.001	0.003 ± 0.0005#	<0.0001	n.s.

All groups, $n = 5$; data are mean ± SD; #$p < 0.05$ for comparisons between alcohol-fed mice within the same dietary group; *$p < 0.05$ for comparisons between dietary group within the same alcohol treatment.

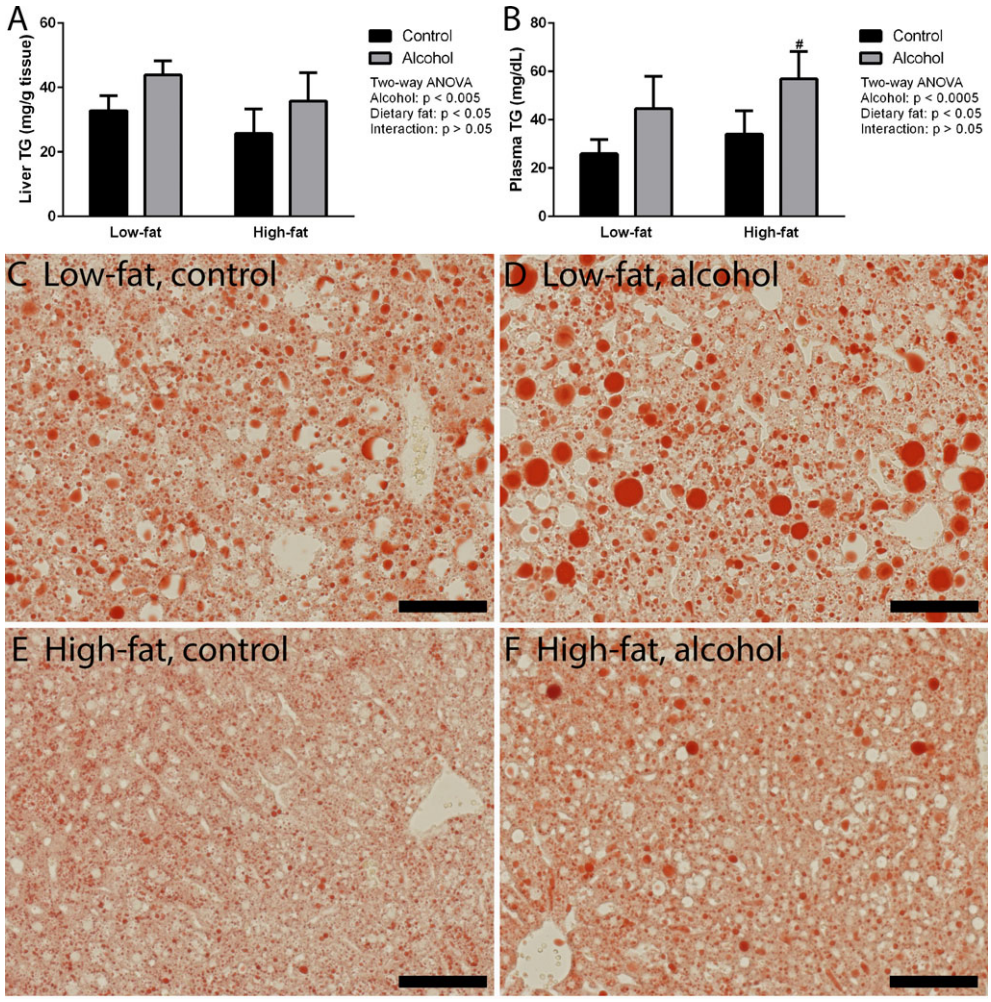

Fig. 2. Effect of consuming alcohol as part of a high-fat or low-fat diet on hepatic and circulating triglycerides (TGs) levels. The concentration of hepatic (**A**) and circulating (**B**) TGs is shown for all experimental mice. Corresponding to the biochemical measures of hepatic TG concentration, representative images of Oil Red O-stained liver sections are shown for mice in the low-fat control (**C**), low-fat alcohol (**D**), high-fat control (**E**), and high-fat alcohol (**F**) groups are shown. (**A**, **B**) All data are shown as mean ± SD; data analyzed by 2-way ANOVA; # represents a significant posttest between animals consuming the same amount of dietary fat. **C**-**F**: Scale bar = 100 μm.

subgroup analysis of the concentration of hepatic saturated fatty acids (SFA; Fig. 3*B*), monounsaturated fatty acids (MUFA; Fig. 3*C*), and polyunsaturated fatty acids (PUFA; Fig. 3*D*). We did not observe any significant effect of alcohol or diet on hepatic SFA or MUFA concentration, but the high-fat diet was associated with significantly higher hepatic PUFAs levels than the low-fat diet. Alcohol also significantly increased the concentration of hepatic PUFAs in groups

consuming both a low-fat and high-fat diet. Given the striking differences observed in the concentration of hepatic PUFAs, we chose to present the data for the following PUFAs: linoleic acid (C18:2; Fig. 3*E*), linolenic acid (C18:3; Fig. 3*F*), eicosapentaenoic acid (C20:5; Fig. 3*G*), and docosahexaenoic acid (C22:6; Fig. 3*H*). In all cases, 2-way ANOVA revealed significant effects of alcohol and dietary fat content that were consistent with the changes observed in total hepatic PUFA concentration.

In addition to hepatic FFA levels, we also analyzed circulating FFA levels by LC/MS. We did not observe any striking changes in total circulating FFAs levels, or in terms of the relative contribution of SFAs, MUFAs, and PUFAs to this total. The concentration of each of the 17 FFA species we measured is presented in Table S3.

