

n-3 fatty acids combined with flavan-3-ols prevent steatosis and liver injury in a murine model of NAFLD

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Abstract

Non-alcoholic fatty liver disease (NAFLD) affects 25% of adults and at present no licensed medication has been approved. Despite its complex patho-physiology, dietary strategies aiming at delaying or preventing NAFLD have taken a reductionist approach, examining the impact of single components. Accumulating evidence suggests that n-3 LC-PUFAs are efficacious in regulating lipogenesis and fatty acid oxidation. In addition, plant derived flavonoids are also emerging as a dietary strategy for NAFLD prevention, with efficacy attributed to their insulin sensitising and indirect antioxidant effects. Based on knowledge of their complementary molecular targets, we aimed to demonstrate that the combination of n-3 LC-PUFA (n-3) and flavan-3-ols (FLAV) prevents NAFLD. In a high-fat high-fructose (HF/HFr) fed C57Bl/6J mouse model, the independent and interactive impact of n-3 and FLAV on histologically defined NAFLD, insulin sensitivity, weight gain, intestinal and hepatic gene expression, intestinal bile acids were examined. Only the combination of FLAV and n-3 (FLAVn-3) prevented steatosis as evidenced by a strong reduction in hepatocyte ballooning. Whilst FLAV reduced body (-28-30%), adipose tissue (-45-50%) weights and serum insulin (-22-25%) as observed following an intra-peritoneal glucose tolerance test, n-3 downregulated the expression of *Srebf1* and the lipogenic genes (*Acaca*, *Fasn*). Significant impacts of interventions on intestinal bile acid metabolism, farnesoid X receptor (*Fxr*) signaling in the intestine and liver, and hepatic expression of fatty acid transporters (*Fabp4*, *Vldlr*, *Cd36*) were also evident. FLAVn-3 may be a novel intervention for NAFLD. Future research should aim to demonstrate its efficacy in the prevention and treatment of human NAFLD.

Keywords: fish oil; flavonoids; *Srebp-1c*, bile acids, NASH.

1. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) associated with obesity, has a global prevalence of 25% in adults [1]. It is a spectrum of progressive liver damage ranging from benign steatosis, to non-alcoholic steatohepatitis (NASH) characterised by necro-inflammation and hepatocyte injury [2]. In about 10% of patients, NASH leads to fibrosis and cirrhosis and an increased risk of liver failure and hepatocellular carcinoma [3]. Increased CVD incidence is the most prevalent clinical feature of NAFLD [4].

The pathogenic progression of NAFLD is characterised by a loss of insulin sensitivity and hepatocyte accumulation of fat, which can induce lipotoxicity through oxidative stress and a pro-inflammatory state, leading to cellular damage [5]. Emerging evidence indicates that alterations in bile acid metabolism and associated farnesoid X nuclear receptor (*Fxr*) signalling also contribute to the development of NAFLD and obesity [6].

At present no licensed medication or surgical procedure have been approved for NAFLD. Available rodent data is encouraging regarding the efficacy of fish derived n-3 fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) in NAFLD [7], regulating lipogenesis and fatty acid oxidation (*via Srebp-1c* and *Ppara*, respectively) [8]. However, in human randomised controlled trials (RCT), fish oil supplementation has shown mixed findings [9], with largely marginal effects on histologically defined NAFLD [10] and no impact on insulin action. Flavan-3-ols (FLAV), a class of plant bioactive flavonoid compounds found in cocoa, tea and berries, are also emerging as a dietary strategy for NAFLD prevention, with efficacy attributed to their insulin sensitising, indirect antioxidant and anti-inflammatory effects [11].

Despite the complexity of the NAFLD patho-physiology, to date dietary strategies which target NAFLD have taken a reductionist approach, examining the impact of single interventions. Here, based on their likely complementarity molecular targets, we examined for the first time the combined effect of n-3 and FLAV on NAFLD development using a high-fat high-fructose (HF/HFr) mouse model. NAFLD severity was established by human histological NAFLD activity scoring (NAS) of the hepatic tissue, with insulin sensitivity

assessed by intra-peritoneal glucose tolerance testing, targeted gene expression profiling and biochemical analyses performed in order to gain a mechanistic insight.

2. MATERIALS AND METHODS

2.1 Study approval

All experimental procedures and protocols used in this study were reviewed and approved by the Animal Welfare and Ethical Review Body (AWERB) and were conducted within the provisions of the Animals (Scientific Procedures) Act 1986 (ASPA).

2.2 Animal experimental design and dietary treatments

Male C57BL/6J mice aged 4 weeks (n=50, Charles River Laboratories (Margate, UK) were maintained in 12 h light/dark cycle, under conditions of constant temperature (21 ± 2 °C) and humidity ($55 \pm 10\%$). After feeding a standard RM3 diet (Special Diet Services, Essex, UK) for two weeks, mice were assigned to five experimental groups (n=10 per group) and allowed free access to a control diet (C), high fat/high fructose (HF/HFr) diet, or a HF/HFr diet supplemented with cocoa powder (HF/HFr + FLAV, Chococru®, London, UK), fish oil (HF/HFr + n-3; EPAX, Oslo, Norway) or a combination of both (HF/HFr + FLAVn-3) (Research Diet Inc., New Brunswick, USA) for 16 weeks (**Supplementary Table S1-S3**). The FLAV and n-3 supplemented diets contained 667 µg/g cocoa flavan-3-ol monomers (epicatechin and catechin), and 6.67 mg EPA+DHA/g, which provided the animals with daily doses of 60, 7, 400 and 280 mg/kg body weight of (-)-epicatechin, (+)-catechin, EPA and of DHA respectively. Using allometric scaling this equated to physiologically relevant human (60 Kg body weight) equivalent doses (HED) of 255mg of epicatechin, 30mg catechin, 1.7g EPA and 1.2g DHA per day [12], which are the levels found in 30 g of FLAV-rich cocoa [13] and one large portion of oily fish [14], respectively. Food intake was assessed twice a week and food pellets were replaced every other day to avoid oxidation of the bioactive components. At week 16, animals were sedated and blood was collected by cardiac

puncture followed by trans-cardiac perfusion of ice-cold saline containing heparin (10 units/ml). Plasma/serum samples were isolated by centrifugation at 2000 x g for 10 min and samples were snap-frozen and stored at -80° C. Livers were rapidly removed, rinsed with ice-cold NaCl (150 mmol/L), blotted and weighed. A lobe from each liver was preserved in formalin for histological analyses, and the remainder anatomical lobes were either snap-frozen in liquid nitrogen or transferred into an RNA preservation solution (Qiagen, Manchester, UK) for gene expression analysis.

