Analysis of the liver lipidome reveals insights into the protective effect of exercise on high fat diet induced hepatosteatosis in mice.


1. Baker IDI Heart & Diabetes Institute, 75 Commercial Road, Melbourne, VIC, 3004 Australia.
2. Department of Nutrition, Exercise and Sports (NEXS), University of Copenhagen, 13 Universitetsparken, Copenhagen, 2100, Denmark.

Running head: Protective effects of exercise on hepatosteatosis.

†Address for correspondence: Darren C Henstridge
Cellular and Molecular Metabolism Laboratory
Baker IDI Heart and Diabetes Institute
75 Commercial Rd, Melbourne 3004 Victoria, Australia
Ph: +61 3 8532 1708, Fax: +61 3 8532 1100
Email: darren.henstridge@bakeridi.edu.au

Word Count Abstract: 250, Word Count Main Text: 4977

Copyright © 2015 by the American Physiological Society.
ABSTRACT

The accumulation of lipid at ectopic sites including the skeletal muscle and liver is a common consequence of obesity and is associated with tissue-specific and whole-body insulin resistance. Exercise is well known to improve insulin resistance by mechanisms not completely understood. We performed lipidomic profiling via mass spectrometry in liver and skeletal muscle samples from exercise trained mice, to decipher the lipid changes associated with exercise-induced improvements in whole body glucose metabolism. Obesity and insulin resistance was induced in C57BL/6J mice by high fat feeding for four weeks. Mice then underwent an exercise training program (treadmill running) five days a week (Ex) for four weeks or remained sedentary (Sed). Compared with Sed, Ex displayed improved (P<0.01) whole-body metabolism as measured via an oral glucose tolerance test. Deleterious lipid species such as diacylglycerol (DG) (P<0.05) and cholesterol esters (P<0.01) which accumulate with high fat feeding were decreased in the liver of trained mice. Furthermore, the ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE) (the PC/PE ratio), which is associated with membrane integrity and linked to hepatic disease progression was increased by training (P<0.05). These findings occurred without corresponding changes in the skeletal muscle lipidome. A concomitant decrease (P<0.05) was observed for the fatty acid transporters CD36 and FATP4 in the liver, suggesting exercise stimulates a coordinated reduction in fatty acid entry into hepatocytes. Given the important role of the liver in the regulation of whole body glucose homeostasis, hepatic lipid regression may be a key component by which exercise can improve metabolism.

KEYWORDS: Hepatic steatosis, exercise, lipids, fatty acid transporters, skeletal muscle, insulin resistance.
ABBREVIATIONS

ACC: acetyl-CoA carboxylase
ALT: alanine aminotransferase
AMPK: AMP-activated protein kinase
AST: asparate aminotransferase
β-HAD: β-hydroxyacyl-CoA dehydrogenase
CD36: fatty acid transporter CD36 (Cluster of Differentiation 36), also known as FAT (fatty acid translocase)
CPT1A: carnitine palmitoyltransferase 1A
CS: citrate synthase
gDG: diacylglycerol
DGAT1: diacylglycerol O-acyltransferase 1
Ex: exercise training
FASN/FAS: fatty acid synthase
FATP4: fatty acid transporter 4
Gcgr glucagon receptor
Gck glucokinase
GLP-1: glucagon-like peptide-1
HFD: high fat diet
IHL: intrahepatic lipid
LBM: lean body mass
Mlxipl: carbohydrate-responsive element-binding protein (protein name ChREBP))
NAFLD: non-alcoholic fatty liver disease
NASH: non-alcoholic steatohepatitis
NC: normal chow diet
PC: phosphatidylcholine

PE: phosphatidylethanolamine

*PNPLA3*: patatin-like phospholipase domain-containing 3 gene

PPARalpha: peroxisome proliferator-activated receptor alpha

SCD1: stearoyl-CoA desaturase 1

Sed: sedentary

*Srebf1*: sterol regulatory element binding factor -1 (protein name SREBP1-c)),

TG: triacylglycerol (TG)
Introduction

It is now recognized that the current worldwide obesity epidemic has resulted in the increased prevalence of “metabolic disease clusters” including Type 2 Diabetes, fatty liver disease and certain cancers (21). As body weight increases, expanded adipose tissue depots are no longer able to optimally utilize or store excess lipid, resulting in the accumulation of lipid metabolites at ectopic sites including the skeletal muscle and liver. This peripheral intramyocellular and intrahepatic (IHL) lipid accumulation is associated with tissue-specific and whole body insulin resistance and, in the case of the liver, is referred to as hepatosteatosis or non alcoholic fatty liver disease (NAFLD).

NAFLD has become the most common liver disorder in the 21st century and now affects one third of adults and an increasing number of children (9). NAFLD has a progressive disease spectrum that is initiated by simple steatosis that arises from an imbalance between triglyceride accumulation and removal, either via VLDL export into blood or fatty acid oxidation within the liver (9). If NAFLD continues to remain unchecked it may result in non-alcoholic steatohepatitis (NASH), cirrhosis and eventually hepatocellular carcinoma (HCC) (38). Genetic risk factors have been identified which predispose individuals to development of NAFLD, most notably, a missense mutation in patatin-like phospholipase domain-containing 3 gene (PNPLA3) (36). Independent of known genetic risk factors, obesity in itself, dramatically increases the risk of IHL accumulation and subsequently the progression of NAFLD. Demonstrating the power of weight loss on liver health, a meta-analysis reported that weight-loss surgery leads to improvement and/or resolution of NASH in ~80% of patients (30). Current therapeutic strategies to treat NAFLD include prescription of insulin sensitizing drugs such as Metformin (6), thiazolidinediones (1, 2) and glucagon-like peptide-1 (GLP-1) receptor agonists (10) which have been shown to be effective in treating NAFLD,
even though some patients with this disorder do not present with insulin resistance. While regular exercise is known to reduce obesity, largely by increasing energy expenditure and lipid oxidation in contracting skeletal muscle, it may also be effective as a treatment for NAFLD independently of weight loss. Studies in patient cohorts have demonstrated an inverse association between physical activity and fitness and NAFLD (8, 31), while training interventions (17, 23, 40) can impact on NAFLD in a positive manner. Similar findings have been made using various rodent models (3) (35).