Altered Hepatic Gene Expression in Response to Alcohol as Part of High-Fat or Low-Fat Diet

To gain insight into potential underlying differences in the pathogenesis of alcoholic steatosis in mice consuming either a low-fat or high-fat diet, we conducted a survey of the

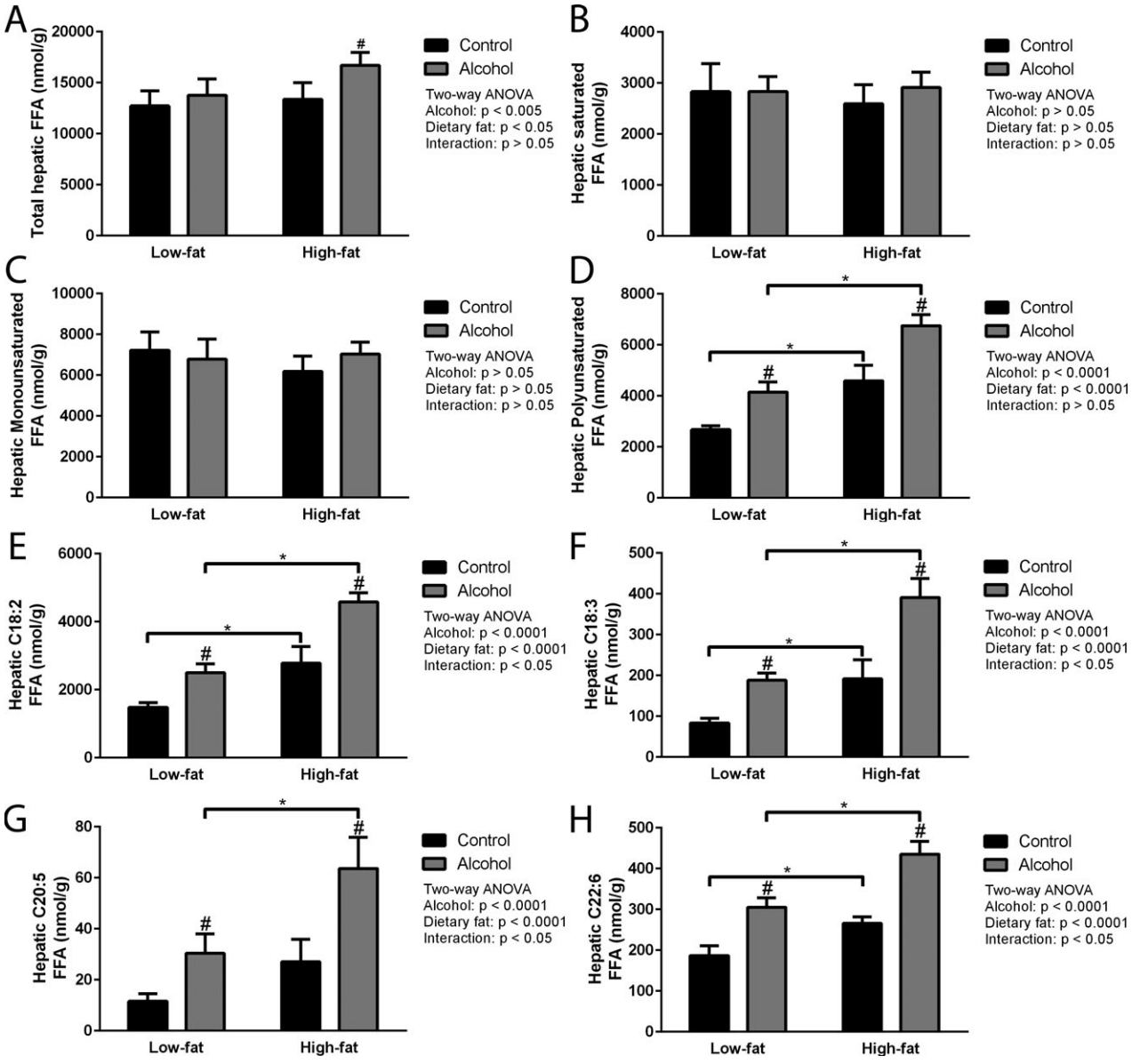

Fig. 3. Effect of consuming alcohol as part of a high-fat or low-fat diet on hepatic FFA levels. Hepatic FFA levels were measured in the liver using LC/MS. The total hepatic FFA levels are presented for each experimental group (**A**). Given the change in total hepatic FFA levels, we also analyzed and presented the subtotals for saturated (**B**), monounsaturated (**C**), and polyunsaturated FFAs (**D**). Based on this subtype analysis, it became apparent that changes in polyunsaturated FFAs underlay the changes in total FFAs; therefore, we also analyzed and presented the concentration of linoleic acid (C18:2; **E**), linolenic acid (C18:3; **F**), eicosapentaenoic acid (C20:5; **G**), and docosahexaenoic acid (C22:6; **H**). All data are shown as mean ± SD; data analyzed by 2-way ANOVA; * represents a significant posttest between animals with the same dietary alcohol content; # represents a significant posttest between animals consuming the same amount of dietary fat.

expression level of *Dgat1* and *Dgat2*, as well as key transcriptional regulators of hepatic lipid metabolism: *Ppara*, *Pparg*, *Srebf1*, and *Chrebp* (Fig. 4). We observed no effect of diet or alcohol on hepatic *Dgat1* expression (Fig. 4*A*). Hepatic *Dgat2* expression was not affected by alcohol, but there was a significant effect of dietary fat content, such that *Dgat2* expression was lower in mice consuming a high-fat diet. Our posttest analysis also revealed significantly lower *Dgat2* expression in the HF-A group versus the LF-A group (Fig. 4*B*). The hepatic expression level of *Ppara* was not affected by diet or alcohol (Fig. 4*C*). On the other hand, hepatic *Pparg* mRNA levels were significantly lower in animals consuming a high-fat diet, with no effect of alcohol (Fig. 4*D*). Our posttest analysis of *Pparg* also showed significantly lower expression in the HF-C group versus the LF-C group, and the HF-A group versus the LF-A group. Like *Dgat2* and *Pparg*, *Srebf1* expression was also significantly lower in the mice consuming a high-fat diet, with posttest analysis revealing significantly reduced expression levels of this gene between control and alcohol-fed mice consuming a high-fat diet (Fig. 4*E*). On the other hand, hepatic expression of *Chrebp* was significantly higher in animals consuming a high-fat diet, but there was no alcohol effect (Fig. 4*F*).

Based on the appearance of larger lipid droplets in mice consuming alcohol as part of a low-fat diet (Fig. 2*D*), and the higher hepatic expression of pro-lipogenic genes in mice on a low-fat diet, we next focused on the different contributions of DGAT1 and DGAT2 to alcohol-induced steatosis in the context of a low-fat and high-fat diet. Because *Dgat2*$^{-/-}$ mice are not viable (Stone et al., 2004), these follow-up studies focused on alcohol feeding studies in *Dgat1*$^{-/-}$ mice.

