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Liraglutide Decreases Hepatic Inflammation and Injury in Advanced Lean Non-Alcoholic Steatohepatitis

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Abstract: Although commonly associated with obesity, non-alcoholic fatty liver disease (NAFLD) is also present in the lean population representing a unique disease phenotype. Affecting 25% of the world's population, NAFLD is associated with increased mortality especially when progressed to non-alcoholic steatohepatitis (NASH). However, no approved pharmacological treatments exist. Current research focuses mainly on NASH associated with obesity, leaving the effectiveness of promising treatments in lean NASH virtually unknown. This study therefore aimed to evaluate the effect of liraglutide (glucagon-like peptide 1 analogue) and dietary intervention, alone and in combination, in guinea pigs with non-obese NASH. After 20 weeks of high-fat feeding (20% fat, 15% sucrose, 0.35% cholesterol), 40 female guinea pigs were block-randomized based on weight into four groups receiving one of four treatments for 4 weeks: continued high-fat diet (HF, control), high-fat diet and liraglutide treatment (HFL), chow diet (4% fat, 0% sucrose, 0% cholesterol; HFC) or chow diet and liraglutide treatment (HFCL). High-fat feeding induced NASH with severe fibrosis. Liraglutide decreased inflammation ($p < 0.05$) and hepatocyte ballooning ($p < 0.05$), while increasing hepatic α-tocopherol ($p = 0.0154$). Dietary intervention did not improve liver histopathology significantly, but decreased liver weight ($p = 0.004$), plasma total cholesterol ($p = 0.0175$), LDL-cholesterol ($p = 0.0063$), VLDL-cholesterol ($p = 0.0034$), hepatic cholesterol ($p < 0.0001$) and increased hepatic vitamin C ($p = 0.0099$). Combined liraglutide and dietary intervention induced a rapid weight loss, necessitating periodical liraglutide dose adjustment/discontinuation, limiting the strength of the findings from this group. Collectively, this pre-clinical study supports the beneficial effect of liraglutide on NASH and extends this notion to lean NASH.

Non-alcoholic fatty liver disease (NAFLD) is estimated to affect about 25% of the world's population [1]. Progression to non-alcoholic steatohepatitis (NASH) promotes liver fibrosis and is an important risk factor for metabolic dysfunction and cardiovascular disease [2–4]. Although often associated with obesity and insulin resistance, NAFLD in lean/non-obese and even non-diabetic individuals is not a rare occurrence, with reported prevalence ranging from 3 to 25% [5,6]. Consequently, lean NAFLD represents a significant, albeit less recognized, end of the phenotypic spectrum of NAFLD [6]. Research efforts are currently engaged in developing novel intervention strategies against NASH; however, no effective pharmacological treatment option is presently available and data supporting reliable effects of lifestyle (e.g. dietary) intervention in lean NAFLD are lacking [7,8]. Furthermore, much of the current clinical and pre-clinical research largely targets obese NASH, potentially neglecting the lean NASH phenotype. Glucagon-like peptide 1 (GLP-1) receptor agonists may hold promise as potential pharmacological treatment options and have been found to improve both hepatic steatosis and oxidative stress in obese mice and rats with steatosis or mild (no/early stage fibrosis) NASH [9–14]. In human beings, an investigator-initiated, randomized, controlled clinical trial recently reported histological resolution of NASH in obese/overweight patients treated for 48 weeks with the GLP-1 receptor agonist liraglutide [15]. This suggests that liraglutide intervention may constitute a treatment option of NASH although the effectiveness in more advanced disease (NASH with severe fibrosis) remains poorly understood, especially in the setting of lean NASH.

The absence of excessive adiposity suggests a phenotypic uniqueness of lean NASH compared to obese NASH and implies that translating results from obese pre-clinical models to the lean phenotype may be challenging. The high-fat diet, as applied in this study, does not cause excessive weight gain compared to a normal chow diet [16,17]. Hence, high-fat-fed guinea pigs do not display an obese phenotype and remain non-obese relative to healthy chow-fed controls. In addition, key histological features of NASH (steatosis, hepatocyte ballooning, inflammation and fibrosis) induced by a high-fat diet are similar in guinea pigs and human beings [17]. This suggests the non-obese NASH guinea pig as a relevant *in vivo* model with high face and predictive validity. Like human beings – but unlike rats and mice – guinea pigs have a LDL-dominant lipoprotein profile and respond to different pharmacotherapies (e.g. statins) similarly to reports from clinical studies [16–19]. The present study therefore aimed to investigate the effects of liraglutide and dietary intervention alone and in

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combination, in our validated guinea pig model [16,17]. Dietary intervention constituted a switch from an unhealthy (high fat) to a healthy (chow) diet similar to a clinical setting. In accordance with the relationship between NAFLD and cardiovascular disease, and recent findings of a cardiovascular protective effect of liraglutide, we also investigated the effect of liraglutide on endothelial function as a secondary end-point [20,21].

Materials and Methods

Animals and experimental design. All animal experimentation was approved by the Animal Experimentation Council under the Danish Ministry of Environment and Food (identification code 2015−15−0201−00621, date of approval: 3 July 2015), and in accordance with the European Legislation of Animal Experimentation 2010/63/EU.