2.3 Intra-peritoneal glucose tolerance test

At the end of week 15, mice were fasted for 16 hours before being administered D-glucose (Sigma-Aldrich, Poole, UK) by intraperitoneal injection (2g/Kg body weight). Blood glucose was measured at 0, 10, 20, 30, 60 and 120 min using a AlphaTRAK 2 glucometer (Abbott Laboratories Ltd., Maidenhead, UK). Additional blood samples (20µl) were collected at baseline, 15 and 120 min into EDTA coated microvette tubes (Sarstedt Ltd., Leicester, UK), and concentrations of insulin were determined using a commercial ELISA kit (Crystal-Chem, Downers Grove, US). Insulin resistance was estimated by the homeostasis model assessment method (HOMA) [fasting plasma insulin (mU/ml) × fasting plasma glucose (mM)/22.5] [15].

2.4 Histological and Biochemical analyses

Formalin-fixed murine liver specimens were processed for hematoxylin and eosin (H&E) staining as described previously [16]. The severity of liver disease was evaluated histologically by two blinded histopathologists, using the NAS scoring system, which is the standard system for reporting the extent of damage in human biopsy samples [17] and represents the combined semi-quantitated pathology score for steatosis (graded 0-3), lobular inflammation (graded 0-3), and hepatocyte ballooning (graded 0-2) and ranges from 0-8 [18]. NAS was calculated for each rodent group. Total lipids were extracted from 500 mg of food pellets or from 300 mg of liver with chloroform/methanol (2:1 v/v) and fatty acids were

analysed as reported previously [19]. The triglyceride content of the livers was carried out using the triglycerides Liquicolor® reagent (Cambridge Life Sciences Ltd, Ely, UK) following the manufacturer's instructions. Lipid peroxidation end-products (LPO) were measured in liver homogenates with a colorimetric assay (Oxford Biomedical Research, Upper-Heyford, UK). AST, ALT and creatinine were quantified using commercially available kits (Werfen, Warrington, UK) on a clinical chemistry analyser IL650. Plasma leptin was measured using the Procarta mouse simplex immunoassay kit (Affymetrix eBioscience, Austria).

2.5 Taqman Low Density Array (TLDA), qRT-PCR

TLDA analysis was carried out on [20] samples from each of the 5 study groups (150ng RNA/TLDA port). Validation of the TLDA data by qRT-PCR was performed on an ABI prism 7500 detection system (Applied Biosystems) as previously described [21] with primer probe sets from Applied Biosystem (**Supplementary Table S4a and Table S4b**).

2.6 Flavan-3-ol metabolites and bile acids analyses

Frozen liver samples (500mg) were lyophilized and ground before being processed according to Bresciani et al [22]. Briefly, proteins from tissue samples were precipitated with 400 µL 20% (v:v) TCA and then extracted with 3 mL of cold methanol. The samples were vortexed vigorously for 5 min, placed in a sonicator bath for 5 min, and then centrifuged (4,000 g for 5 min). A second extraction was performed for each sample with 3 mL methanol as described above. The two supernatants were pooled and dried under vacuum by rotary evaporation. Finally, the pellet was suspended in 150 µL of 50% (v:v) methanol acidified with 1% (v:v) formic acid. Urine samples were defrosted, centrifuged for 5 min at 8,765g, and diluted 1:4 with water containing 0.1% formic acid before analysis.

Flavanol, procyanidin content (by degree of polymerization 1-10) and xanthines (caffeine and theobromine) of the cocoa powder were analysed following published AOAC methods [23, 24]. Flavan-3-ol metabolites in liver and urine samples were analysed using ultra-high

performance liquid chromatography coupled with mass spectrometry (UHPLC-MS/MS) using an UHPLC DIONEX Ultimate 3000 equipped with a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization source (H-ESI; Thermo Fisher Scientific Inc.). For UHPLC, mobile phase A was 0.5% formic acid in water and mobile phase B was acetonitrile containing 0.5% formic acid. Separations were performed with a Kinetex PFP (50 × 2.1 mm) column, 2.6 µm particle size (Phenomenex, Macclesfield, UK). The gradient started with 1%B, keeping isocratic conditions for 1 min, reaching 40%B at 15 min, followed by 2 min at 80% B and then 4 min at the start conditions to re-equilibrate the column. The flow rate was set at 0.3 mL/min, the injection volume was 5 µL, and the column was thermostated at 35°C. The applied method consisted in the selective determination of each target precursor ion by the acquisition of characteristic product ions in the “selected reaction monitoring” (SRM) mode, with negative ionization. The MS worked with capillary temperature at 270 °C, while the source at 200 °C. The sheath gas flow was 50 units, while auxiliary gas pressure was set to 5 units. The source voltage was 3 kV. Ultra-high purity argon gas was used for collision-induced dissociation (CID). Data processing was performed using Xcalibur software from Thermo Scientific. Most of the compounds were quantified by comparison with their corresponding standards; while 5-(3'-hydroxy-phenyl)valeric acid-sulfate, dihydroxyphenyl γ valerolactone-sulfates, and trihydroxyphenyl-γ-valerolactone-sulfate, were quantified as 3'-hydroxyphenyl-γ-valerolactone (M7)-sulfate; (epi)catechin-sulfate, methyl-(epi)catechin-sulfates, and (epi)gallocatechin-sulfate, as quercetin-sulfate; dihydroxyphenyl-valerolactone-glucuronide and dihydroxyphenyl-valerolactone-sulfate-glucuronide as 3'-hydroxyphenyl-γ-valerolactone (M7)-glucuronide; and (epi)catechin-glucuronide, methyl-(epi)catechin-glucuronide, and (epi)catechin-sulfate-glucuronide as quercetin-glucuronide. Urinary excretion of selected metabolites was adjusted for creatinine levels.

The concentration of bile acids in intestinal mucosa samples was analyzed by using an UPLC-H I-Class (Waters, UK) coupled to a Xevo-G2 QToF mass spectrometer (Waters, UK). Electrospray ionization was performed in the negative ionization mode. Chromatographic

separation was performed on a BEH C18 column (2.1 x 100 mm, 1.7 μ m). The mobile phase consisted of water (Eluent A) and acetonitrile (Eluent B) both containing 0.1 % formic acid. Analytes were separated by a linear gradient elution. The injection volume was 2 μ L and the column was maintained at 40 °C. Detection of the bile acids and their glycine and taurine conjugates was performed by exact mass (\pm 0.01 Da). All standards as well as the deuterated internal standard (IS) substance (d4-chenodeoxycholic acid) were purchased from Steraloids (Newport, USA).