Physical Characteristics of Dgat1$^{-/-}$ *Mice Consuming Alcohol as Part of a Low-Fat or High-Fat Liquid Diet*

We performed 2 separate alcohol feeding studies using WT and *Dgat1*$^{-/-}$ mice, the first one used a low-fat liquid diet, and the second used a high-fat liquid diet. The effects of these different dietary regimens on body, liver, WAT, and BAT weight are presented in Table 2 for the alcohol feeding study using a low-fat diet, and in Table 3 for the alcohol feeding study using a high-fat diet. In both studies, we

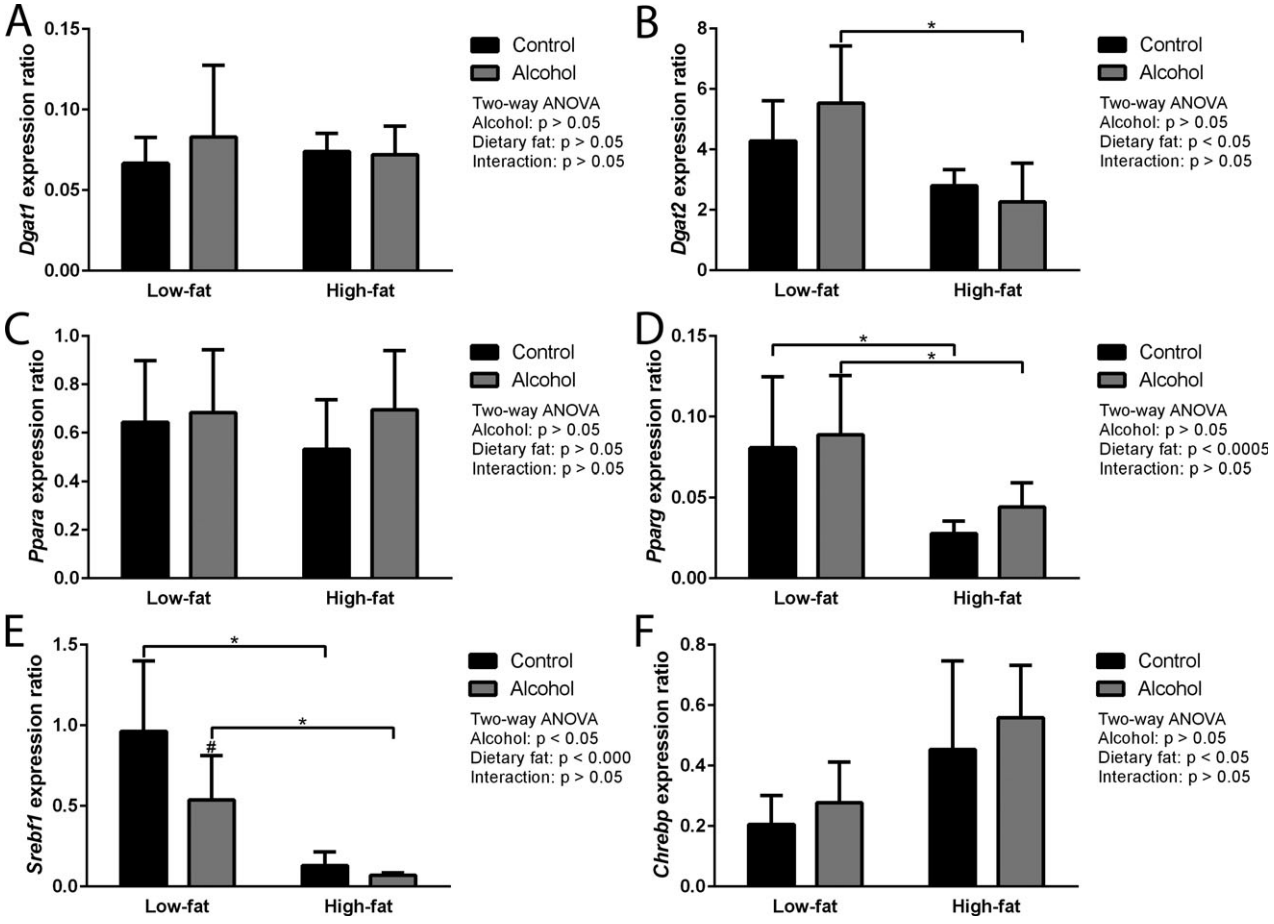

Fig. 4. Altered hepatic gene expression in response to alcohol as part of high-fat or low-fat diet. The relative hepatic gene expression levels, as determined by qPCR, are shown for *Dgat1* (**A**), *Dgat2* (**B**), *Ppara* (**C**), *Pparg* (**D**), *Srebf1* (**E**), and *Chrebp* (**F**). Target gene expression ratios are normalized relative to the reference gene *CypA*. All data are shown as mean ± SD; data analyzed by 2-way ANOVA; * represents a significant posttest between animals with the same dietary alcohol content; # represents a significant posttest between animals consuming the same amount of dietary fat.

observed an effect of genotype on body weight, such that *Dgat1*$^{-/-}$ mice were significantly lighter than WT mice, which is consistent with previous descriptions of these mice (Smith et al., 2000). Although our 2-way ANOVA was not significant, our posttest analysis did show a significant decrease in the body weight of alcohol-consuming WT mice on the low-fat diet, but not in *Dgat1*$^{-/-}$ mice. In terms of absolute liver weight, and its normalized ratio to body weight, the livers of alcohol-consuming mice were larger in the low-fat study, regardless of genotype. Indeed, alcohol significantly increased the liver:body weight ratio in WT and *Dgat1*$^{-/-}$ mice consuming a low-fat diet. While the effects of alcohol in the high-fat study were less pronounced, alcohol significantly increases the liver:body weight ratio in WT mice, but not *Dgat1*$^{-/-}$ mice. Consistent with the results from our first study, the amount of WAT and BAT in alcohol-consuming WT mice was significantly decreased in mice consuming both a low-fat and high-fat diet. *Dgat1*$^{-/-}$ mice had lower amounts of both WAT and BAT (significant genotype effect), and in accord with these lower total levels of adipose tissue, alcohol's effect seemed to be blunted.

Effect of Dietary Fat Content on Hepatic TG Accumulation in Dgat1$^{-/-}$ *Mice*

We measured the concentration of hepatic TGs in the liver of WT and *Dgat1*$^{-/-}$ mice consuming alcohol as part of a low-fat or high-fat liquid diet (Fig. 5). *Dgat1*$^{-/-}$ mice consuming a low-fat diet, like WT mice, had a highly significant increase in hepatic TG concentration with alcohol consumption (Fig. 5*A*). Strikingly, in mice consuming a high-fat diet, only alcohol was associated with a significant increase in hepatic TG concentration in WT mice, but not *Dgat1*$^{-/-}$ mice (Fig. 5*B*). This observation agrees with the alcohol-associated increase in liver:body weight ratio seen in WT mice but not *Dgat1*$^{-/-}$ mice on a high-fat diet described above. *Dgat1*$^{-/-}$ did have lower average hepatic TG levels than WT mice, though this effect was not seen in *Dgat1*$^{-/-}$ mice consuming a low-fat diet.