Forty female Dunkin Hartley guinea pigs (Envigo RMS B.V. Venray 5800 AN the Netherlands) weighing 500–650 g were group-housed in floor pens with wood shavings, hay, straw and environmental enrichment and maintained on a 12-hr light–dark cycle with temperatures between 20 and 24°C. The guinea pigs were fed a high-fat high-sucrose diet (20% fat, 15% sucrose, 0.35% cholesterol) for 20 weeks, after which they were block-randomized based on weight into four groups (n = 10): A high-fat diet (HF) group, which continued on the start-up high-fat diet, a high-fat diet group treated with liraglutide (HFL), a group changed to a chow diet (4% fat, 0% sucrose, 0% cholesterol; HFC) and a group changed to a chow diet and treated with liraglutide (HFCL) for 4 weeks until study termination, at which animals were killed as previously described [16,17]. All diets (Ssniff Spezialdiäten GmbH, Soest, Germany) were stored at −20°C until use (table 1). The exact dietary compositions are given in Table S1 and are similar to previously used high-fat and chow diets [16,17]. Feed aliquots were thawed twice weekly and food intake measured by weighing the amount of food remaining prior to each refill. The dose of liraglutide (Novo Nordisk A/S, Måløv, Denmark) was based on previous literature [9–14] and a small pilot study aiming to induce a 10–15% weight loss. Liraglutide was slowly titrated over a period of 9 days to 30 nmol/kg body-weight (BW) twice a day (BID) and administered by subcutaneous injections. During dose escalation, guinea pigs received 5.4 nmol/kg BW BID for 2 days, then 10.8 nmol/kg BW BID for 2 days, followed by 16.2 nmol/kg BW BID for 3 days and lastly 21 nmol/kg BW BID for 2 days. Groups not treated with liraglutide (HF and HFC) were injected with a corresponding volume of vehicle (50 nM phosphate, 70 mM NaCl, 0.05% Tween 80, pH 7.4).

Expectedly, the combination of liraglutide and dietary intervention induced weight loss in the HFCL group. However, the weight loss occurred more rapidly than predicted and approached 20% of the initial body-weight due to an almost complete cessation of food intake. As 20% weight loss was defined as a humane end-point for this study, this necessitated dose adjustment/discontinuation of the liraglutide treatment in some animals. Dietary intervention was maintained, but treatment with liraglutide was only fully resumed in animals that had regained weight corresponding to a weight loss of <10% of their initial body-weight. During adjustment, doses were 15 nmol/kg BW BID for 2 days, then 21 nmol/kg BW BID for 2 days before being increased to 30 nmol/kg BW BID. Consequently, none of the guinea pigs in the HFCL group received uninterrupted treatment with liraglutide during the 4-week intervention period. Three of 10 guinea pigs in the HFCL group made it back to the original dose.

Table 1.

Dietary composition

Nutrient (%)	High-fat high-sucrose	Chow
Protein	16.7	16.8
Carbohydrates	37.9	47.1
Fat	20.0	4.2
Cholesterol	0.35	–
Sucrose (total amount added to diet)	15.0	–

Plasma samples. Blood samples for the analysis of total cholesterol (TC), triglyceride (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lipoprotein fractions (VLDL, LDL and HDL) were collected from the *vena saphena* in K_3-EDTA microvettes (Sarstedt, Nümbrecht, Germany) prior to ('week 0') and 2 weeks after the initiation of the treatments, as previously described [22]. At these time-points, guinea pigs from each of the four groups were block-randomized based on weight and plasma analysed for either lipids (n = 5) or ALT and AST (n = 5). At euthanasia (week 4), blood samples were collected intracardially using a K_3-EDTA flushed syringe except for samples analysed for free fatty acids (FFA) and alkaline phosphatase (ALP), which were collected in NaF and heparin microvettes (Sarstedt), respectively. Plasma was obtained by centrifuging blood samples at 2000 × *g* for 4 min. at 4°C. AST, ALT, ALP and FFA were analysed on a Cobas 6000 (Roche Diagnostic Systems, Berne, Switzerland) according to the manufacturer's specifications. TC, TG and lipoprotein fractions were analysed at the *Lipoprotein Analysis Laboratory* (Wake Forest School of Medicine, Winston-Salem, NC, USA) as previously described [23]. L-arginine and asymmetric dimethylearginine (ADMA) levels were measured by HPLC [24] and plasma samples for quantification of ascorbic acid (AA) and dehydroascorbic acid (DHA) were stabilized metaphosphoric acid before analysis by HPLC as previously described [25–27]. 8-Isoprostanes were analysed by ELISA according to the manufacturer's specifications (Cayman Chemicals, Ann Arbor, MI, USA). For the determination of dihydrobiopterin (BH_2) and tetrahydrobiopterin (BH_4), blood was stabilized in 0.1% dithioerythritol and centrifuged (2000 × *g*, 4 min., 4°C), yielding a plasma fraction which was analysed by HPLC as described elsewhere [28].

Liver samples.

Lipids and glycogen. The liver was rinsed in ice-cold phosphate-buffered saline (140 mM NaCl, 10 mM phosphate, 3 mM KCl, pH 7.4, Millipore, Billerica, MA, USA) and weighed. TG, TC and glycogen content were analysed on homogenates from the left lateral lobe (*lobus hepatis sinister lateralis*) on a Cobas 6000 according to the manufacturer's specifications, as described previously [16,17]. In short, 1 ml of extraction buffer (0.15 M sodium acetate and 0.75% Triton-X) was added to the frozen samples which were subsequently homogenized. The samples were then placed in a 100°C water bath for two minutes, before cooling on ice and supplemented with 0.5 ml extraction buffer. Five hundred microlitres of homogenate was then centrifuged at 3400 g for 10 min. at 4°C and the supernatant analysed for TG, TC and free glucose. Total glucose was measured in 400 µl homogenate after overnight incubation at room temperature with 20 µl amyloglucosidase (Sigma, St. Louis, MO, USA). Subsequently, glycogen concentrations were calculated by subtracting free glucose from total glucose.