Despite the clear nexus between obesity, liver fat and liver disease, many questions remain regarding the pathogenesis and treatment of this disorder. For example, some patients with NAFLD progress to NASH and cirrhosis, while others do not. One factor that likely influences NAFLD disease progression is the specific lipid species which accumulate in response to nutrient overload. For example, alterations in specific lipid species without changes in overall IHL accumulation protects against high fat diet (HFD)-induced hepatic insulin resistance in a mouse lacking a long-chain fatty acid elongase, elongation of long-chain fatty acids family member-6 (Elov6) (28). This suggests that the ratio of certain lipid species may be more important than the total amount that has accumulated (5). In addition, not only the individual lipid species, but also the relative ratio of certain lipids, may play a role in liver pathology. Accordingly, the ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE) is associated with membrane integrity and liver failure (25).

As lipid species of different fatty acid compositions may be important regulators of cellular proliferation (12), inflammation (27) and ER stress-induce apoptosis (4), it is important to delineate the specific lipid signature of NAFLD, as it could provide insights into the signaling pathways relevant in the progression of the condition. In a comparison of murine with human...
liver tissue samples, mice maintained on a HFD provide a reproducible model of human
NAFLD in regards to the specificity of diacylglycerol (DG) and other lipid species in the
liver (14). Currently, an analysis of the precise lipid composition at the species level in the
skeletal muscle and liver following exercise training has not been described. Accordingly, to
thoroughly investigate the impact of both a HFD and exercise training on IHL and
intramyocellular accumulation, we performed a comprehensive lipodomic analysis on a broad
array of lipid classes in mice. Of significance, we show that a HFD increases a wide array of
liver triacylglycerol (TG), DG and cholesterol ester species, which are reversed by exercise
training. Moreover, while the HFD decreased the PC/PE ratio, this was also reversed by
exercise training.
Materials and Methods

Mouse studies.

All experiments were approved by the Alfred Medical Research Education Precinct Animal Ethics Committee and animals were provided humane care in line with the “Principles of laboratory care” (NIH publication no. 85-23, revised 1985) and in accordance with the National Health and Medical Research Council of Australia Guidelines on Animal experimentation. Animals were administered their prescribed diet and water *ad libitum* and housed in a temperature controlled environment (~22°C) with a 12 hour light–dark cycle.

Eight week old male mice (C57Bl/6J background) were fed either regular normal chow diet (NC) (14.3 MJ/kg, 76% of kJ from carbohydrate, 5% fat, 19% protein, Specialty Feeds, Glen Forrest, Western Australia, Australia) or for the fat-feeding studies, a HFD (19 MJ/kg, 36% of kJ from carbohydrate, 43% fat [42.7% saturated, 35.1 monounsaturated and 21.7% polyunsaturated FAs], 21% protein, Specialty Feeds) for a total of 8 wks. Mice were divided into either sedentary control or exercise groups after 4 wks of the respective diets so that there was not a significant difference in body weight between the groups at the start of the exercise training regime. Mice allocated to the exercise training groups completed a 4 wk period of treadmill exercise training (Ex) while the remaining mice maintained their non-training sedentary lifestyle (Sed).

Exercise Testing.

For exercise tests, mice completed a 2 day familiarization protocol that consisted of progressively increasing the intensity and duration of treadmill running (Columbus Instruments, Columbus, OH, USA). All experiments were performed at 10:00am and food was withdrawn from mice 2h prior to running. An incremental exercise test was performed which consisted of mice running at 10 m/min for 3 min. The velocity was increased by 4
m/min every 3 min until fatigue. This was defined as spending >10 sec at the base of the treadmill despite manual encouragement. The exercise testing determined the speed in which the mice were trained.

**Exercise Training.**

Mice were trained on the treadmill using the progressive overload principle for five days a week for 30, 40, 45, 50 and 60 min respectively. In week 1, the speed of the treadmill was set at 50% of the maximum speed the mice reached in their exercise test. In week 2 this was increased to 60%, week 3 to 70% and week 4 to 80%. The mice in the sedentary group had their cages removed from their holding room and placed next to the treadmill while the exercise groups trained. All mice were able to complete the exercise program besides two mice (one on a chow diet and one on a HFD) that could not complete the fourth week at 80% and were instead trained for a second week at 70%. To ensure no acute effects of exercise on our measurements, training was withheld on the days of testing and sacrifice.

**Metabolic testing**

*Body composition analysis.* Fat and lean mass was determined using the EchoMRI 4-in-1 (Echo Medical Systems, Houston, Tx, USA).