2.7 Statistics

The impact of treatment was compared by one-way ANOVA followed by protected multiple comparison tests (i.e., Bonferroni and Tukey-Kramer) or Kruskal-Wallis followed by Dunn's Multiple Comparison Test. Histology data and components of the NAS (steatosis amount, lobular inflammation, and ballooning) were analysed using a Mann-Whitney U test. For the IPGTT data, a repeated measures ANOVA was conducted with time and dietary groups as independent variables. One-way ANOVA with Duncan's post hoc test was applied to compare values relating to tissue accumulation or urinary excretion of flavan-3-ol metabolites. When flavan-3-ol metabolites were detected only for the HF/HFr + FLAV and HF/HFr + FLAVn-3 groups, a *t*-test instead of one-way ANOVA was applied. For all tests, the bilateral alpha risk was set as $\alpha = 0.05$. Principal component analysis (PCA) with varimax was also performed. The statistical analysis was conducted using the SPSS package (version 16.0; SPSS Inc., Chicago, IL, USA).

3. RESULTS

3.1 Flavan-3-ols and n-3 fatty acids supplemented diets protect the liver from developing the biochemical and histological features of NASH

Following 16 weeks of HF/HFr feeding, mice developed steatosis and hepatocyte ballooning, while livers from control-fed mice showed normal histological hepatocyte architecture and no evidence of steatosis (**Figure 1A**). Mice fed with FLAV and n-3 displayed a reduction in the lipid droplet size and hepatocyte damage induced by HF/HFr. Upon conversion of the histological images into semi-quantitative data, relative to HF/HFr, FLAVn-3 supplementation significantly decreased both steatosis and hepatocyte ballooning (**Table 1**). Reduction in hepatocyte ballooning was also observed in presence of n-3 also, but to a lesser extent. Fatty liver development and liver function was also assessed by liver TAG and serum ALT and AST. Consistent with the histology, HF/HFr was associated with significant increases in all three markers (**Figure 1B-D**), which were ameliorated by FLAV and n-3 supplementation.

3.2 The weight gain associated with HF/HFr feeding is in part negated by flavan-3-ol supplementation

At 16 weeks, body weight gain and liver weight were 50% and 33% higher respectively in the HF/HFr mice compared to the control animals (**Figure 2A and Figure 2B**). FLAV and FLAVn-3 supplementation reduced weight gain by 28-30% with no differences in body weight associated with n-3 supplementation. No difference in cumulative food intake between groups was evident (average 2.55 ± 0.08 g/d, data not shown). A 4-fold increase in inguinal adipose tissue mass (**Figure 2C**) along with a 10-fold higher plasma leptin levels (**Figure 2D**) were also observed in mice fed a HF/HFr relative to control diet. Such increased levels were partly attenuated by FLAV and FLAVn-3 feeding (**Figure 2C-D**).

3.3 Flavan-3-ol but not n-3 maintains insulin sensitivity in fed mice challenged with a HF/HFr diet

HF/HFr diet raised fasted blood glucose, plasma insulin and HOMA-IR relative to control, indicative of decreased insulin sensitivity (**Figure 3A-C**). In the IPGTT, HF/HFr feeding raised glucose concentrations at 60 and 120 min relative to control (**Figure 3A**). For insulin, 2- and 3-fold higher concentrations were observed at 15 min and 120 min respectively in the

HF/HFr group (**Figure 3B**). At 120 min, FLAV and FLAVn-3 reduced insulin levels by 35% indicative of a FLAV associated amelioration of the loss of insulin sensitivity induced by HF/HFr. Additionally, only the FLAVn-3 supplementation decreased HOMA-IR. (**Figure 3C**).

3.4 Supplementation with n-3 fatty acids and flavan-3-ols modulates the expression of genes involved in hepatic lipid accumulation and fibrosis.

HF/HFr upregulated genes associated with hepatic lipid accumulation with an almost 2-fold increase in the expression of the lipogenic genes, acyl-CoA carboxylase (*Acaca*) and fatty acid synthase (*Fasn*). Likewise, 1.5-6 fold increases in the fatty acid/lipoprotein receptors, fatty acid binding protein 4 (*Fabp4*) and very-low-density-lipoprotein receptor (*Vldlr*) were evident, along with an up-regulation of sterol regulatory element-binding protein 1c (*Srebf1*), the main transcription factor modulating lipogenesis (*Acaca* and *Fasn*). n-3 (+/- FLAV) decreased *Acaca*, *Fasn* and *Srebf1* expression with FLAV (+/- n-3) down-regulating *Fabp4* and the *Vldlr* expression. Although no impact of HF/HFr feeding on fibrosis-related genes was observed, FLAV and n-3 down-regulated collagen 1A1 (*Col1a1*), *Col1a2*, matrix metalloproteinase (Mmp) 2, and tissue inhibitor of MMP 2 (*Timp2*), with an increase in *Timp3* evident following combined FLAVn-3 exposure (**Figure 4 and Supplementary Table S5**).

3.5 Flavan-3-ols but not n-3 fatty acids alter bile acid metabolism leading to upregulation of CYP7A1 in mice fed a HF/HFr diet

HF/HFr diet had a negligible impact on total ileal bile acids (**Figure 5A**), with a modest increase in the proportion in the conjugated form (**Figure 5B**). Supplementing HF/HFr with FLAV alone or in combination with n-3 not only enhanced the percentage of conjugated forms (**Figure 5B**) but also reduced the content of hyodeoxycholic acid (HDCA) (**Figure 5C**), decreased the ratio HDCA to muricholic acids (MCA) (**Figure 5D**), increased the level of FXR-agonist bile acids and tended to increase the content of FXR-antagonist species (**Figure 4E**). The magnitude of these widespread effects were most pronounced for FLAVn-

3. Although statistically not significant, it is important to note that the balance of FXR bile acid activators and inhibitors was about 40% more antagonistic in mice fed FLAV, n-3, and FLAVn-3 than in their counterparts offered HF/HFr and control diet. Compared to HF/HFr, all treatments decreased FXR signalling as revealed by a downregulation of *Shp* mRNA in the ileum and liver (**Figure 6A, D**). The greatest inhibitory action was evident with FLAV, which is consistent with the response to intervention of other FXR target genes in the liver (*Cyp7a1* and *Cyp8b1*, **Figure 6B, C**) and the intestine (*Tgr5* and *Gcg*, **Figure 6E, F**) and *Fgf15* (**Supplementary Figure S1**).

3.6 Supplementation with n-3 fatty acids and flavan-3-ols results in significant increases in hepatic tissue EPA, DHA and flavan-3-ol metabolite concentrations

In the n-3 groups a 1.7 and 4.0 absolute increase in percentage EPA and DHA was evident relative to the HF/HFr, with no impact of FLAV on fatty acid status (**Supplementary Table S6**). Furthermore, n-3 feeding increased tissue lipid hydroperoxide (LPO) levels by approximately 2-fold relative to the other groups.