Markers of oxidative stress. Vitamin C, L-arginine and ADMA were quantified in liver homogenates as stated above. Hepatic levels of glutathione (GSH) and oxidized glutathione (GSSG) were measured according to Hissin and Hilf [29] and malondialdehyde (MDA) was measured as described [30]. Superoxide dismutase (SOD) was measured using colorimetry (Randox Superoxide Dismutase (Ransod assay), Randox Laboratories Ltd, Crumlin, UK) according to the manufacturer's specifications. α-Tocopherol and γ-tocopherol were analysed by stabilizing liver homogenates with butylated hydroxytoluene and otherwise proceeding as described by Burton *et al.* [31].

Histology. Sections of the left lateral lobe (*lobus hepatis sinister lateralis*) were fixed in 4% paraformaldehyde in phosphate-buffered saline for 72 hr at 4°C, after which the tissue was stored in 1% paraformaldehyde at 4°C prior to embedding in paraffin [16,17]. Tissue samples were cut into 2- to 4-μm sections and stained with haematoxylin and eosin and Masson's trichrome and scored in a blinded fashion in accordance with the semi-quantitative scoring scheme suggested by Kleiner *et al.* [32]. The degree of steatosis, ballooning (degenerating) hepatocytes and fibrosis were scored in the entire liver section. Steatosis was graded as 0 (<5%), 1 (5–33%), 2 (>33–66%) or 3 (>66%) and ballooning as 0 (not present), 1 (few ballooning hepatocytes) or 2 (many/prominent ballooning hepatocytes). Lobular inflammation was scored in five lobuli (each defined by the presence of at least two portal areas surrounding a central vein) and assessed based on the number of inflammatory foci (defined as at least three inflammatory cells in close proximity of each other). Inflammation was scored as 0 (not present), 1 (<2 foci), 2 (2–4 foci) and 3 (>4 foci). Portal inflammation was assessed by individually evaluating all portal areas for inflammation (defined by the presence of ≥10 inflammatory cells) and scored as 0 (no inflammation in any portal area) or 1 (inflammation in at least one portal area). The presence of intracellular lipid depositions was previously verified, in this model, by Oil Red O staining [16]. Fibrosis was evaluated on Masson's trichrome-stained sections as either 0 (not present), 1 (perisinusoidal *or* periportal), 2 (perisinusoidal *and* periportal), 3 (bridging from central vein to central vein, central vein to portal vein and/or portal vein to portal vein) or 4 (cirrhosis). To assess disease severity, the NAFLD activity score was calculated as the unweighted sum of steatosis, ballooning and inflammation, ranging from 0 to 8 [32].

Endothelial dysfunction. Endothelial function was investigated in coronary arteries from HF and HFL. After euthanasia, the heart was isolated and placed into cold physiological buffer (117.8 mM NaCl, 4.0 mM KCl, 2.0 mM $CaCl_2$, 0.9 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 20 mM $NaHCO_3$ and 5.0 mM glucose). The left anterior descending coronary artery was cleaned of surrounding myocardial tissue and cut into segments (approximately 2 mm long) that were mounted in wire-myograph organ chambers (Danish Myo Technology, Aarhus, Denmark) filled with 5 ml physiological buffer (37°C, perfused with 5% CO_2 in air). The segments were progressively stretched to their optimal internal circumference (IC1) equal to 90% of the internal circumference (IC100) under a passive transmural pressure at 100 mmHg (13.3 kPa). After an equilibrium period for 30 min., the segments were challenged twice with 60 mM potassium (similar composition as the physiological buffer, with NaCl exchanged by KCl in an equimolar basis). Only segments with potassium-induced contraction >1 mN/mm were included in the study. Vasodilator responses were determined by adding increasing concentration of the muscarinic acetylcholine receptor agonist carbachol (from 10^{-9} to 3×10^{-5} M) (Sigma-Aldrich, St. Louis, MO, USA) to 60 mM potassium pre-contracted segments. To elucidate the carbachol–vasodilator responses, the carbachol concentration–response curves were obtained in the absence (controls) or in the presence of either the cyclooxygenase inhibitor indomethacin (10^{-4} M) (Sigma-Aldrich, St. Louis, MO, USA) or the endothelial nitric oxide synthase inhibitor L-NAME (10^{-5} M) (Sigma-Aldrich). Active tension was calculated by subtracting the passive tension from the potassium-induced active tension. Carbachol-induced tension was normalized to the potassium-induced active tension.

Statistical analysis. All statistical analyses were performed in SAS Enterprise Guide 7.1 (SAS Institute Inc, Cary, NC, USA) and graphs were made with GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). Weight, energy intake and endothelial function was analysed by mixed linear model, while plasma TG, VLDL-TG, TC, VLDL-C, LDL-C, HDL-C, ALT and AST were analysed with an ANCOVA with baseline (week 0) as covariate. All other continuous data were analysed with ANOVA. Multiple comparisons were corrected by Tukey's post hoc test after the ANCOVA or ANOVA. Continuous data are presented as means with standard deviations (S.D.) while ordinal data (liver histology) were analysed with Kruskal–Wallis test followed by Dunn's post hoc test [33] and presented as medians. Data with inhomogeneous variances (plasma TG, VLDL-TG, TC, VLDL-C, LDL-C, HDL-C, ALT, AST, L-arginine/ADMA and liver L-arginine/ADMA) were log-transformed before analysis and then back-transformed and presented as geometric means with 95% confidence intervals. A p-value below 0.05 was considered statistically significant.