*Oral glucose tolerance tests (OGTT).* OGTT were performed in 5h fasted mice. Blood glucose was measured using a glucometer (Accu-Check, Roche, Castle Hill, NSW, Australia) at 0, 15, 30, 45, 60, 90 and 120 min after oral administration of glucose (2g/kg LBM) on blood obtained from the tail tip.
Biochemical Analysis

**Plasma Analysis.** Liver function enzymes, Asparate Aminotransferase (AST) and Alanine Aminotransferase (ALT), were measured by enzymatic reaction (Australian Specialized Animal Pathology, Mulgrave, Vic, Australia). Plasma Insulin was measured using an ALPCO Insulin ELISA (ALPCO Diagnostics, Salem, Massachusetts, USA) Intra-assay variation 3.1-9.3 CV%, Inter-assay variation 5.55-11.49 CV%.

Western blotting. Muscle and liver samples were lysed, protein concentration determined and resolved by SDS page as previously described (19). Immunoblotting was performed using the following primary antibodies: anti-SCD1, anti-pAMPK\(^{Thr172}\), anti-pACC1/2\(^{Ser79}\), tACC (Streptavidin-HRP), anti-NFκβp65, anti-pJNK1/2\(^{Thr183/Tyr185}\), tJNK1/2, anti-pIKK1/2\(^{Ser180/Ser181}\), anti-tIKK1/2, anti-CD36, anti-NLRP3, and IL-1β (R&D Systems, MN, USA), anti-FATP4 (kindly donated by Prof. Joachim Füllekrug, University Hospital of Heidelberg, Germany), anti-AMPK\(^{α2}\) (kindly donated by Dr. Graham Hardie, University of Dundee, Scotland, UK), anti-DGAT1 (Novus Biologicals, Littleton, CO, USA) anti-CPT1A (Alpha Diagnostics International, San Antonio, Tx, USA) anti-Caspase 1 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) and anti-β-actin (Sigma Aldrich, St Louis, MO ,USA). Protein quantification was normalized to GAPDH, β-actin or total protein (Coomassie stain).

RNA extraction and Real time quantitative PCR. Total RNA was isolated from liver with Tri Reagent® (Sigma Aldrich, St Louis, MO, USA) and reverse transcribed to cDNA with the use of random hexamers. Real-time PCR was performed on a 7500 fast sequence detector (Applied Biosystems, Foster City, CA, USA). Each assay included a no-template control, a no-reverse transcriptase control. Oligos for GCGR (Mm00433546_m1), CD36
(Mm01135198_m1), FABP1 (Mm00444340_m1), SREBF1 (Mm01138338), GCK
(Mm00439129), FASN (Mm01253292), Mlxipl (Mm02342723), DGAT1
(Mm00515643_m1), SCD1 (Mm00772290_m1), CPT1A (Mm00550438_m1), PPARα
(Mm00440939), PGClα (Mm01208835_m1), SL27A1 (FATP1) (Mm00449511_m1), Got
2 (FABPpm) (Mm02342495_m1), CD11c / Itgax (Mm00498698_m1), F4-80 / Emr1
(Mm00802530_m1), IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1) and TNF-α
(Mm00443258_m1) were obtained from, Applied Biosystems, TaqMan®. The relative
concentrations of measured mRNA were determined by plotting the threshold cycle (Ct)
versus the log of the serial dilution points, and the relative expression of the gene of interest
was determined after normalization to the housekeeper gene eukaryotic translation elongation
factor 2 (Eef2).

Muscle and liver metabolites and enzymes.

Lipidomics. Lipid content was determined in the skeletal muscle or liver using methods
previously described (19) (29) (41). Briefly, samples (20-30 mg wet weight) were
homogenized in 100 μl PBS buffer, pH 7.47. Lipids were extracted from 25 (muscle)-50
(liver) μg of protein using 20 volumes of chloroform:methanol (2:1) in a single phase
extraction process, recovering all lipids in a single phase suitable for liquid chromatography–
mass spectrometry analysis. Lipid analyses were performed by liquid chromatography,
electrospray ionisation–tandem mass spectrometry using an HP 1200 liquid chromatography
system combined with a PE Sciex API 4000 Q/Trap mass spectrometer with a turbo-
ionspray source (350°C) and Analyst 1.5 data system. Lipid classes investigated included
triacylglycerol (TG), diacylglycerol (DG), ceramide, sphingomyelin, cholesterol ester,
Phosphatidylcholine’s (PC), Phosphatidylethanolamine’s (PE) and cardiolipin. All individual
lipid species of each class of lipid (8 classes) were summed to give the total pool for each
lipid class. Due to the large number of species detected not all of these individual species are presented in the figures.

**Oxidative enzymes.** $\beta$-hydroxyacyl CoA dehydrogenase ($\beta$-HAD) and citrate synthase (CS) activity was measured in 5–10 mg of skeletal muscle as previously reported (7, 18).