Potential flavan-3-ols metabolites were monitored using LC-MS methods with five microbial FLAV degradation products [25] detected in the liver samples (**Supplementary Table S7 and S8**). In particular, a significant increase in hydroxyphenylvalerolactones, originating from the C-ring-opening of flavan-3-ols by the colonic microflora followed by further lactonization, was observed. In addition, urine samples were analysed and 37 compounds identified, including phase II and colonic metabolites which varied significantly between groups (**Supplementary Table S9**). n-3 fatty acid consumption modified the metabolism of flavan-3-ols, enhancing the excretion of phase II conjugates of flavan-3-ol monomers and hydroxyphenylvalerolactones while decreasing the excretion of phenolic acid conjugates which is suggestive of enhanced FLAV absorption (**Supplementary Table S9, Figure S2 and Figure S3**).

4. Discussion

Liver disease is the third most common cause of premature death in the UK [26]. Due to the increased incidence of obesity, NAFLD is now more prevalent than alcohol-induced liver disease [2] with 67% of overweight and 94% of obese individuals presenting this condition [27]. The HF/HFr regime was selected as a physiologically relevant model for human NAFLD development and resulted in steatosis and hepatocyte ballooning indicative of early NASH. In this study we demonstrate that combined supplementation with FLAVn-3 prevented steatosis and hepatocyte ballooning, lowered weight and adipose tissue gain and improved insulin sensitivity. Our findings are similar to previous data demonstrating that 8% cocoa powder for 10 weeks significantly reduced the rate of body weight gain, as well as final body weight and retroperitoneal WAT weight compared to HFD-fed controls [28]. Such effects may be related to increased faecal fat excretion [29], with cocoa extracts and their component polyphenols shown to inhibit the activity of pancreatic lipase and secreted phospholipase A2, which may reduce dietary fat absorption [30]. Individual feeding of FLAV and n-3 identified numerous physiological and molecular mechanisms which are likely to have acted synergistically to prevent NASH in the FLAVn-3 group. Previous studies have reported the hypoglycemic and insulin sensitising effects of cocoa polyphenols. For example, a proanthocyanidin-rich cocoa liquor extract suppressed a HFD-induced hyperglycemia through the activation of AMP-activated protein kinase α , and the translocation of glucose transporter 4 in mice [20]. In addition, short-term administration of dark chocolate improved insulin resistance in terms of improved HOMA-IR and quantitative insulin sensitivity check index (QUICKI) in healthy subjects [31]. Whilst cocoa polyphenols may play a large part in the observed effects, emerging evidence is also suggestive that caffeine contribute to the weight loss [32] although its intake could explain some but not all of the diabetes-risk reduction and weight change in humans [33]. In this study, FLAV ameliorated the HF/HFr induced obesity, the aetiology of which is likely related to changes in bile acid metabolism [34]. Whilst most bile acids enter the gut conjugated to taurine or glycine, these are

deamidated by microbial bile salt hydrolases (BSH) in the distal small intestine and, if not absorbed and transported back to the liver, are further metabolized by gut microbiota [35]. Recent studies in obese mice showed that partial inhibition of BSH by antibiotics [36] results in the intestinal accumulation of conjugated bile acids, and in particular of the FXR antagonists tauro-muricholic acid (T-MCA) [37] and tauro-ursodeoxycholic acid (TUDCA) [38], therefore reducing steatosis and body weight gain partly via repressed expression of hepatic *Srebf1* [36]. Recent evidence suggest that elevated FXR antagonistic bile acids along with FXR antagonist that specifically inhibit intestine FXR, play a role in the treatment of NAFLD [39]. The role of intestinal FXR in body weight reduction, insulin resistance and fatty livers was firmly established in mice lacking FXR that are metabolically resistant to HFD-induced metabolic disease [40]. Furthermore, liver-specific *Shp* deletion, a target of FXR, prevented hepatic steatosis in animal fed a HFD and Western diet whilst the global double knockdown of *Shp* and *Fxr* protected against weight gain, glucose intolerance and hepatic steatosis [41]. Accordingly, our results indicate that FLAV, and in particular when combined with n-3, partially suppressed bile salt deconjugation and the subsequent microbial conversion of MCA into HDCA resulting in an increase of FXR bile acids antagonist which favoured its signalling inhibition in the intestine and liver. More precisely, we demonstrate that the FLAV-triggered repression of hepatic *Shp* translated into enhanced expression of *Cyp7a1*, the rate-limiting enzyme in the bile acid synthesis pathway, whose overexpression in the liver of mice counteracted diet-induced obesity, steatosis, and insulin resistance [42]. In support of this statement, the finding that FLAV suppressed the expression of *Tgr5* and *Gcg* mRNA levels in the small intestine, suggests that the secretion of glucagon-like peptide-1 via intestinal *Tgr5* did not account for the metabolic improvements caused by FLAV. Additionally, FLAV resulted in lower fasting insulin and insulin concentrations following the IPGTT, which along with the increased expression of *Cyp7a1* is thought to underlie the impact on steatosis and hepatocyte ballooning evident following the FLAVn-3 treatment. Given the collinearity between the FLAV induced changes in weight and insulin responses, the weight independent influence of FLAV on insulin sensitivity and NASH development

cannot be established. The weight gain associated with HF/HFr was accompanied by elevated plasma leptin. Leptin is secreted in proportion to white adipose tissue mass and is essential in the regulation of energy homeostasis, glucose and lipid metabolism functions [43]. The role of leptin in the progression of NAFLD remains unknown, but clinical data summarised in a meta-analysis (evaluating 33 studies and 2612 individuals), reported that circulatory leptin levels were associated with the severity of NAFLD [44]. Obesity is commonly associated with hyperleptinemia that can progress to leptin resistance with attenuated hypothalamic leptin signalling which fails to reduce the excess of adiposity [45, 46]. Here we showed that Flavn-3 reduced the weight gain induced by the HF/HFr diet, and was associated with lower circulating leptin levels, which may suggest improved leptin signalling. In agreement with this observation. In agreement with this observation and our FLAV induced lowering of leptin concentrations, Park *et al.* [47] recently reported that a flavonoid-rich grape extract reduced leptin, which together with our results suggest that altered leptin metabolism may partly underlie the FLAV induced reduction in NAFLD. These novel findings expand the state-of-the-art regarding the mechanism underlying the benefits of flavonoids in NAFLD [11].

De novo lipogenesis has been approximated to be 5-fold greater in NAFLD compared to normal individuals [48]. Our gene expression profiles indicate that reduced lipogenesis due to lower *Acaca* and *Fasn* expression along with their regulatory transcription factor *Srebf1* underlies the observed n-3 induced reduced steatosis, which is consistent with previous observations [49, 50]. A significant reduction in *Nr1h3*, the gene encoding the liver X receptor alpha (*Lxra*), a *Srebf1* upstream regulator, induced by n-3 supports this observation (See **Supplementary Table S4**). ~~No significant impact of treatment on carnitine palmitoyltransferase I (*Cpt1*) was evident, indicating that increased mitochondrial fatty acid oxidation did not significantly contribute to the reduced steatosis.~~ Increased fatty acid flux from adipose tissue and uptake of fatty acids as lipoproteins or complexed to albumin also contributes to hepatic TAG load in NAFLD. In particular, the lipoprotein and fatty acid receptors *Vldlr* and *Fabp4* have been implicated in NAFLD progression [51]. The observed

reduced expression of these receptors following both n-3 and FLAV supplementation is also likely to significantly contribute to the reduced steatosis and TAG concentration observed. Of interest is that because FLAV, but not n-3, failed to reduce the expression of *Srebf1*, a target gene of *Fxr* that controls fatty acid synthesis [52], such an explanation could also account for the synergistic effect of combining FLAV with n-3 in preventing NALFD development.