Results

Body-weight and energy intake.

All groups displayed similar weight gain prior to liraglutide and dietary intervention. Liraglutide treatment resulted in weight loss of HFL and HFCL compared to HF (HFL/HFCL *versus* HF $p = 0.046/p = 0.0009$, $p < 0.0001/p < 0.0001$, $p < 0.0001/p < 0.0001$ and $p < 0.0001/p < 0.0001$ at weeks 21, 22, 23 and 24, respectively) and HFC (HFL/HFCL *versus* HFC $p = 0.0461/p = 0.0122$, $p = 0.0009/p < 0.0001$, $p = 0.0027/p < 0.0001$ and $p = 0.0004/p = 0.0003$ at weeks 21, 22, 23 and 24, respectively). The change from high-fat to chow diet in the HFC group seemed to result in weight stagnation, but the weight was not significantly different from that of HF animals (fig. 1A). The average daily energy intake was decreased for HFL compared to HF at weeks 1 ($p < 0.0001$), 2 ($p < 0.0001$) and 4 ($p = 0.0018$) post-intervention and in HFC and HFCL compared to HF at weeks 1 ($p < 0.0001$ and $p < 0.0001$, respectively), 2 ($p < 0.0001$ and $p < 0.0001$, respectively), 3 ($p = 0.005$ and $p = 0.0007$, respectively) and 4 ($p < 0.0001$ and $p < 0.0001$, respectively). Energy intake was lower in HFL compared to HFC at week 1 post-intervention ($p = 0.01$) and lower in HFCL compared to HFL and HFC at weeks 1 ($p < 0.0001$ and $p < 0.0001$, respectively) and 2 post-intervention ($p < 0.0001$ and $p < 0.0001$, respectively) (fig. 1B).

Liver histology and status.

High-fat feeding induced NASH and severe (grade 3) fibrosis with close resemblance to human histopathology, that is bridging fibrosis and a pericellular/perisinusoidal (chicken wire) fibrotic architecture originating from the central veins alongside macro- and microvesicular steatosis, inflammation and hepatocyte ballooning. Liraglutide decreased hepatocyte ballooning in HFL compared to HF ($p < 0.05$) and inflammation in HFL and HFCL compared to HF ($p < 0.05$; fig. 2A,B). Although not reaching statistical significance, hepatocyte ballooning was reduced in HFC and HFCL compared to HF. Hepatic steatosis was lowered after dietary intervention in HFC and HFCL compared to HFL ($p < 0.05$) and also tended to be decreased compared to HF (fig. 2C). Bridging fibrosis was observed in most HF animals (80%) and was not significantly affected by treatments (fig. 2D). Portal inflammation was not significantly different among groups, although a nominal reduction was observed by

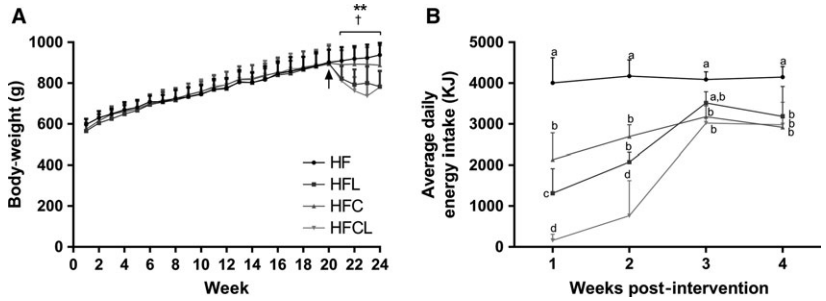

Fig. 1. Body-weight and energy intake. (A) Body-weight increased similarly in all groups prior to intervention (indicated by the arrow) after which the body-weight stagnated in HFC and decreased significantly in HFL and HFCL compared to HF and HFC. (B) The average daily energy intake followed the changes in body-weight and decreased in HFL, HFC and HFCL compared to HF (B). (A) $^{**}p < 0.01$ HFL/HFCL *versus* HF $^{\dagger}p < 0.05$ HFL/HFCL *versus* HFC. Groups with different subscripts differs significantly from each other at the designated time-point with at least $p < 0.05$. Means with S.D. n = 10.

treatment with liraglutide in HFL compared to HF (fig. 2E). NAFLD activity scores were reduced in all treatment groups, reaching statistical significance in HFCL compared to HF ($p < 0.05$; fig. 2F).

In HFL, HFC and HFCL, the absolute liver weight was decreased compared to HF animals (Table S2). Normalized to body-weight only HFC ($p = 0.004$) and HFCL ($p = 0.0311$) animals displayed significantly reduced liver weights compared to HF. Likewise, hepatic cholesterol levels were decreased in HFC and HFCL compared to HF ($p < 0.0001$ and $p < 0.0001$, respectively) and HFL ($p = 0.0363$ and $p = 0.0307$, respectively), but not in HFL compared to HF. Hepatic TG levels did not differ between groups. Liver glycogen was not different between HF, HFL and HFC, but reduced in HFCL compared to HF ($p = 0.0335$; table 2). ALT and AST was elevated in HFCL compared to HF ($p < 0.0001$ and