To corroborate our biochemical measures of hepatic TG concentration, we also assessed the extent of Oil Red O staining in histological liver sections taken from our experimental mice. Consistent with the data presented in Fig. 5, the most striking effects were in the mice consuming a low-fat diet (Fig. 6). The representative images presented in Fig. 6 clearly show more Oil Red O staining in the livers of alcohol-fed mice, with apparent lipid accumulation in both WT and *Dgat1*$^{-/-}$ mice. High magnification images of H&E-stained sections show large lipid droplets, consistent with our data presented in Fig. 2. On the other hand, while WT mice consuming alcohol as part of a high-fat diet had noticeably more Oil Red O staining in their liver, this effect was notably absent in *Dgat1*$^{-/-}$ mice, which appeared to have low levels of staining in both the control and alcohol-fed groups (Fig. 7).

In addition to hepatic TGs, we also measured TG levels in the plasma, as well as plasma and hepatic FFA concentrations. No striking effects of alcohol or genotype were observed for either of these parameters and the data are presented in Table S4.

DISCUSSION

Our initial goal was to compare alcohol-induced hepatic TG accumulation in mice consuming low-fat and high-fat formulations of the Lieber-DeCarli liquid diet, a common experimental approach to induce ALD (Bertola et al., 2013; De La Motte Hall et al., 2001). Based on our initial findings, subsequent work focused on hepatic TG synthesis induced by alcohol, revealing a preferential role for DGAT1 in the context of an alcohol-containing high-fat diet, but not low-fat diet. Below, we discuss differences in hepatic lipid metabolism in mice consuming liquid diets with these different macronutrient composition, and how the different DGAT enzymes play distinct roles in alcohol-induced hepatic TG accumulation depending on dietary macronutrient content.

If we take the concentration of hepatic TG as our primary end point, then the effects of a low-fat versus high-fat diet were surprisingly similar. Although alcohol consumption was associated with significantly higher levels of hepatic TGs, there was no significant difference between alcohol-consuming mice fed either a low-fat or high-fat diet.

Table 2. Physical Characteristics of WT and *Dgat1*$^{-/-}$ Mice Consuming Alcohol as Part of a Low-Fat Diet

					p-Value (2-way ANOVA)	
	WT control	WT alcohol	*Dgat1*$^{-/-}$ control	*Dgat1*$^{-/-}$ alcohol	Genotype	Alcohol
Body weight (g)	32.9 ± 2.33	29.9 ± 1.57	28.6 ± 2.53*	29.6 ± 1.71	n.s.	<0.05
Liver weight (g)	1.33 ± 0.24	1.53 ± 0.19	1.07 ± 0.15	1.56 ± 0.12$^{\#}$	n.s.	<0.005
WAT weight (g)	0.48 ± 0.10	0.33 ± 0.07$^{\#}$	0.25 ± 0.08*	0.22 ± 0.05	<0.0001	<0.05
BAT weight (g)	0.13 ± 0.03	0.09 ± 0.02$^{\#}$	0.08 ± 0.02*	0.07 ± 0.01	<0.005	<0.05
Liver:body ratio	0.04 ± 0.005	0.05 ± 0.006$^{\#}$	0.04 ± 0.005	0.05 ± 0.005$^{\#}$	n.s.	<0.0001
WAT:body ratio	0.015 ± 0.003	0.011 ± 0.002$^{\#}$	0.009 ± 0.002*	0.007 ± 0.001*	<0.0001	<0.01
BAT:body ratio	0.004 ± 0.0009	0.003 ± 0.0006	0.003 ± 0.0006*	0.003 ± 0.0004	<0.01	<0.05

All groups are $n = 5$ to 7; data are mean ± SD; $^{\#}p < 0.05$ for comparisons between diets within the same genotype; $^{*}p < 0.05$ for comparisons between genotypes within the same diet.

Table 3. Physical Characteristics of WT and *Dgat1*$^{-/-}$ Mice Consuming Alcohol as Part of a High-Fat Diet

					p-Value (2-way ANOVA)	
	WT control	WT alcohol	*Dgat1*$^{-/-}$ control	*Dgat1*$^{-/-}$ alcohol	Genotype	Alcohol
Body weight (g)	34.8 ± 2.37	29.8 ± 1.99$^{\#}$	26.5 ± 1.54*	28.5 ± 1.91	<0.0001	n.s.
Liver weight (g)	1.43 ± 0.12	1.35 ± 0.16	1.09 ± 0.08*	1.27 ± 0.15	<0.005	n.s.
WAT weight (g)	0.74 ± 0.16	0.43 ± 0.16$^{\#}$	0.16 ± 0.05*	0.18 ± 0.06*	<0.0001	<0.05
BAT weight (g)	0.17 ± 0.05	0.08 ± 0.02$^{\#}$	0.07 ± 0.01*	0.05 ± 0.006	<0.0001	<0.0001
Liver:body ratio	0.04 ± 0.002	0.05 ± 0.003$^{\#}$	0.04 ± 0.003	0.04 ± 0.003	n.s.	<0.01
WAT:body ratio	0.021 ± 0.004	0.014 ± 0.005$^{\#}$	0.006 ± 0.002*	0.006 ± 0.002*	<0.0001	<0.05
BAT:body ratio	0.005 ± 0.001	0.003 ± 0.0006$^{\#}$	0.003 ± 0.0005*	0.002 ± 0.0002	<0.0001	<0.0001

All groups are $n = 4$ to 9; data are mean ± SD; $^{\#}p < 0.05$ for comparisons between diets within the same genotype; $^{*}p < 0.05$ for comparisons between genotypes within the same diet.