Finally, although not evident in the liver, an n-3 induced increase in the apparent bioavailability (based on higher urinary excretion) of select flavan-3-ol metabolites was observed which could contribute to the greater efficacy of FLAVn-3 relative to FLAV alone. This finding is consistent with Giunta et al. [53] who observed an n-3 induced increase in blood and brain green tea flavonoid concentrations in mice.

In conclusion combined supplementation with FLAVn-3 is highly effective in preventing NAFLD. The additive anti-lipogenic (n-3 effect) and insulin sensitising actions associated with reduced weight gain (FLAV effect) is likely to be responsible for the greater effects relative to either dietary component fed in isolation (**Figure 6**). The ability of this combined dietary approach to reverse steatosis or the more pathological NASH and fibrosis stages of the disease remains to be established and should be explored in clinical trials.

Authors' contributions: DV and AMM initiated, designed and obtained funding for the research. DV and IRR conducted the animal study, data acquisition and analysis. SR and SD performed the liver histology. DB and JG performed the TLDA experiments. NT and SPT conducted the fatty acids and lipid peroxides analyses. DDR and PM analysed the flavan-3-ols in the liver and urine samples. IRI provided expertise in bile acid metabolism, signalling and conducted analysis. DV, IRR, IRI and AMM wrote the paper. All authors critically revised the manuscript for intellectual content and approved the final manuscript.

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Figure legends

Figure 1. Hepatic biochemical and histological evidence of steatohepatitis in the liver of mice fed with a high fat/high fructose diet and the amelioration induced by the individual and combined impact of 16 weeks intervention with flavan-3-ols and n-3 fatty acids. A) Representative H&E-stained images of liver from mice fed with the different diets under 20x magnification. B) Liver TAG; C) Serum ALT; D) Serum AST. Values are expressed as mean \pm SEM. $n \geq 7$ (per group). Different superscripts indicate statistical differences between diet groups, ** $p < 0.01$; *** $p < 0.001$; vs the control group. NS, not significant

Figure 2. Effect of flavan-3-ols and n-3 fatty acids on body weight gain, liver and adipose tissue weight and leptin levels in mice fed a high fat/high fructose diet. (A) Body weight gain (0-16 weeks). (B) Liver weight (C) Inguinal adipose tissue weight (D) Plasma leptin (16 weeks). Values are expressed as mean \pm SEM. $n \geq 7$ (per group). Different superscripts indicate statistical differences between diet groups.

Figure 3. Effect of flavan-3-ols and n-3 fatty acids on insulin and glucose response in mice fed a high fat/high fructose diet. (A) Glucose and (B) insulin concentrations during the intraperitoneal glucose tolerance test (15 weeks). (C) Homeostatic model assessment for insulin resistance (HOMA-IR) (15 weeks). Values are expressed as mean \pm SEM. $n \geq 7$ (per group). Different superscripts indicate statistical differences between diet groups, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; vs the control group. # depicts statistical significance ($p < 0.05$) when compared to the control group (fasted state).

Figure 4. Taqman Low Density Array (TLDA) and real time quantitative PCR (qRT-PCR) analysis of liver samples. Impact of the intervention treatments on the gene expression modulation of A) Fatty acid uptake, B) Lipogenesis and C) Fibrosis. Values are expressed as mean \pm SEM. $n \geq 7$ (per group). Different superscripts indicate statistical differences between diet groups.

Figure 5. Flavan-3-ols and n-3 fatty acids alter intestinal bile acid metabolism and signature in mice fed a high fat/high fructose diet. Concentration of (A) total bile acids; (B) proportion of conjugated bile acids; (C) content of hyodeoxycholic acid (HDCA); (D) ratio HDCA to tauro-muricholic acids (α MCA + β MCA); and (E) concentration of FXR bile acid activators or inhibitors in the small intestine. Values are least square means \pm SEM, $n = 7-8$ per treatment. Different superscripts indicate statistical differences between diet groups, $P < 0.001$. CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; T-UDCA, tauro ursodeoxycholic acid.

Figure 6. Flavan-3-ols and n-3 fatty acids inhibit FXR signalling in the intestine and liver of mice fed a high fat/high fructose diet. Gene expression of FXR target genes in the liver (A-C) and illeum (D-F). Values are expressed as mean \pm SEM. $n \geq 7$ (per group). Different superscripts indicate statistical differences between diet groups, $P < 0.05$. *Shp*, small herodimer partner; *Cyp*, cytochrome P450; *Tgr5* (aka *Gpbar1*, G protein-coupled bile acid receptor 1); *Gcg*, glucagon.

Figure 7. Proposed mechanisms underlying the effect of flavan-3-ols (FLAV), n-3 fatty acids (n-3) and their combination (FLAVn-3) in NAFLD prevention. a) n-3 increases the bioavailability of FLAV in the intestine. b) FLAV increase the pool of *Fxr* bile acids (BA) antagonists which reduce its downstream effector (SHP) and in turn upregulates *Cyp7a1*. c) FLAV and/or its metabolites reduce the higher insulin and leptin plasma levels and d) overweight induced by HF/HFr diet. e) n-3 supresses TAG accumulation in the liver by downregulating de novo lipogenesis (DNL). f) FLAVn-3 reduces the fatty acids efflux into the liver through downregulation of lipoprotein (VLDLr) and fatty acids receptors (FABP4). g) FLAVn-3 downregulates genes involved in fibrosis (FIBR).