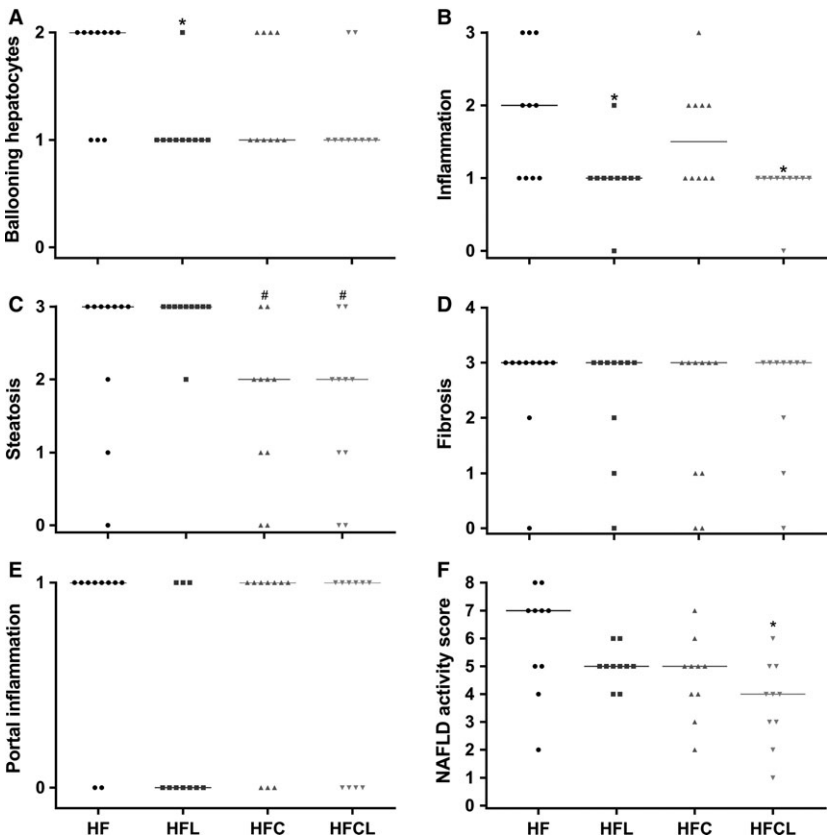

Fig. 2. Liver histology and disease severity. (A) HFL displayed significantly fewer ballooning (degenerative) hepatocytes compared to HF animals, (B) while HFL and HFCL reduced the degree of inflammation. (C) Dietary intervention reduced hepatic steatosis compared to HFL and tended to reduce steatosis grade compared to HF. (D) Bridging fibrosis was observed in most animals and was not significantly affected by the interventions. (E) Portal inflammation appeared to be reduced in HFL, but did not reach statistical significance compared to HF. (F) The NAFLD activity score was reduced in HFCL and a tended to be reduced in HFL and HFC. $^{*}p < 0.05$ *versus* HF. $^{\#}p < 0.05$ *versus* HFL. Individual values with medians (line). n = 10.

$p = 0.0243$), HFL ($p < 0.0001$ and $p = 0.0134$) and HFC ($p < 0.0001$ and $p = 0.0389$) 2 weeks after intervention, but did not differ between groups at any other time-points (fig. 3). ALP did not differ between the groups (table 2).

Hepatic oxidative stress.

Total levels of the water-soluble antioxidant vitamin C were higher in HFC livers compared to HF ($p = 0.0099$), HFL ($p < 0.0001$) and HFCL ($p = 0.0014$) despite identical vitamin C content in the diets, whereas levels of the oxidized form of vitamin C, DHA, did not differ between groups. Liraglutide increased the fat-soluble antioxidants α-tocopherol in HFL compared to HF ($p < 0.0154$), HFC ($p = 0.0023$) and HFCL ($p = 0.0012$) and γ-tocopherol compared to HFC ($p = 0.013$). MDA, a marker of lipid oxidation, was increased in HFCL compared to HF ($p < 0.0001$), HFL ($p = 0.0021$) and HFC ($p = 0.017$). There were no differences in hepatic antioxidants SOD, GSH and GSSG, %GSSG, or the oxidative stress markers L-arginine, ADMA and the L-arginine/ADMA ratio between groups (table 2).

Dyslipidaemia.

Plasma TC, TG and fractionated (VLDL-TG, VLDL-C, LDL-C and HDL-C) lipoprotein levels confirmed an equal degree of dyslipidaemia in all groups before intervention (week 0). Measurements were obtained at two additional time-points; weeks 2 and 4 (termination) after intervention (table 3) (for reference TC = 1.16 [0.92–1.45] mM, LDL-C = 0.99 [0.74–1.31] mM and TG = 1.02 [0.80–1.31] in guinea pigs fed a chow diet for 25 weeks [17]). HFL animals displayed an increase in VLDL-TG ($p = 0.0254$) compared to HFCL at week 2, but was otherwise not significantly different from the other groups. In the HFC group, TC was decreased after 2 and 4 weeks compared to both HF ($p = 0.0005$ and $p = 0.0175$, respectively) and HFL ($p < 0.0001$ and $p = 0.0056$, respectively) animals on both time-points. A similar pattern was observed in HFCL, although the reduction in TC only reached statistical significance compared to HFL animals at week 2 ($p = 0.0006$). After 2 and 4 weeks, VLDL-C ($p < 0.0001$ and $p = 0.0034$) and LDL-C ($p < 0.0001$ and $p = 0.0063$) were decreased in HFC animals compared to HF. In the HFCL group, VLDL-C was decreased compared to HF at week 2 ($p = 0.0183$) and compared to HFL at weeks 2 ($p < 0.0001$) and 4 ($p = 0.023$), whereas LDL-C was decreased at week 2 compared to HFL ($p < 0.0001$). VLDL-TG was also found to be decreased in HFCL animals compared to HFL animals at week 2 ($p < 0.0254$). At week 4, free fatty acids (FFA) were significantly increased in HFL compared to HF ($p = 0.0217$) and HFC ($p = 0.0396$) and in HFCL compared to HF ($p = 0.0412$) (table 4). On all other time-points and measures of dyslipidaemia, groups did not differ significantly.