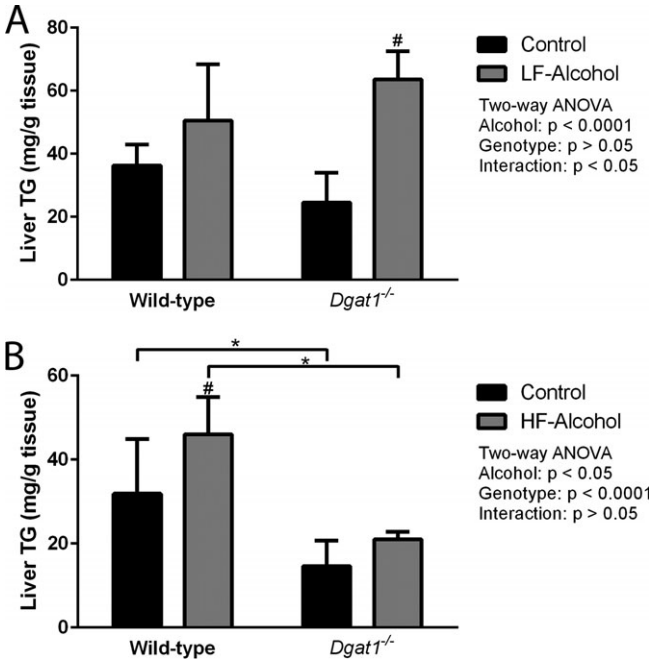

Fig. 5. Effect of dietary fat content on alcohol-induced TG accumulation in *Dgat1*$^{-/-}$ mice. The concentration of hepatic triglycerides (TGs) is shown for WT and *Dgat1*$^{-/-}$ mice consuming a low-fat (LF; **A**) and high-fat liquid diet (HF; **B**). All data are shown as mean ± SD; data analyzed by 2-way ANOVA; * represents a significant posttest between animals with the same dietary alcohol content; # represents a significant posttest between animals consuming the same amount of dietary fat.

However, a closer look at the data revealed several differences between these different dietary approaches that suggest unique underlying mechanisms resulting in excess hepatic TG accumulation. For example, mice consuming the low-fat liquid diet had significantly higher hepatic TG levels, a finding that was echoed in the higher relative liver size of these mice. Increased liver weight and more hepatic steatosis are consistent with a previous comparison of the low-fat and high-fat formulations of the Lieber-DeCarli liquid diet, although this prior work did not study the effects of alcohol (Li et al., 2015b). In addition to higher hepatic TG levels, the pattern of lipid accumulation was strikingly different, such that mice consuming alcohol as part of a low-fat diet had visibly larger lipid droplets, as evidence by our Oil Red O staining, the significance of which will be discussed below.

Another interesting difference observed in our comparison of the low-fat and high-fat liquid diets was hepatic FFA composition. In terms of total hepatic FFA concentration, alcohol-consuming mice on a high-fat liquid diet had significantly elevated levels of FFAs. When we dissected this difference further, we found no differences in SFAs and MUFAs, but significant increases in PUFAs. Interestingly, alcohol-consuming mice on both a low-fat and high-fat diet had significantly elevated levels of PUFAs in their livers, but this increase was significantly potentiated in mice consuming a high-fat diet. When we looked at individual PUFA species, an identical pattern was observed; for example, levels of linoleic acid (C18:2), linolenic acid (C18:3), eicosapentaenoic acid (C20:5), and docosahexaenoic acid (C22:6) were significantly elevated in alcohol-consuming mice, with the highest levels observed in mice on the high-fat diet. Thus, increased total hepatic FFA concentration in mice consuming alcohol as part of a high-fat diet was caused by significant increases in multiple species of PUFA. Considering the relatively higher levels of fatty acids in the high-fat formulation of the liquid diet, this result is perhaps unsurprising, but why these fatty acids accumulate in the liver is unclear. The observed accumulation of PUFAs in the liver of alcohol-fed mice is consistent with our previous work (Clugston et al., 2011) and that of others (Clugston et al., 2017). The molecular mechanism for this phenomenon is unclear and warrants further investigation. Possible explanations include preferential uptake of PUFAs into the liver, increased synthesis of PUFAs by the liver, or their decreased metabolism. The notion that alcohol increases hepatic desaturase activity has been reported (Narce et al., 2001; Pawlosky et al., 2009), but the molecular mechanisms for this have not been elucidated.

Although PUFAs are considered beneficial in terms of cardiovascular health (Jump et al., 2012; Rizos et al., 2012), there is an older literature suggesting that dietary fish oils rich in PUFAs can exacerbate ALD (Morimoto et al., 1994; Nanji et al., 1994). Indeed, as recently reviewed by Kirpich and colleagues (2016) in the context of alcoholism, dietary SFA is considered protective, whereas dietary unsaturated fatty acids are linked with worsened ALD, a finding echoed