**Data and Statistics.**

Data were analyzed by two-way analysis of variance with Tukey post-hoc tests (SigmaStat v3.5, Systat Software, Inc.) where all four groups were included for analysis at once (NC-Sed, NC-Ex, HFD-Sed and HFD-Ex). Logarithmic transformation was applied to end points not meeting normality assumptions. * indicates a diet effect, † indicates an exercise effect. In the figures, a straight line represents a main effect while line’s with ticks represents an interaction between those two specific groups. All data are presented as mean ± standard error of the mean (SEM). Statistical significance was set at P<0.05.
Results

Exercise training limits the HFD induced increase in adipose tissue mass

We characterized the body composition of mice before and after completion of the training or sedentary protocols. As expected, after 4 wks of dietary intervention, the mice fed a HFD were heavier, had greater fat mass, less lean mass and a higher body fat percentage (Fig. 1A, D, G, J). Following the completion of the 4 wk exercise intervention, HFD-Sed mice were heavier, had greater fat mass, less lean mass and a higher body fat percentage compared with all other groups (Fig 1B, E, H, K), demonstrating that the exercise training was effective in slowing the progression of weight gain while consuming a HFD. To quantify the precise weight differentials during the 4 wk exercise intervention, weights were analyzed as a change between the 4 and 8 wk time points. NC-Ex accumulated less fat mass and consequently had a lower body fat percentage than NC-Sed (Fig. 1F, L). HFD-Ex had lower body mass, fat mass and body fat percentage compared with HFD-Sed (Fig 1C, F, L) while there was a small difference in lean mass between the NC-Ex and HFD-Ex groups (Fig 1I). The lower weight and fat mass was not due to alterations in eating behavior (Fig 2A).

Exercise training improves exercise capacity, muscle oxidative capacity and HFD-induced glucose intolerance and hyperinsulinemia

To examine the effect of our training protocol on exercise capacity, we performed acute exercise tests before and after training. Prior to training mice fed NC had similar exercise capacity as those on the HFD, indicating the 4 wk of high fat feeding did not impact on exercise capacity (Fig. 2B). While training increased the exercise capacity of mice on both NC and HFD, the mice on NC tended to gain a greater benefit from the training protocol with a 35% improvement in exercise capacity compared with 17% with the HFD (Fig 2B). To assess a biochemical marker of exercise adaptation, we measured the maximal activities of
two important mitochondrial oxidative enzymes in mixed gastrocnemius muscle. The activities of β-hydroxyacyl CoA dehydrogenase (β-HAD) and citrate synthase (CS) were elevated in the Ex groups independent of diet (Fig. 2C, D). Together, the functional improvement in exercise capacity and the biochemical alterations in these oxidative enzymes suggested that the exercise protocol was of sufficient duration and intensity to derive a training effect.

To assess the impact of diet and exercise on whole body glucose homeostasis, we performed an OGTT. The HFD promoted glucose intolerance (Fig 2E, F) compared with NC, however the HFD-Ex group displayed improved glucose tolerance compared with the HFD-Sed mice (Fig. 2E, F). Furthermore, HFD induced hyperinsulinaemia was attenuated in the HFD-Ex group (Fig 2G). As previous observations have emphasized the importance of defective hepatic glucose metabolism in the development of impaired glucose tolerance and, insulin resistance (24) we measured plasma markers of liver function. Alanine transaminase (ALT) and Aspartate transaminase (AST) two markers of liver damage were increased by the HFD (Fig 2H, I). However, the HFD-Ex group had reduced levels of these markers compared with their respective controls (Fig 2H, I). Taken together, the exercise protocol led to a large improvement in exercise capacity in the NC fed mice with minimal impact on body composition and glucose homeostasis. While the mice on a HFD had less improvement in exercise capacity, however, the exercise training was largely protective against the deleterious effects of high fat feeding in regards to fat accumulation and glucose intolerance.

Exercise training does not affect HFD-induced lipid accretion in skeletal muscle

As skeletal muscle is the organ responsible for locomotion and energy utilization during exercise, we hypothesized that this would be the organ most affected in terms of lipid
accumulation with training. Accordingly, we first performed lipidomic analysis on skeletal muscle from all groups of mice for eight classes of lipids; TG, DG, ceramide, sphingomyelin, cholesterol ester, PC, PE and cardiolipin. Our analysis revealed a significant increase in total TG, DG and ceramide with the HFD (Fig 3A-C) with multiple individual lipid species altered (Fig 3D, E, 4A). Contrary to our hypothesis, this increase was not attenuated by exercise training (Fig 3A-E, 4A), however the TG in the NC Ex group were lower that the NC Sed group, predominantly due to changes in the TG 16:1_16:1_18:1, TG 16:1_18:1_18:1, TG 16:1_18:1_18:2 and TG 18:1_18:1_18:1 species (Fig 3A, D). Sphingomyelin, cholesterol ester and PC were neither affected by HFD nor exercise training (Fig 4B, C, E, 5A, B), while there was a trend for the HFD to increase PE levels (Fig 4D), and cardiolipin levels (Fig 5C, D) and decrease the PC/PE ratio (Fig 4F). Analysis of skeletal muscle fatty acid transporters demonstrated an increase in response to the HFD for slc27a1 (FATP-1) and a trend for an increase with diet for cd36 and got2 (FABP-pm) (Fig 5E), while exercise training reduced the HFD-induced expression of slc27a1. Together, these data demonstrate that the improvements in metabolic homeostasis observed with the HFD-Ex group are unlikely to be due to alterations in intramyocellular lipids.