Plasma markers of oxidative stress.

Plasma vitamin C levels were significantly lower in HFL and HFCL compared to HF and HFC ($p < 0.05$), whereas DHA and the DHA/vitamin C ratio (Table S2) were equal between groups. Circulating levels of L-arginine, ADMA (Table S2), the L-arginine/ADMA ratio and 8-isoprostanes were not different between groups. The BH_2/BH_4 ratio was increased in HFCL compared to HF ($p = 0.0237$) and HFC ($p = 0.0124$; table 4).

Endothelial dysfunction.

The acetylcholine receptor agonist carbachol induced vasodilation that was completely abolished by the nitric oxide synthase

Table 2.

Liver status at euthanasia (week 4 post-intervention)

	HF	HFL	HFC	HFCL
Relative liver weight (%)	5.72 ± 1.89	4.61 ± 1.24	3.53 ± 0.83**	4.00 ± 1.13*
Cholesterol (μmol/g tissue)	40.8 ± 9.18	33.3 ± 8.12	23.4 ± 6.99***,#	23.1 ± 6.83***,#
Triglycerides (μmol/g tissue)	52.5 ± 11.3	57.4 ± 8.85	47.2 ± 18.6	40.1 ± 16.5
Glycogen[1] (μmol/g tissue)	55.7 (38.8–80.0)	32.9 (22.9–47.2)	28.4 (19.7–40.7)	18.5 (12.2–28.1)**
Plasma ALP (U/l)	47.0 ± 10.1	48.6 ± 7.56	56.4 ± 10.4	59.5 ± 13.4
Total vitamin C (nmol/g tissue)	500 ± 95.5	422 ± 102	661 ± 94.3**,###	465 ± 134††
DHA (nmol/g tissue)	9.44 ± 15.9	9.00 ± 7.85	3.14 ± 7.65	7.42 ± 4.67
α-Tocopherol (nmol/g tissue)	25.5 ± 10.0	41.8 ± 17.6*	21.9 ± 8.31##	20.7 ± 6.90##
γ-Tocopherol (nmol/g tissue)	1.27 ± 0.75	2.13 ± 1.25	0.95 ± 0.54#	1.23 ± 0.47
GSH (nmol/g tissue)	3902 ± 644	3999 ± 761	3569 ± 368	3804 ± 674
GSSG (nmol/g tissue)	209 ± 77.3	246 ± 62.5	208 ± 81.5	208 ± 60.6
% GSSG[2]	5.13 ± 1.96	6.02 ± 2.12	5.41 ± 1.65	5.33 ± 1.66
MDA (nmol/g tissue)	136 ± 29.9	174 ± 52.6	192 ± 54.2	265 ± 67.0***,##,†
SOD (U/g tissue)	1669 ± 231	1803 ± 599	2168 ± 419	2117 ± 624
L-arginine/ADMA[1]	621 (351–1101)	909 (513–1609)	364 (206–646)	433 (245–767)

ADMA, asymmetric dimethylarginine; ALP, alkaline phosphatase; DHA, dehydroascorbic acid; GSH, glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde; SOD, superoxide dismutase.

[1]Geometric means with 95% confidence intervals.

[2]Calculated as GSSG/(GSH + GSSG). n = 10.

*$p < 0.05$, **$p < 0.01$ ***$p < 0.001$ *versus* HF. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ *versus* HFL. †$p < 0.05$ ††$p < 0.01$ *versus* HFC. Means with S.D.

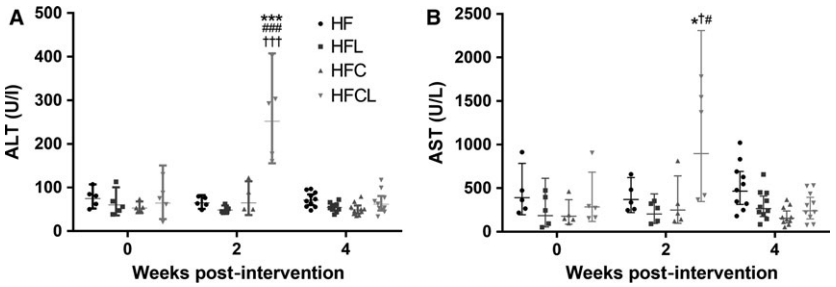

Fig. 3. Liver enzymes. (A) ALT and (B) AST were increased in HFCL compared to the other groups two weeks after initiating the intervention ($p < 0.001$ and $p < 0.05$, respectively), but remained similar between groups at all other time-points. Geometric means with 95% confidence intervals. n = 5–10. Previous reported values for chow-fed controls ALT: 37.0 (29.2–46.9) U/l, AST: 43.6 (30.7–61.8) U/l [17].

inhibitor N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) ($p < 0.001$ at all concentrations of carbachol) in both HF and HFL. The cyclooxygenase inhibitor, indomethacin, also reduced carbachol-induced vasodilation, although this was only significant for the HF group at carbachol concentrations of 10^{-7} M ($p = 0.0035$), 3×10^{-7} M ($p < 0.0001$) and 10^{-6} M ($p = 0.0338$; fig. 4).

Discussion

Treatment with the GLP-1 analogue liraglutide in guinea pigs reduced NASH progression by ameliorating hepatic inflammation and hepatocyte damage (ballooning), while dietary intervention decreased dyslipidaemia and tended to reduce hepatic steatosis and ballooning. These results imply the applicability of GLP-1 treatment in lean NASH. The differential effect on liver histology and circulating lipids of the two applied interventions suggests a potential benefit of combined treatment in lean NASH, albeit the limitations of the present study weakens conclusions regarding such effects.