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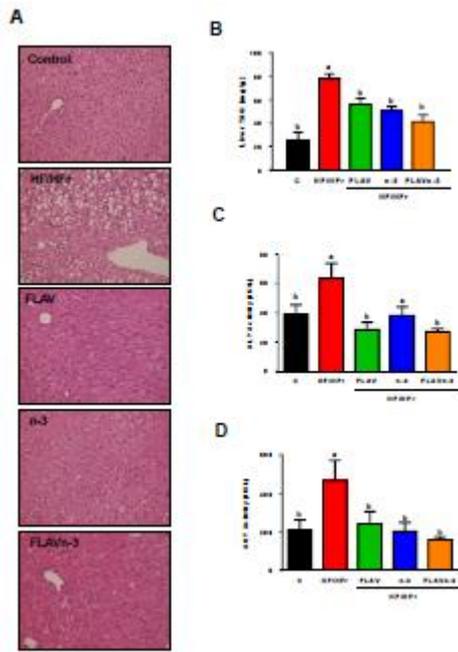


Fig. 1

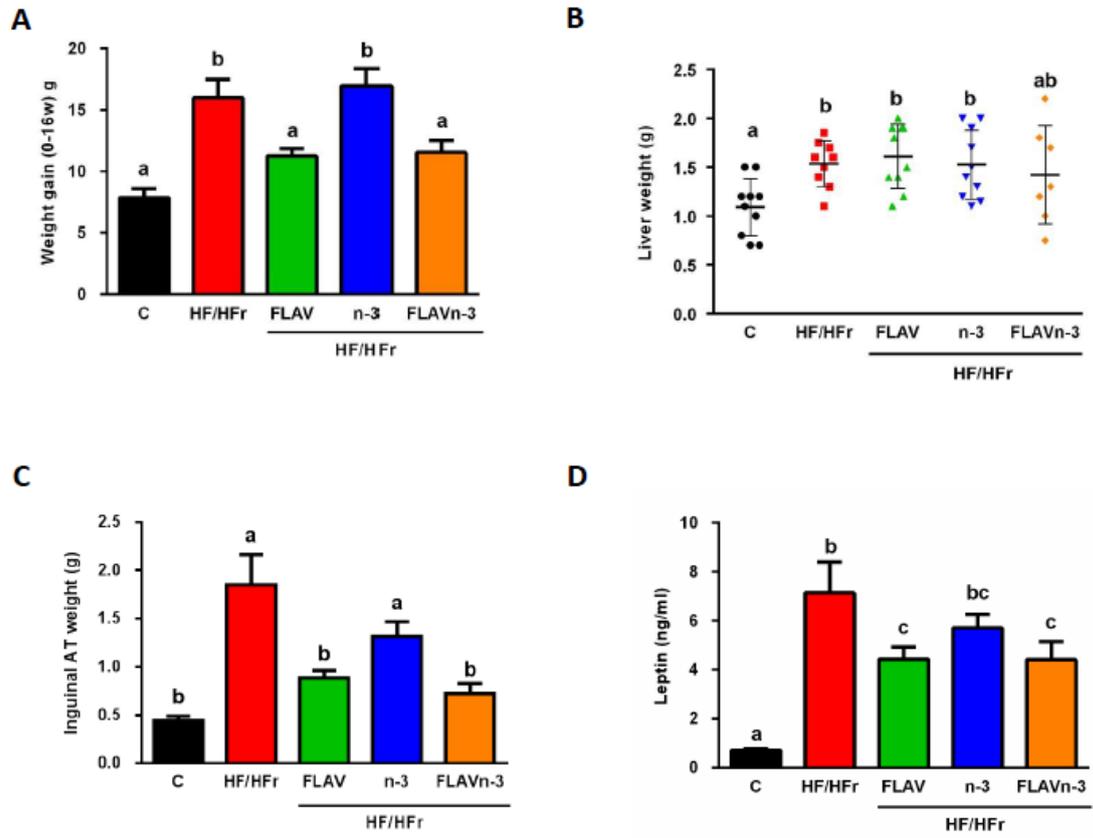


Fig. 2

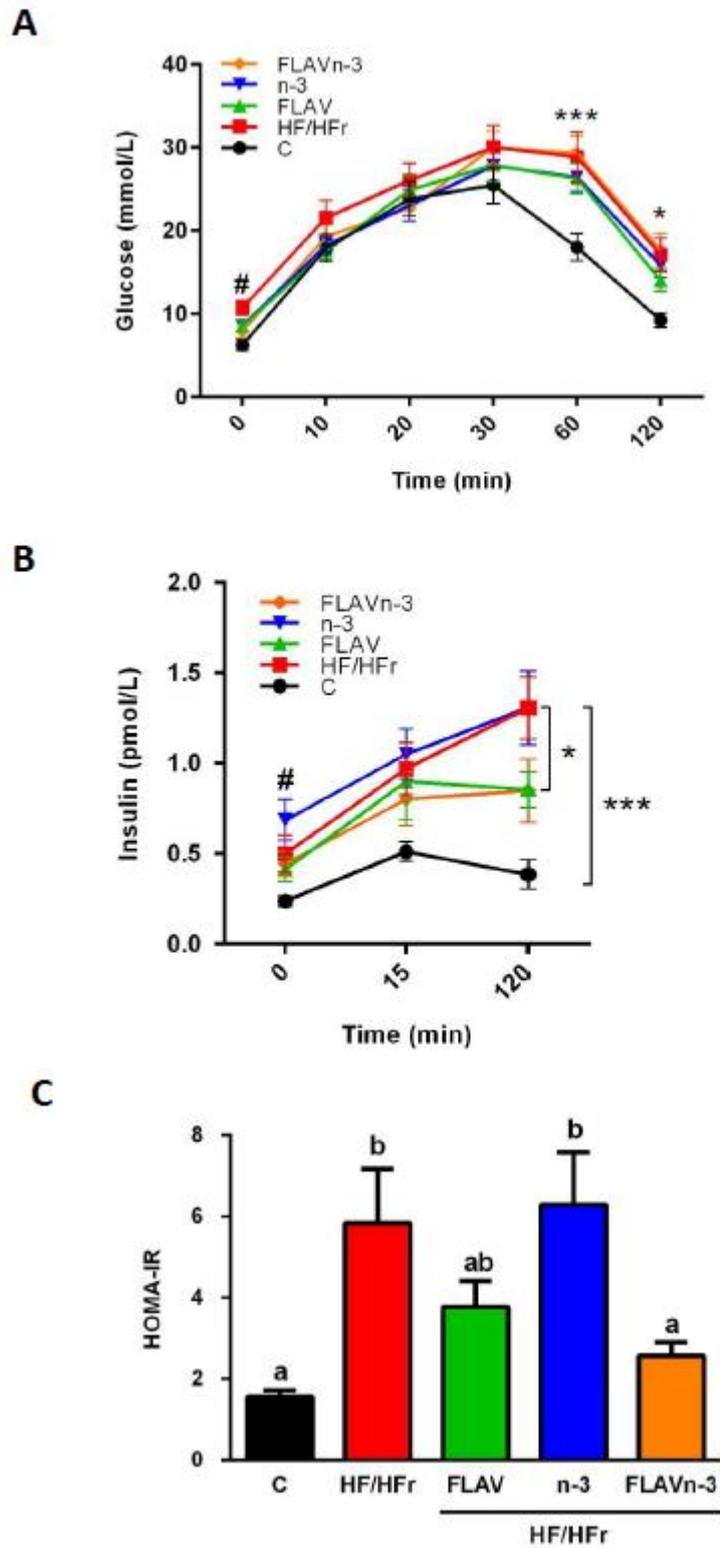


Fig. 3

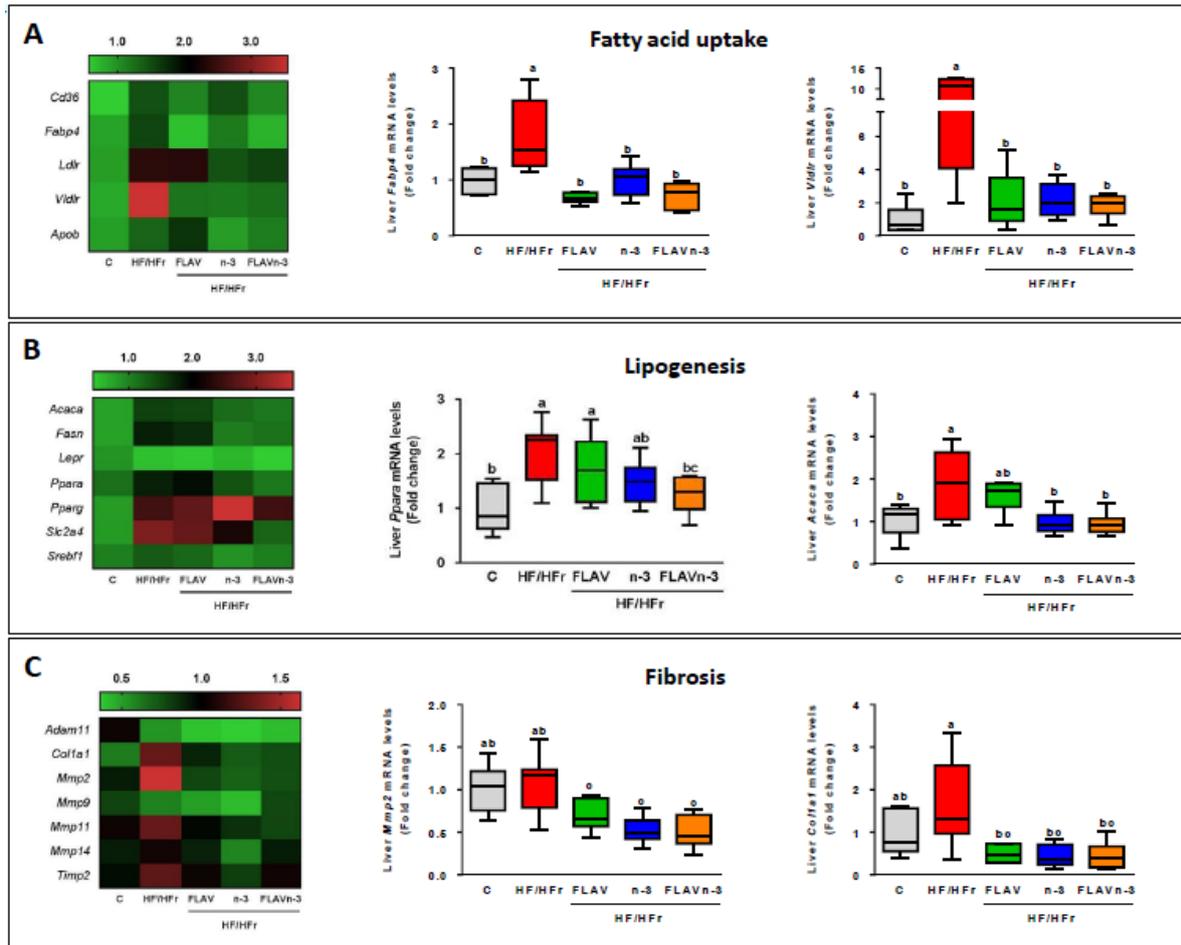


Fig. 4

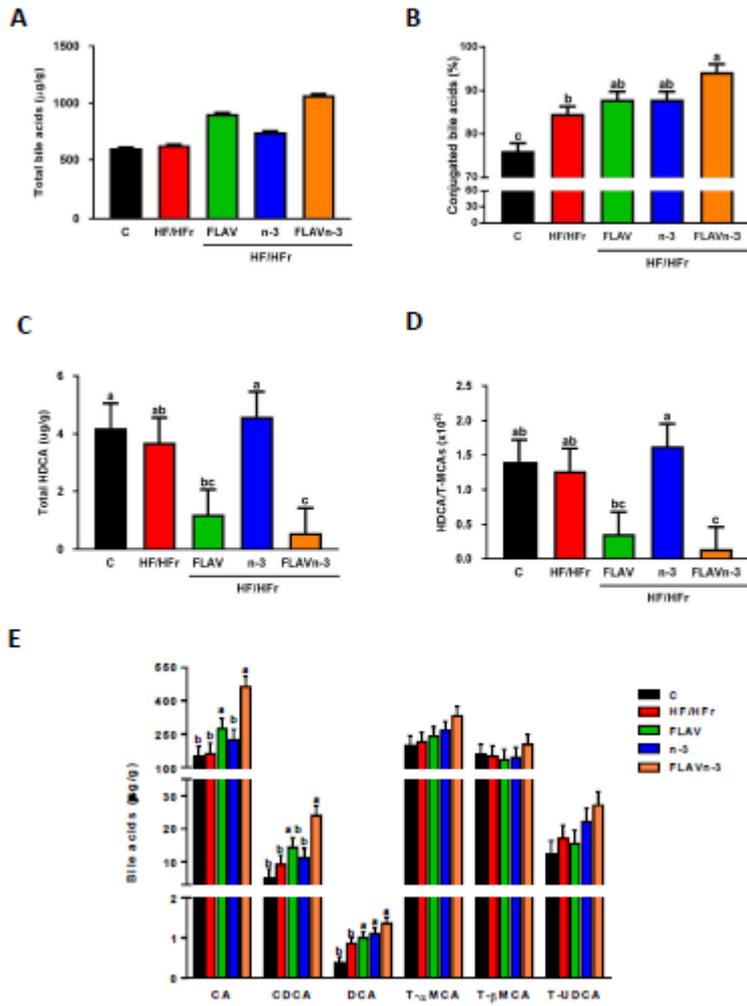


Fig. 5

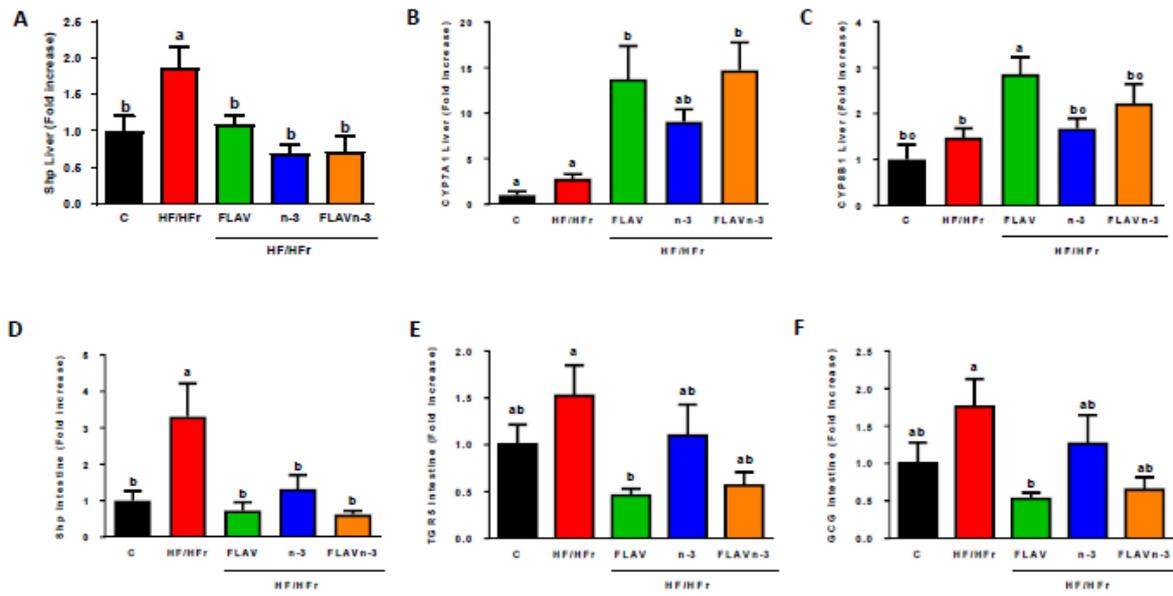


Fig. 6

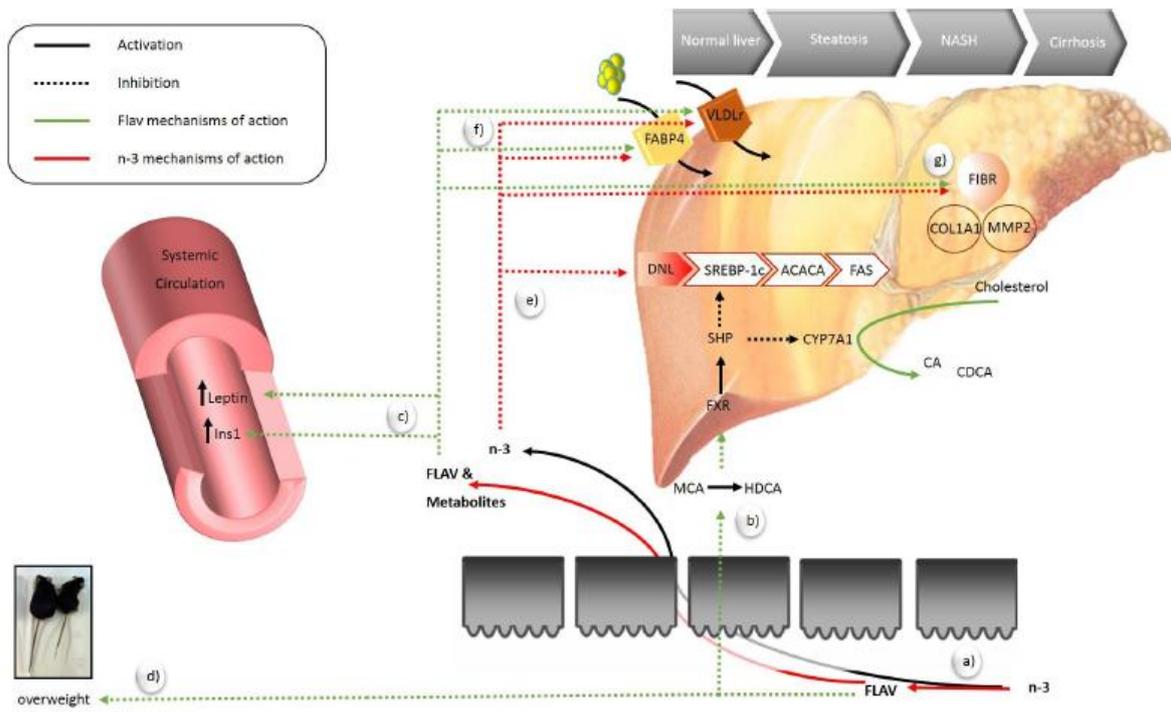


Fig. 7

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Table 1. Histological changes following 16 weeks high fat high fructose (HF/HFr) diet supplemented or not with flavanols (FLAV), long-chain n-3 PUFAs (n-3) or a combination of both (FLAVn-3). Components