Analysis of the liver lipidome reveals differential regulation of lipid species by diet and exercise training

We have previously demonstrated that lipid accumulation in the liver closely relates to alterations in insulin sensitivity during high fat feeding and that hepatosteatosis and liver insulin resistance occurs within one week of such a dietary intervention in C57Bl/6 mice (39). In addition, relatively little is known regarding the effect of high fat feeding and/or exercise training on the liver lipidome, liver inflammation or function. Accordingly, we next performed a comprehensive liver lipidomic analysis on tissues from our mice enrolled in the
exercise training program. In contrast with the pattern observed in skeletal muscle, TG levels were elevated in the HFD-Sed mice, indicative of hepatosteatosis, but this was attenuated with exercise (Fig 6A). The same general pattern was observed for total liver DG (Fig 6B). Analysis of the molecular species of TG demonstrated a generalized significant increase in all species with HFD (Fig 6D). These all tended to be decreased in the HFD-Ex group, but none reached statistical significance (Fig 6D). While TG are thought to be generally inert lipids, DG are thought to be a more deleterious lipid class by acting as second messengers to disrupt signal transduction processes (37) and by activating inflammatory signaling cascades via activation of protein kinase C (PKC) isoforms (15). Numerous DG species were increased in the HFD-Sed group, but decreased in the HFD-Ex group (Fig 6E). Ceramides are thought to be amongst the most pathogenic of all lipid species because they promote inflammatory signaling cascades and impair vital cell signaling processes (20). However, somewhat unexpectedly, total liver ceramide accumulation was decreased with the HFD and was not impacted by exercise (Fig 6C). Further analysis of the ceramide species revealed an increase in the ceramide 18:0 and ceramide 20:0 species, but a decrease with the longer chain ceramide 24:0 and ceramide 24:1 species with HFD (Fig 6F). As the longer chain species contributed more to the total pool of ceramide in these samples, this resulted in the decrease in total ceramide levels in response to HFD. Much like the ceramide data, sphingomyelin levels were lower with the HFD (Fig 7A) but closer inspection of the species data (Fig 7B) revealed that some species were elevated (sphingomyelin 32:1) and others decreased (sphingomyelin 38:2). There was no major effect of exercise training on hepatic sphingomyelin levels. As discussed, maintaining the PC/PE ratio is important for liver NASH progression and liver membrane integrity (25). In the current study, the hepatic PC/PE ratio decreased with high fat feeding (Fig 7E) due to a decrease in PC levels (Fig 7D) rather than an increase in PE levels (Fig 7C). Importantly, exercise training in HFD mice resulted in a
small but significant increase in the PC/PE ratio compared with HFD-Sed mice (Fig 7E).

Finally, cholesterol ester levels were robustly elevated with HFD and significantly decreased with exercise training (Fig 8A, C) while total liver cardiolipin levels were not altered by diet or exercise (Fig 8B), although some species were elevated or decreased with HFD (Fig 8D).

Exercise training decreases the expression of fatty acid transporters in the liver

Lipid storage in hepatocytes is determined by the balance between hepatocyte fatty acid uptake, lipid synthesis, oxidation and exportation. Hence, we next examined these pathways to ascertain a possible mechanism to the protective effects of exercise training on IHL accumulation. We first performed RT-PCR analysis of a number of genes involved in these different processes. High fat feeding increased expression of the glucagon receptor (Gcgr), the fatty acid transporter Cd36 (Cluster of Differentiation 36), also known as FAT (fatty acid translocase), Srebf1 (gene of sterol regulatory element binding protein-1c; SREBP1-c), glucokinase (Gck), carnitine palmitoyltransferase 1A (Cpt1A) while decreasing Mlxipl (Carbohydrate-responsive element-binding protein; ChREBP), Stearoyl-CoA desaturase-1 (Scd1) and Peroxisome proliferator-activated receptor alpha (Ppar-alpha) (Fig 9A). Exercise training decreased the expression of Gcgr, Cd36, Gck, diacylglycerol O-acyltransferase 1 (Dgat1), and Cpt1A while there was a tendency for exercise training to increase Fabp1 expression (p = 0.05). The effect of exercise was most notable in its ability to decrease HFD-induced Cd36 and Gck expression (Fig 9A).

To further characterize potential mechanisms of the phenotype, we also investigated various markers at the protein level. Analysis of CD36 protein levels validated the mRNA findings with a significant reduction in protein expression with exercise (Fig 9B, C). Quantification of another fatty acid transporter (FATP4) also demonstrated a decrease with exercise (Fig 9B,
indicating that there may be a generalized decrease in hepatic fatty acid transporters with exercise training. DGAT1 protein expression levels were not different (Fig 9B, E) and did not align with findings at the mRNA level (Fig 9A) indicating either a lag time between transcription and translation or non-transcriptional control of these proteins (i.e. post-translational modifications). Hepatic SCD1 protein levels did correspond tightly with the mRNA data, with a significant decrease with HFD, and a trend (p=0.06) for a further decrease with exercise training on the HFD (Fig 9A, F). Analysis of proteins linked to hepatic β-oxidation demonstrated an increase in the ratio of phosphorylated Acetyl-CoA carboxylase (ACC) to total ACC (Fig 9G, H), which was due to a reduction in total ACC rather than an increase in the phosphorylation status per se (Fig 9I, J). Similar to studies in rats that had voluntarily exercised (34, 35), these changes in ACC, were independent of changes in AMP-activated protein kinase (AMPK), a key kinase regulator of ACC (Fig 6G, K) while no effect was seen for diet or exercise for CPT1A protein expression (Fig 6G, L).