The need to study the effectiveness of treatment modalities on lean NASH and its associated comorbidities is underlined by the relatively high prevalence and unique phenotype of this disease [5,6]. Previous studies from our laboratory have recorded that the high-fat feeding regime also applied in the current study does not induce excessive weight gain compared to chow, nor promotes excessive deposition of body fat compared to chow-fed controls, confirming the guinea pig as a non-obese (lean) animal model of NAFLD and NASH [16,17]. The present findings indicate that liraglutide exerts similar effects on NASH histopathology in lean guinea pigs as previously reported in clinical and pre-clinical settings utilizing obese individuals and animals [9–15,34,35]. Notably, the beneficial effects of treatment with liraglutide occurred in the presence of severe (grade 3) fibrosis. This is interesting as univariate analysis of individuals treated with liraglutide for 48 weeks, found that patients with severe (grade 3–4) fibrosis at baseline were less likely to respond to liraglutide [15]. In support of this, switching spontaneous hypertensive stroke prone rats from a high-fat diet to a low-fat diet only reversed hepatic steatosis and down-regulated inflammatory genes and liver enzymes if the intervention took place before the development of fibrosis [36]. The presence of advanced NASH in the guinea pigs therefore could render them less responsive to treatment. Nevertheless, liraglutide decreased hepatic inflammation and hepatocyte ballooning, but it is currently not clear if these anti-inflammatory effects result from direct actions on immune cells or are secondary to metabolic and weight loss effects [37]. However, the absence of hepatocyte GLP-1 receptors implies that histological improvements are mediated by indirect effects of liraglutide [38,39]. Studies in obese murine NASH models have suggested that liraglutide decreases endoplasmic reticulum stress and TNF-α levels in the liver, both of which are believed to play key roles in NASH [11,13]. It remains to be investigated if similar mechanisms underlie the effect of liraglutide in lean NASH. The histological improvement by liraglutide was associated with enhanced hepatic α-tocopherol (vitamin E) content. The positive effects of liraglutide may then be linked to vitamin E associated effects as randomized clinical trials have found vitamin E to improve NASH histology in both adults and children [40,41].

As other included markers of hepatic redox status were not affected by liraglutide in the current study, it may be suggested that the effect of liraglutide is not exclusively linked to reduction in hepatic oxidative stress [42]. Based on both clinical and pre-clinical studies [9–15,34,35], we expected liraglutide to decrease hepatic steatosis, but surprisingly steatosis grade and hepatic triglyceride content were similar between liraglutide-treated animals and high-fat controls. FFA are implicated in the pathogenesis of NASH and increased supply of fatty acids, due to enhanced lipolysis in the adipose tissue, may directly cause steatosis [4]. Liraglutide-treated animals exhibited an increase in circulating FFA, likely resulting from the weight loss. These FFA may then have been taken up by the liver preventing a potential antisteatotic effect of liraglutide. Prolonging the treatment duration to ensure weight stability after the initial weight loss may then be necessary to reduce hepatic steatosis. In mice with steatosis, liraglutide increased mRNA levels of microsomal triglyceride transfer protein (MTTP) and apoB, suggesting that enhanced hepatic lipid export is involved in decreasing liver lipid levels after liraglutide treatment [9]. In human beings and rats, advanced disease appears to compromise hepatic lipid out-flow, as the expression of MTTP was decreased in NASH [43–45]. The unchanged hepatic triglyceride levels in the current study could reflect the presence of advanced NASH at the time of

Table 3.

Plasma lipids prior to and two and four weeks after intervention

Group	Week	TG (mM)	VLDL-TG (mM)	TC (mM)	VLDL-C (mM)	LDL-C (mM)	HDL-C (mM)
HF	0	0.63 (0.44–0.90)	0.55 (0.33–0.90)	7.93 (4.33–14.5)	0.10 (0.06–0.19)	7.49 (4.00–14.0)	0.09 (0.03–0.30)
	2	0.62 (0.35–1.09)	0.55 (0.31–1.00)	8.52 (4.95–14.7)	0.14 (0.08–0.22)	8.02 (4.59–14.0)	0.16 (0.04–0.57)
	4	0.39 (0.28–0.53)	0.32 (0.23–0.46)	6.06 (3.95–9.30)	0.10 (0.06–0.16)	5.76 (3.71–8.94)	0.15 (0.11–0.22)
HFL	0	0.58 (0.36–0.94)	0.50 (0.29–0.87)	10.8 (4.79–24.1)	0.19 (0.08–0.44)	10.1 (4.44–23.0)	0.23 (0.06–0.80)
	2	0.79 (0.43–1.48)	0.69 (0.34–1.41)	15.3 (12.0–19.4)	0.61 (0.34–1.11)***	14.1 (11.2–17.8)	0.24 (0.10–0.55)
	4	0.42 (0.34–0.52)	0.33 (0.26–0.41)	11.3 (8.04–16.0)	0.27 (0.12–0.58)	10.8 (7.68–15.1)	0.18 (0.11–0.28)
HFC	0	0.32 (0.25–0.42)	0.24 (0.16–0.38)	7.68 (5.00–11.0)	0.09 (0.04–0.24)	7.23 (4.64–11.3)	0.21 (0.15–0.30)
	2	0.30 (0.22–0.42)	0.22 (0.15–0.33)	2.78 (2.11–3.66)***,###	0.06 (0.04–0.09)*,###	2.53 (1.91–3.37)***,###	0.09 (0.06–0.12)
	4	0.31 (0.25–0.39)	0.22 (0.18–0.27)	2.77 (1.80–4.26)*,##	0.04 (0.03–0.06)*,##	2.62 (1.68–4.09)*,##	0.07 (0.05–0.11)
HFCL	0	0.46 (0.29–0.73)	0.34 (0.20–0.59)	9.15 (5.47–15.3)	0.12 (0.05–0.29)	8.76 (5.28–14.5)	0.12 (0.02–0.58)
	2	0.40 (0.27–0.59)	0.24 (0.16–0.35)#	4.83 (2.68–8.68)###	0.06 (0.03–0.10)*,###	4.49 (2.40–8.38)###	0.08 (0.02–0.26)
	4	0.27 (0.12–0.63)	0.18 (0.07–0.46)	3.77 (2.40–5.92)	0.05 (0.03–0.08)#	3.61 (2.28–5.73)	0.08 (0.05–0.13)