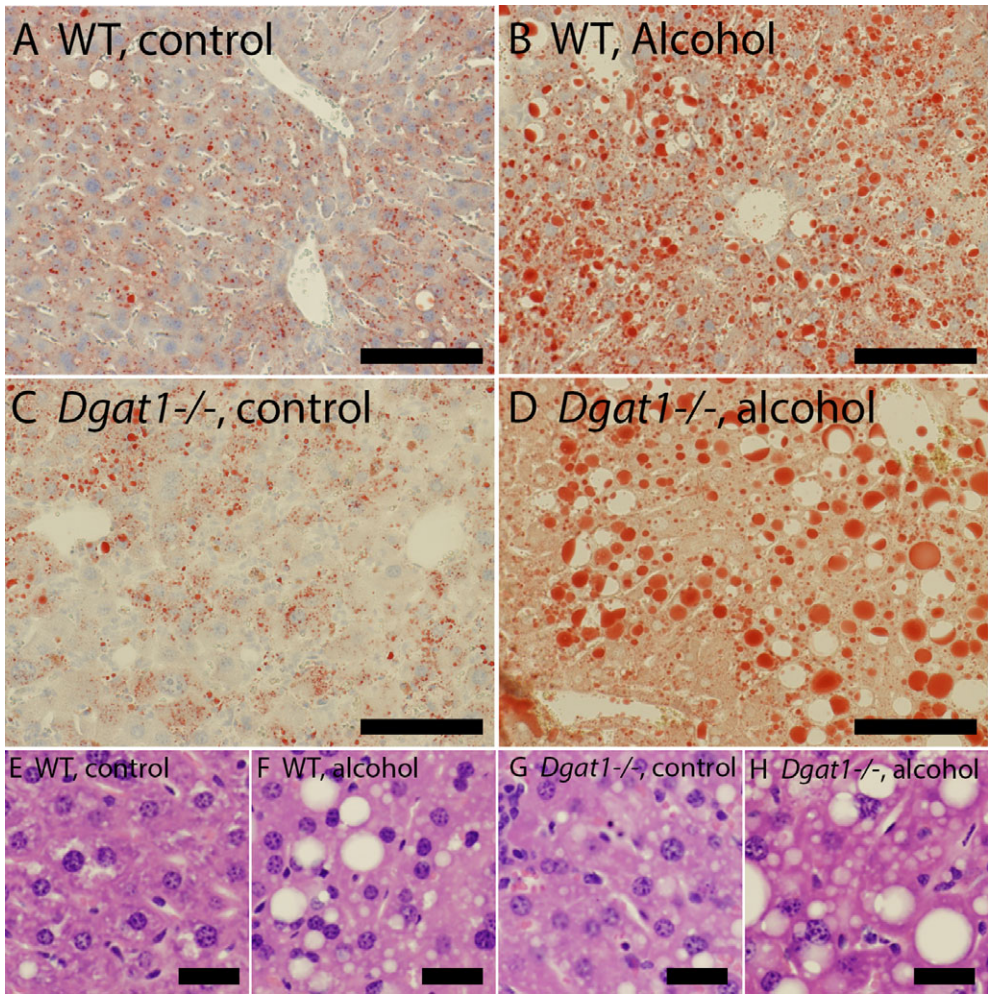

Fig. 6. Effect of consuming alcohol as part of a low-fat diet on hepatic lipid accumulation in *Dgat1*$^{-/-}$ mice. Representative images of Oil Red O-stained liver sections are shown for mice in the WT control (**A**), WT alcohol (**B**), *Dgat1*$^{-/-}$ control (**C**), and *Dgat1*$^{-/-}$ alcohol (**D**) groups, being fed a low-fat liquid diet. Higher magnification images highlight differences in lipid droplet size between groups: WT control (**E**), WT alcohol (**F**), *Dgat1*$^{-/-}$ control (**G**), and *Dgat1*$^{-/-}$ (**H**) alcohol. (**A**–**D**) scale bar = 100 μm, (**E**–**F**) scale bar = 50 μm.

in animal and epidemiological studies. At the molecular level, the protective effects of SFAs have been linked to the induction of SIRT1, which is associated with decreased signaling through SREBP-1 and a resultant decrease in expression of the pro-lipogenic genes SCD1 and FAS (You et al., 2008). Understanding why PUFAs accumulate in the liver of alcohol-fed mice and what impact this might have on the pathogenesis of ALD is an important future direction.

Given the diet-dependent effect of hepatic TG concentration, and the apparent difference we observed in lipid droplet size between alcohol-fed mice consuming a low-fat versus high-fat diet, we focused on the roles of DGAT1 and DGAT2 in alcohol-induced hepatic TG synthesis. When we studied the relative mRNA expression level of *Dgat1* and *Dgat2*, we did not observe any effects of alcohol, although we did note that *Dgat2* expression was significantly higher in mice consuming a low-fat diet. The expression of other transcriptional regulators of hepatic lipid metabolism followed a similar pattern, with significantly elevated levels of *Pparg* and *Srebf1* in the mice on a low-fat diet, except for *Chrebp*, which was significantly elevated in the liver of mice on a high-fat diet. In the liver, PPARγ is an important transcriptional regulator of lipid metabolism, the activation of which is typically associated with increased TG synthesis (Gavrilova et al., 2003). Similarly, signaling through SREBP-1 is an important regulator of hepatic lipid metabolism, primarily in association with the control of de novo lipogenesis (Ferre and Foufelle, 2010). While there is no evidence that SREBP-1 can control either DGAT1 or DGAT2 expression, both genes are thought to be up-regulated via PPARγ (Yen et al., 2008). Accordingly, we interpreted our gene expression data to indicate that the low-fat diet stimulated a lipogenic gene program that favored de novo lipogenesis, and an increase in *Dgat2* expression. This pattern is consistent with other work studying NAFLD caused by high-carbohydrate diets (Moore et al., 2014).

The relative effects of signaling via SREBP1 and ChREBP in mice consuming low-fat and high-fat liquid diets