**Exercise training dampens the expression of inflammatory mediators in the liver**

As inflammation has been linked to ectopic lipid-induced impairments in the insulin signaling cascade in insulin sensitive tissues such as the liver and skeletal muscle, we analyzed important inflammatory markers in the liver samples. Although there was no dietary effect (there was a trend for an increase with HFD for Tnf-α), analysis revealed a main effect for a decrease in gene expression of Cd11c, IL-1β, IL-6, and Tnf-α with exercise, while F4-80 expression was not different (Fig 10A). The phosphorylation of c-Jun N-terminal kinases (JNK) was increased with HFD (Fig 10B, C) and tended to be decreased with exercise (p=0.058). However, I kappa B kinase (IKK) phosphorylation (Fig 10B, D) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) protein expression (Fig 10B, E) were unaltered by HFD or exercise. As IL-1β mRNA levels were decreased with exercise, we
investigated protein markers of inflammasome activation. Caspase 1, NLRP3 and IL-1β itself were unaltered by either diet or exercise (Fig 10F, G). Together, although there was only a modest inflammatory phenotype in the hepatocytes of this mouse model, the results suggest an ability of exercise to dampen hepatic inflammation pathways.
**Discussion**

In this study, we demonstrate that specific hepatic lipid species accumulation is ameliorated by exercise training. Moreover, exercise training resulted in a generalized decrease in markers of inflammation and cell damage in high fat fed animals. Interestingly, these effects were independent of any corresponding changes in intramuscular lipid levels. Together, our data provide evidence that exercise training may be a viable therapeutic intervention to prevent, or at least slow the progression of, NAFLD associated with nutrient overload.

An important finding from the present study was the increase observed in the PC to PE ratio in the liver which accompanied exercise training. PC and PE are major phospholipids in mammalian membranes and contribute to the regulation of membrane fluidity. This ratio is a key regulator of cell membrane integrity and a decrease in the ratio plays a role in the progression of hepatic steatosis to steatohepatitis (25). It is likely that abnormally high or low levels of the PC/PE ratio can be detrimental in the setting of NAFLD and that maintenance of a balance between these metabolites is potentially a key factor in preventing the incidence of NAFLD and preventing deterioration to NASH (22). We observed a HFD-induced decrease in this ratio that was offset by an increase in the exercise groups. Consequently, exercise may be one way in which a PC/PE balance may be restored.

Numerous DG species were elevated in the liver with the HFD and improved with exercise (Fig 6E). The particular species that were altered coincide with the findings of Gorden and colleagues who analysed the DG species in the livers of C57Bl/6 mice fed a HFD for 12 weeks and the DG species from biopsies obtained from patients with hepatic steatosis (14). All 7 DG species (similar fatty acid composition) that we present to be elevated in the current study were also found to be elevated in the high fat fed livers in this previous paper (14). Furthermore, 4 of these 7 species were also found to be significantly elevated in the human
biopsy samples indicating that a HFD provides a reproducible model of NAFLD in regards to DG species accumulation (14). The improvements, therefore, observed with exercise training may have significant relation to the human condition. Interestingly, exercise training may also impact upon cholesterol homeostasis in the liver, as there was a decrease in accumulated cholesterol esters, which have previously been shown to be increased with high fat feeding (3). Together, these changes in hepatic lipid levels correlated with the overall health of the liver given the marked improvement in liver function as measured by AST and ALT levels (Figure 2H,I).

In the current study we observed no effect of exercise on skeletal muscle HFD-induced lipid accumulation. This could be due to a number of possibilities. The myocytes could be having their lipid replenished due to a 1:1 replacement of lipid oxidized during exercise with lipid replaced via fatty acid uptake due to the ample availability of the lipid supplied in the HFD. Although the lipid levels in the skeletal muscle were not altered, an increase in lipid flux through the muscle may be the mechanism via which the liver is protected. The muscle may simply act as a lipid sink, sequestering circulating fatty acids and glucose away from the liver. In this respect, improved muscle insulin sensitivity may also be playing a role. The reversal of muscle insulin resistance, with one bout of exercise, has been shown to decrease postprandial hepatic de novo lipogenesis and hepatic triglyceride synthesis in insulin resistant individuals (32). This has the effect of diverting ingested carbohydrate away from the liver and into the muscle, thereby reducing lipid that accumulates by hepatic de novo lipogenesis (32).

Alternatively, another possibility exists whereby the contraction of the skeletal muscle may be initiating tissue cross talk where a secreted substance from the muscle a “myokine” may
be released and then act directly on the liver. For instance the myokine may signal to the hepatocytes to decrease the levels of the fatty acid transporters CD36 and FATP4 to provide more availability of fat to the skeletal muscle or stimulate a co-ordinated reduction in inflammatory gene profile. Recently a liver-muscle axis was described whereby a hepatic de novo lipogenesis-derived lipid metabolite (PC 18:0_18:1) mediates inter-organ communications between the liver and the skeletal muscle (26). This PPARδ-dependent hepatic pathway utilizes a circulating lipid (PC 18:0_18:1) to modulate fat use by the muscle and thus coordinates metabolic function between the organs (26). Given these finding of communication from the liver to the skeletal muscle, it is plausible that the exercising skeletal muscle may also communicate to the liver via a similar axis. Further specific studies would be needed to “capture” the release of such a myokine or lipokine to prove this hypothesis.

Lipid accumulation in hepatocytes increases the livers susceptibility to injury by oxidative stress and inflammatory cytokines (11). Indeed, the “two hit” hypothesis was coined to describe the temporal changes associated with the development of NASH. The first “hit”; the development of liver steatosis, increases susceptibility of the liver to injury mediated by “second hits” such as cytokines and mitochondrial dysfunction (13). While it must be noted that our HFD intervention was not of sufficient duration to increase hepatic inflammatory markers, there was an exercise-induced general decrease in many of these markers. Thus, the possibility exists that in disease conditions where inflammatory pathways are elevated in the liver, exercise may be useful in decreasing inflammatory tone.