of the NAS (steatosis amount, lobular inflammation, and ballooning) mean scores are expressed as mean \pm standard deviation and significance was obtained using the Mann-Whitney U test for $P < 0.05$.

	Control	HF/HFr	HF/HFr		
			+ Flav	+ n-3	+ FLAVn-3
Steatosis					
Grade 0 (< 5 %)	7	3	7	8	9
Grade 1 (5-33 %)	0	3	2	2	1
Grade 2 (34-66 %)	0	4	0	0	0
Grade 3 (>66 %)	0	0	0	0	0
<i>Mean Score</i>	0.0 ± 0.0^b	1.1 ± 0.8^a	0.2 ± 0.4^{ab}	0.2 ± 0.2^{ab}	0.1 ± 0.3^b
Lobular Inflammation					
Grade 0 (none)	5	1	5	6	8
Grade 1 (<2)	2	9	4	4	2
Grade 2 (2-4)	0	0	0	0	0
Grade 3 (>4)	0	0	0	0	0
<i>Mean Score</i>	0.2 ± 0.4	0.6 ± 0.3	0.3 ± 0.4	0.3 ± 0.3	0.3 ± 0.5
Hepatocyte Ballooning					
Grade 0 (none)	7	1	5	8	10
Grade 1 (few)	0	2	3	2	0
Grade 2 (many)	0	7	1	0	0
<i>Mean Score</i>	0.0 ± 0.0^b	1.6 ± 0.7^a	0.6 ± 0.7^{ab}	0.2 ± 0.2^b	0.0 ± 0.0^b
NAS Score (0-8)	0.2 ± 0.2^b	3.3 ± 0.5^a	1.1 ± 0.2^b	0.7 ± 0.1^b	0.4 ± 0.2^b

Highlights

- Although highly prevalent, no treatment exists for NAFLD
- Flavonoids (FLAV) and n-3 fatty acids are emerging as dietary strategies
- FLAV and n-3 reduced steatosis, body weight gain and insulin resistance in mice
- FLAV ameliorated bile acid metabolism and n-3 downregulated lipogenic genes
- FLAVn-3 should be tested for efficacy in human NAFLD

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