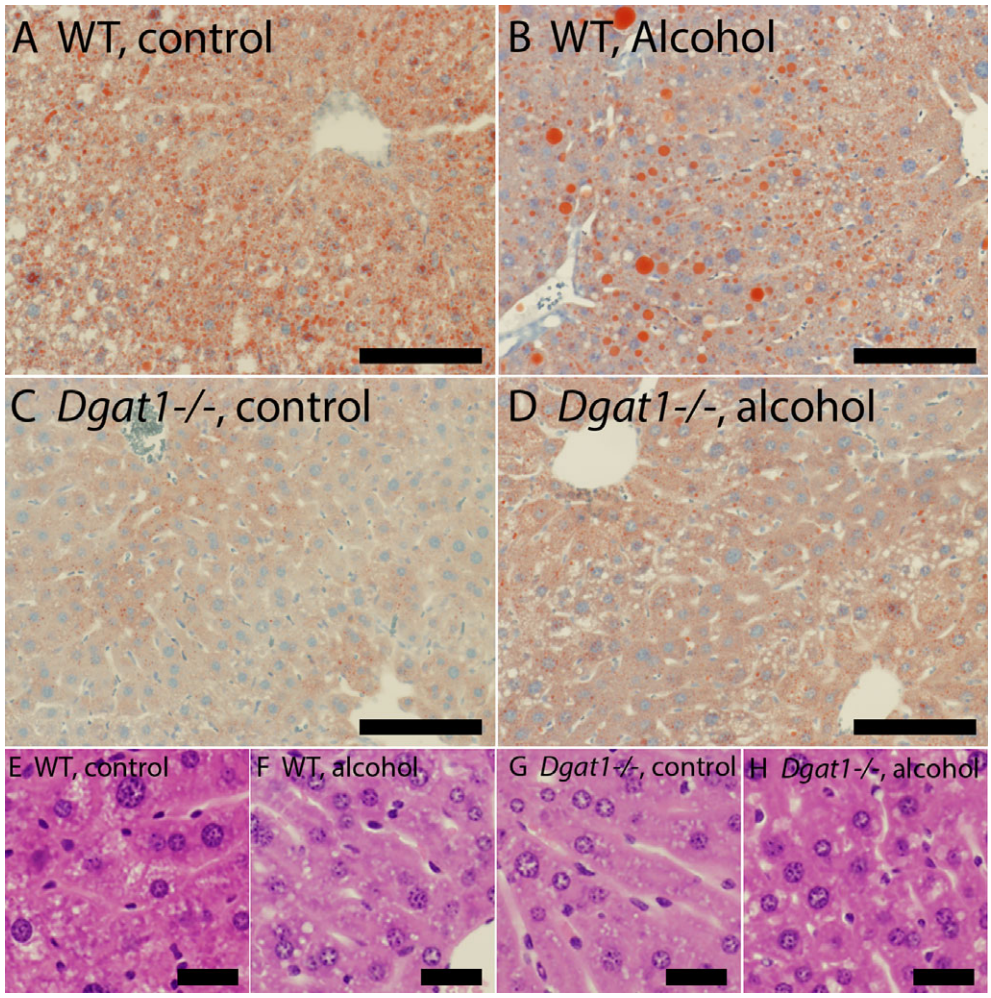

Fig. 7. Effect of consuming alcohol as part of a high-fat diet on hepatic lipid accumulation in *Dgat1*$^{-/-}$ mice. Representative images of Oil Red O-stained liver sections are shown for mice in the WT control (**A**), WT alcohol (**B**), *Dgat1*$^{-/-}$ control (**C**), and *Dgat1*$^{-/-}$ alcohol (**D**) groups, being fed a high-fat liquid diet. Higher magnification images highlight differences in lipid droplet size between groups: WT control (**E**), WT alcohol (**F**), *Dgat1*$^{-/-}$ control (**G**), and *Dgat1*$^{-/-}$ (**H**) alcohol. (**A**–**D**) scale bar = 100 μm, (**E**–**F**) scale bar = 50 μm.

containing alcohol have previously been studied. Liangpunsakul and colleagues (2013) found increased nuclear localization of ChREBP in alcohol-fed mice consuming a low-fat, liquid diet signals an increase in de novo lipogenesis via FAS and ACC. This is similar to data from Ronis and colleagues (2014) who reported a similar finding in rats fed alcohol intragastrically. Our analysis of *Chrebp* mRNA levels revealed increased expression in the livers of mice consuming a high-fat diet, although we did not observe any significant changes in the expression of *Fasn* or *Acc* (data not shown). This result is similar to that observed by Ronis and colleagues (2014) who reported higher ChREBP expression in rats consuming a high-fat diet, albeit we did not observe a significant alcohol effect. Interestingly, our results for *Srebf1* expression closely match Ronis and colleagues (2014), such that they observed decreased expression in response to alcohol and higher dietary fat content. Moreover, these results are consistent with another study showing decreased *Srebf1* expression and no corresponding change in *Fasn* expression (You et al., 2008). Another possible explanation for the lipogenic effect of the low-fat diet in the absence of increased ACC expression is that activity of this enzyme was increased allosterically by elevated levels of citrate, as previously described (Singh et al., 2008). While our primary focus in this article was on hepatic lipids, the transcriptional regulation of the different dietary responses is the focus of future studies.

Higher expression levels of *Dgat2* in mice consuming a low-fat diet, as well as the presence of large lipid droplets in mice in the low-fat, alcohol group drew our attention to the potentially different roles that DGAT1 and DGAT2 might play in hepatic TG accumulation in response to dietary fat content and alcohol. As introduced, it is known that DGAT2 contributes to a pool of TGs that is preferentially stored in expanded lipid droplets, whereas DGAT1 contributes to smaller, initial lipid droplets (Li et al., 2015a). It is also known that DGAT2 prefers endogenous fatty acids derived from de novo lipogenesis, whereas DGAT1 prefers

exogenous fatty acids (Qi et al., 2012). With this literature in mind, we derived 2 working models to explain our data. First, the low-fat formulation of the Lieber-DeCarli liquid diet is associated with increased hepatic de novo lipogenesis, with the resultant endogenously produced fatty acids incorporated into TG preferentially by DGAT2. Second, the high-fat formulation of the Lieber-DeCarli liquid diet is associated with an influx of exogenous fatty acids into the liver, which are preferentially incorporated into TG by DGAT1.

The link between the low-fat diet and de novo lipogenesis rests in the fact that this diet has a relatively high-carbohydrate content compared to the high-fat diet. Indeed, others have shown that a high-carbohydrate diet will produce greater steatosis than a high-fat diet, with corresponding markers of increased hepatic de novo lipogenesis (Li et al., 2015b; Pierce et al., 2016; Schwarz et al., 2003). Thus, in our model, we propose that increased carbohydrate flux into the liver serves as a substrate for de novo lipogenesis, with the resultant fatty acids channeled toward TG synthesis catalyzed by DGAT2. The first point here is important to emphasize. While the different formulations of the Lieber-DeCarli liquid diets are commonly referred to as being either high- or low-fat, this is perhaps a misnomer. Our results show that the low-fat diet led to comparable TG accumulation in alcohol-consuming mice, but it is likely the high amount of carbohydrates in this diet that leads to increased de novo lipogenesis as a pathway toward increased TG synthesis. In this regard, it may be more accurate to refer to these diets as low-fat/high-carbohydrate and high-fat/low-carbohydrate. As future research focuses on the molecular mechanisms of alcoholic steatosis, dietary macronutrient composition should be an important consideration as it is possible that different results could be obtained using different diets.

There is an important point to be made here regarding the method of alcohol delivery. While ours is the first comparison of alcohol-consuming mice fed low-fat and high-fat liquid diets, Ronis and colleagues (2014) compared different dietary fat and carbohydrate ratios in rats using intragastric infusion of alcohol. Because of methodological differences (e.g., species, alcohol feeding protocol, and dietary macronutrient composition), we must be cautious in comparing the results of these studies; however, several points of agreement between both studies provide support for our conclusions. Most notably, Ronis and colleagues (2014) observed that rats consuming alcohol and a high-carbohydrate diet had higher hepatic TG levels and had obviously larger hepatic lipid droplets than animals consuming alcohol and a high-fat diet. This is in agreement with our results and others (Li et al., 2015b), and supports the notion that low-fat/high-carbohydrate diets are more lipogenic than high-fat/low-carbohydrate diets.