A limitation of the current study is that only one type (running) and intensity (50-80% of max) of exercise was examined and for a short duration of time (4 weeks). Whether different exercise or exercise of less intensity exerts similar effects over time (such as resistance
exercise or walking) is unknown. Encouragingly, resistance exercise training in a group of patients with NAFLD reduced total liver fat independently of weight loss (16) suggesting multiple exercise types may be beneficial in the setting of NAFLD.

In conclusion, our data suggest that exercise training is an important component of the treatment regime for the prevention of NAFLD progression. This is important considering NAFLD has been described as “the next global epidemic” (33). Future studies may wish to investigate various exercise training principles to elucidate the most effective types of exercise in terms of duration, intensity and frequency in relation to its impact on hepatic lipid levels. Further, as exercise compliance is the limiting factor to exercise prescription, the effectiveness of each intervention and method of delivery should be studied, to ensure the exercise training will be conducted frequently enough to benefit the liver.
Financial Support

This study was supported by grants from the National Health and Medical Research Council of Australia (NHMRC Project Grant 1004441 to MAF) and the Victorian Government Operational Infrastructure Support Program. DCH is supported by a National Heart Foundation (NHF) Biomedical Postdoctoral Fellowship and Australian Diabetes Society (ADS) Skip Martin Fellowship. ABJ is supported by the Augustinus Foundation and the Lundbeck Foundation. BK is supported by The Danish Council for Independent Research and the UNIK initiative grant. MAF is a Senior Principal Research Fellow of the NHMRC. PJM is a Senior Research Fellow of the NHMRC. Authors have no conflict of interest to declare relating to this work.

Acknowledgements

We wish to acknowledge Deb Ramsey and her team at the AMREP AS for their assistance with the animal studies. We are also grateful to Prof. Joachim Füllekrug for providing the FATP4 antibody.

Author Contributions

DCH and MAF designed research; DCH, ABJ, TG, MJK, EE, JMW, HLK, BK, PJM performed and/or analyzed research; DCH, ABJ and MAF wrote the paper and all authors contributed to the writing of the final submitted version.
References


**Figure Legends**

**Fig 1.** Characteristics of sedentary (Sed) and exercise trained (Ex) mice fed a NC or HFD. (A) Body weight after 4 wks NC or HFD, (B) body weight after 8 wks (including 4wks training) and (C) change in body weight during training period. (D) Fat mass after 4 wks NC or HFD, (E) fat mass after 8 wks (including 4wks training) and (F) change in fat mass during training period. (G) Lean mass after 4 wks of NC or HFD, (H) lean mass after 8 wks of NC or HFD (including 4wks of training) and (I) change in lean mass during training period. (J) Body fat percentage after 4 wks of NC or HFD, (K) body fat percentage after 8 wks of NC or HFD (including 4wks of training) and (L) change in body fat percentage during training period. n = 8-10 per group. * P<0.05, ** P<0.01, *** P<0.001 for dietary effect, † P<0.05, †† P<0.01, ††† P<0.001 for exercise effect (Sed vs. Ex). Straight line represents a main effect while the line with ticks represents an interaction between those two groups. Data are mean ± SEM.

**Fig. 2.** Food consumption, exercise capacity, oxidative enzymes and glucose metabolism in Sed or trained (Ex) mice. (A) Food intake displayed as energy intake over a 24h period, n = 4 mice per group averaged over a week. (B) Running time during an exercise capacity test prior to (Pre) and following (Post) training. (C) CS and (D) β-HAD activities in muscle, n = 8-10. (E) Blood glucose levels during an OGTT and (F) incremental area under the curve, n = 7-10. (G) Plasma insulin levels following a 5h fast expressed as a percentage change from the relative NC control group, n = 7-8. (H) Plasma ALT and (I) AST levels after a 5h fast displayed as a percentage change from the relative NC control group, n = 5-8. * P<0.05, *** P<0.001 for dietary effect, † P<0.05, †† P<0.01, ††† P<0.001 for exercise effect (Sed vs. Ex or training effect). ## P<0.01 for significant difference between HFD-Sed and HFD-Ex at 15, 30 and 45min of the OGTT. Data are mean ± SEM.
**Fig. 3.** Lipidomic analysis of skeletal muscle in sedentary (Sed) or trained (Ex) mice. (A) Skeletal muscle total TG levels relative to NC Sed =1. (B) Skeletal muscle total DG levels relative to NC Sed =1. (C) Skeletal muscle total ceramide levels relative to NC Sed =1. (D) Individual TG molecular lipid species relative to NC Sed =1. (E) Individual DG molecular lipid species relative to NC Sed =1. \( n = 7-10 \) per group. * \( P<0.05 \), ** \( P<0.01 \), *** \( P<0.001 \) for dietary effect (NC vs. HFD), † \( P<0.05 \), † † \( P<0.01 \), for exercise effect (Sed vs. Ex). Straight line represents a main effect while the line with ticks represents an interaction between those two groups. Data are mean ± SEM.

**Fig 4.** Lipidomic analysis of skeletal muscle in sedentary (Sed) or trained (Ex) mice. (A) Individual ceramide molecular lipid species relative to NC Sed =1. (B) Total sphingomyelin levels relative to NC Sed = 1. (C) Individual sphingomyelin molecular lipid species relative to NC Sed =1. (D) Total phosphatidylethanolamine (PE) levels (E) Total phosphatidylcholine (PC) levels (F) and skeletal muscle PC/PE ratio, \( n = 7-10 \) per group. * \( P<0.05 \), ** \( P<0.01 \), *** \( P<0.001 \) for dietary effect (NC vs. HFD), † \( P<0.05 \), † † \( P<0.01 \), for exercise effect (sedentary vs. exercise). Straight line represents a main effect while the line with ticks represents an interaction between those two groups. Data are mean ± SEM.

**Fig. 5.** Lipidomic analysis of skeletal muscle in sedentary (Sed) or trained (Ex) mice. (A) Total cholesterol ester levels. (B) Individual cholesterol ester lipid species relative to NC Sed =1 (cholesterol ester 22:5 not detected for NC EX group). C) Total cardiolipin levels. (D) Individual cardiolipin molecular lipid species relative to NC Sed. \( n = 7-10 \) per group. (E) Skeletal muscle gene profiling, mRNA expression for genes involved in fatty acid transportation, \( n = 6-9 \). * \( P<0.05 \) for dietary effect ** \( P<0.01 \) for dietary effect *** \( P<0.001 \) for
dietary effect (NC vs. HFD), † P<0.05, for exercise effect. Straight line represents a main
effect. Data are mean ± SEM.

**Fig. 6.** Lipidomic analysis of hepatic triacylglycerol, diacylglycerol and ceramide in Sed or
trained (Ex) mice. (A) Hepatic total TG. (B) Hepatic total DG. (C) Hepatic total ceramide.
(D) Individual hepatic TG molecular lipid species. (E) Individual hepatic DG molecular lipid
species. (F) Individual hepatic ceramide molecular lipid species (all relative to NC Sed =1). n
= 6-10 per group. * P<0.05, ** P<0.01, *** P<0.001 for dietary effect, † P<0.05, †† P<0.01, for
exercise effect. Data are mean ± SEM.

**Fig. 7.** Lipidomic analysis of hepatic ceramide, sphingomyelin, phosphatidylcholine and
phosphatidylethanolamine in Sed or trained (Ex) mice. (A) Hepatic total sphingomyelin. (B)
Individual hepatic sphingomyelin molecular lipid species. (C) Hepatic total
phosphatidylethanolamine (PE), (D) hepatic total phosphatidylcholine (PC) and (E) liver
PC/PE ratio (all relative to NC Sed =1). n = 7-10 per group. * P<0.05, ** P<0.01, *** P<0.001
for dietary effect, † P<0.05, †† P<0.01, for exercise effect. Data are mean ± SEM.

**Fig. 8.** Lipidomic analysis of hepatic cholesterol ester and cardiolipin in Sed or trained (Ex)
mice. (A) Hepatic total cholesterol ester. (B) Hepatic total cardiolipin. (C) Individual hepatic
cholesterol ester molecular lipid species. (D) Individual hepatic cardiolipin molecular lipid
species (all relative to NC Sed =1) n = 7-10 per group. * P<0.05, ** P<0.01, *** P<0.001 for
dietary effect, † P<0.05, †† P<0.01, for exercise effect. Data are mean ± SEM.
Fig. 9. Hepatic gene profiling and protein expression in Sed or trained (Ex) mice. (A) mRNA expression for genes involved in hepatic fatty acid uptake, glycolysis, lipogenesis and fatty acid oxidation, $n = 6-10$. (B) Western blotting of proteins involved in hepatic fatty acid uptake and synthesis and quantification of (C) $Cd36$, (D) $Fatp4$, (E) $Dgat1$ and (F) $Scd1$, (G) Western blotting of proteins involved in hepatic fatty acid oxidation and quantification of (H, I, J) ACC, (K) AMPK and (L) CPT1A, $n = 8-10$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ for dietary effect, † $P<0.05$, †† $P<0.01$, ††† $P<0.001$ for exercise effect. Data are mean ± SEM.

Fig. 10. Markers of hepatic inflammation in Sed or trained (Ex) mice. (A) mRNA expression for inflammatory genes. (B) Western blotting of inflammatory proteins. Quantification of components of the inflammatory cascade (C) JNK, (D) IKK and (E) NFκB. (F) Blotting and (G) quantification of components of inflammasome activation, $n = 8-10$. ** $P<0.01$ for dietary effect, † $P<0.05$, †† $P<0.01$, ††† $P<0.001$ for exercise effect. Data are mean ± SEM.
Figure 5

(A) Muscle Cholesterol Ester (relative to NC SED = 1)

(B) Muscle Cholesterol Ester species (relative to NC SED = 1)

(C) Muscle Cardiolipin (relative to NC SED = 1)

(D) Muscle Cardiolipin species (relative to NC SED = 1)

(E) mRNA expression (relative to NC SED = 1)

SLC27A1, Cd36, Got2
Figure 7

A) Liver Sphingomyelin (relative to NC SED = 1)

B) Liver Sphingomyelin species (relative to NC SED = 1)

C) Liver PEs (relative to NC SED = 1)

D) Liver PCs (relative to NC SED = 1)

E) Liver PC to PE Ratio (relative to NC SED = 1)
Figure 10

A) mRNA Expression (relative to NC Sed = 1)

CD11c  IL-1β  IL-6  TNF-α  F4-80

NC Sed  NC Ex  HFD Sed  HFD Ex

B) Protein Expression

pJNK1/2  tJNK1/2  pIKK1/2  tIKK1/2  NFκBp65

C) Liver pJNK1/2

D) Liver pIKK1/2

E) Liver NFκBp65

F) Protein expression

Caspase1  IL1-β  NLRP3  β-actin

G) Liver Protein Expression (relative to β-actin)

Caspase1  IL-1β  NLRP3