In the second part of our study, we decided to test our working model by feeding *Dgat1*$^{-/-}$ mice alcohol in the context of a low-fat and high-fat diet. Based on our model, we predicted that *Dgat1*$^{-/-}$ mice would be resistant to alcoholic steatosis in the context of a high-fat diet because they would be unable to convert exogenously sourced fatty acids into TG in the absence of DGAT1. Conversely, we predicted that *Dgat1*$^{-/-}$ mice would still develop alcoholic steatosis in the context of a low-fat diet, because the endogenously produced fatty acids could be incorporated into TG by DGAT2. Our results from 2 separate feeding studies in *Dgat1*$^{-/-}$ mice are consistent with our working model. When we fed *Dgat1*$^{-/-}$ mice alcohol in the context of a high-fat diet, these mice were protected from alcohol-induced hepatic TG accumulation. On the other hand, when we fed *Dgat1*$^{-/-}$ mice alcohol in the context of a low-fat diet, they accumulated significantly more hepatic TGs than control mice. These results lead us to the conclusion that dietary macronutrient composition determines the relative contribution of DGAT1 to the development of alcoholic steatosis, such that in the context of a high-fat diet, it is the primary contributor to TG synthesis, whereas in the context of a low-fat diet, it is less important, with DGAT2 presumably synthesizing the TG that accumulates in the liver of these mice.

The finding that *Dgat1*$^{-/-}$ mice are resistant to alcoholic steatosis is consistent with observations from these mice when fed a high-fat diet or when DGAT1 activity is inhibited by other means (Sachdev et al., 2016; Smith et al., 2000; Villanueva et al., 2009; Yamaguchi et al., 2008). Despite this existing data in the context of NAFLD, our study is the first to show that *Dgat1*$^{-/-}$ mice are resistant to alcoholic steatosis in the context of a high-fat diet. To our knowledge, resistance to NAFLD has not been studied in *Dgat1*$^{-/-}$ mice when consuming a low-fat/high-carbohydrate diet and certainly not in the context of chronic alcohol consumption. The finding that *Dgat1*$^{-/-}$ mice develop alcoholic steatosis in the context of a low-fat diet, but not a high-fat diet, is an important observation, helping to uncover the importance of substrate preference between DGAT1 and DGAT2 in the development of fatty liver. Indeed, our work provides important in vivo evidence to support the notion that DGAT1 prefers exogenous fatty acids as a substrate, whereas DGAT2 prefers endogenously synthesized fatty acids, at least in the context of chronic alcohol consumption.

If we consider alcoholic steatosis in humans, then our study is important because it is the first to highlight the role of dietary macronutrient composition in determining the relative contribution of DGAT1 and DGAT2 to the pathogenesis of alcoholic steatosis. Given that pharmacological modulators are being developed for both enzymes (e.g., Imbriglio et al., 2015; Meyers et al., 2015), dietary macronutrient composition should be an important variable when testing the efficacy of these drugs. Indeed, while it is known that heavy drinkers may consume as much as 50% of their caloric intake as alcohol (Lands and Zakhari, 1991), the potential impact of where the balance of the caloric intake comes from (i.e., fat vs. carbohydrate) has not been studied. Interestingly, current clinical guidelines for nutritional therapy in ALD include a diet with 50 to 65% of calories coming

from carbohydrates and 25 to 35% of calories coming from fat (Kirpich et al., 2016).

While our primary focus was on the effect of dietary fat content on alcohol-induced changes in hepatic lipid metabolism, we also assessed markers of hepatic inflammation and fibrosis (Fig. S1). These data show that we did not observe any strong differences between alcohol-consuming mice on either a low-fat or high-fat diet. We saw no significant effects on the hepatic expression level of the inflammatory markers *Tnfa* or *Tgfb1*, although hepatic *Ccl2* expression was increased in alcohol-consuming mice, particularly on a high-fat diet. In terms of fibrosis markers, there were no significant effects on the expression of *Col1a1* or *Acta2*, although both showed a trend for increased expression in the alcohol-fed mice on a high-fat diet. Thus, there may be some evidence to suggest that animals fed alcohol and a high-fat diet tend to have more liver injury, but our data do not strongly support this conclusion.

A limitation of this study is our emphasis on the role of DGAT1, without equal attention to DGAT2. As such, while we are confident of our conclusions regarding the role of DGAT1 in alcoholic steatosis, those regarding DGAT2 are more inferential. As introduced, $Dgat2^{-/-}$ mice are not viable; therefore, it was not possible for us to conduct the same feeding studies in $Dgat2^{-/-}$ mice that we did in $Dgat1^{-/-}$ mice. Future work exploring a liver-specific deletion of *Dgat2* would circumvent this limitation but was beyond the scope of the current work. We should also recognize the limitations of our animal feeding protocol, which utilized Lieber-DeCarli liquid diets. While this model is well established for the study of alcoholic steatosis, it tends not to produce advanced alcoholic liver injury (e.g., fibrosis; De La Motte Hall et al., 2001). Given that the focus of this study was on hepatic lipid metabolism and steatosis, we remain confident in our conclusions.

In conclusion, in mice chronically consuming alcohol, the low-fat and high-fat formulations of the Lieber-DeCarli liquid diets produce comparable levels of hepatic TG accumulation, though this effect is seemingly mediated by distinct pathways of lipid metabolism in the liver. In terms of TG synthesis, we show that DGAT1 is required for the development of alcoholic steatosis in the context of a high-fat diet but is dispensable in the context of a low-fat diet. Our data indicate dietary macronutrient composition is an important determinant of which DGAT enzyme primarily contributes to alcohol-induced hepatic TG synthesis. This finding is an important consideration when studying the pathogenic mechanisms underlying ALD, as well as developing potential therapeutic interventions to prevent alcoholic steatosis.

ACKNOWLEDGMENTS

This work was supported by the following U.S. National Institutes of Health grants: K99 AA022652 (RDC), R21 AA020561 (L-SH), R21 HL112233 (L-SH), R21 AA021336 (WSB), R01 DK068437 (WSB), R01 DK101251 (WSB), and HL45095 (IJG). RDC was also supported by an operating grant from the Canadian Institutes of Health Research (CIHR), Institute of Nutrition, Metabolism and Diabetes (PJT 156226). MJT was supported by a Canadian Natural Sciences and Engineering Research Council (NSERC), Alexander Graham Bell Canada Graduate Scholarship-Doctoral. AS (undergraduate), CTE and SK (MSc students) contributed to this work as part of their training at the Columbia University Medical Center. L-SH's current affiliation is at the Division of Cardiovascular Sciences, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD. The content of this manuscript is the sole responsibility of the authors and does not represent the official view of the National Institutes of Health. The authors have no conflict of interest to declare.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Markers of liver injury in mice consuming alcohol as part of a low-fat or high-fat liquid diet.

Table S1. Primer sequences used for qPCR.

Table S2. Breakdown of hepatic FFA composition.

Table S3. Breakdown of plasma FFA concentration.

Table S4. Hepatic and plasma lipid levels in alcohol-consuming *Dgat1*$^{-/-}$ mice.