TC, total cholesterol; TG, triglycerides.
Geometric means with 95% confidence intervals. n = 5 at weeks 0 and 2. n = 10 at week 4.
*$p < 0.05$, ***$p < 0.001$ *versus* HF. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ *versus* HFL.

intervention, as well as the relatively short intervention period in which weight stability was not obtained. In murine models where liraglutide decreased steatosis, the animals were obese and glucose intolerant/insulin-resistant [9–14,46,47]. The effect of liraglutide in these animals could, in part, be ascribed to a reduction in hyperglycaemia and subsequent prevention of glucose-induced *de novo* lipogenesis [16,17,19,48]. The absence of glucose intolerance and obesity in the lean guinea pig NASH model [16,17] is in accordance with the observation that insulin resistance is not universally present in lean NASH patients [8] and may help account for the unaffected hepatic steatosis after liraglutide treatment. The absence of glucose intolerance also allows the current study to dissociate a hepatic effect of liraglutide from an effect promoted by liraglutide's ability to improve glucose metabolism. In addition, steatosis rather than NASH *per se* may have been present in the previously described murine models as only a single study confirmed NASH histologically [13]. Furthermore, fibrosis was described as mild in another mouse study [47], rendering the reported effects targeting steatosis and possibly early stages of NASH in these animal models. Whether intervention at an earlier disease stage, and/or longer duration of treatment, will exert more profound effects on the steatosis grade in lean NASH, remains to be investigated.

Dyslipidaemia was improved by dietary intervention reducing circulating levels of TC, VLDL-C and LDL-C in HFC and HFCL groups compared to HF. However, lipid concentrations were still higher compared to previously reported levels from healthy control guinea pigs fed a chow diet for 25 weeks [17]. The effect was most pronounced in HFC, likely due to the rapid weight loss of HFCL expected to affect lipid metabolism and increase hepatic MDA [49]. Hepatic histopathology was considerably improved after dietary intervention but did not reach statistical significance. Reports from rats have shown that switching to a normal low-fat diet for 8 weeks after 8 weeks of high-fat diet ameliorated dyslipidaemia as well as hepatic steatosis and inflammation [50]. Thus, prolonged treatment duration may be necessary to reverse NASH via dietary intervention, especially considering the advanced disease stage in the current model, exhibiting hepatic fibrosis, which was not apparent in the rat model [50]. Furthermore, two animals of the HF group presented a very low steatosis grade. This was highly unexpected in comparison with our previous guinea pig studies [16,17], where a high degree of steatosis was consistently found in high-fat-fed groups. However, as HFC and HFCL animals displayed a significantly lower steatosis grade compared to HFL animals, the lack of significance when comparing to the HF group is most likely due to these isolated animals representing biological variation or a yet undetermined underlying cause. Indeed, HFC ($p < 0.05$) and HFCL ($p < 0.05$) had a lower degree of steatosis compared to HF when excluding these two animals from the statistical analysis. However, no experimental justification could support this exclusion; hence, the statistical analysis was based on the full sample size. Oxidative stress is a key finding in both patients and animals with NAFLD [3]. After dietary intervention, hepatic oxidative stress was reduced, evident by increased vitamin

Table 4.
Plasma lipids and markers of oxidative stress at euthanasia (week 4 post-intervention)

	HF	HFL	HFC	HFCL
Total vitamin C (μM)	44.3 ± 15.6	26.2 ± 10.8*	41.5 ± 9.23$^{\#}$	23.1 ± 12.6**,†
DHA (μM)	0.30 ± 1.52	0.28 ± 0.59	−0.49 ± 0.44	−0.08 ± 0.38
FFA (mM)	0.36 ± 0.09	0.53 ± 0.13*	0.37 ± 0.08$^{\#}$	0.52 ± 0.18*
8-Isoprostanes (ng/l)	55.2 ± 17.2	57.7 ± 22.3	54.3 ± 10.3	51.1 ± 15.8
L-arginine/ADMA[1]	169 (142–197)	204 (150–258)	166 (139–193)	207 (160–254)
BH_2/BH_4	0.15 ± 0.04	0.20 ± 0.06	0.14 ± 0.02	0.21 ± 0.06*,†

DHA, dehydroascorbic acid; FFA, free fatty acids.
[1]Geometric means with 95% confidence intervals. n = 10. ADMA: asymmetric dimethylarginine. BH_2: dihydrobiopterin. BH_4: tetrahydrobiopterin. *$p < 0.05$, **$p < 0.01$ *versus* HF. $^{\#}p < 0.05$ *versus* HFL. $^{\dagger}p < 0.05$ *versus* HFC. Means with S.D.

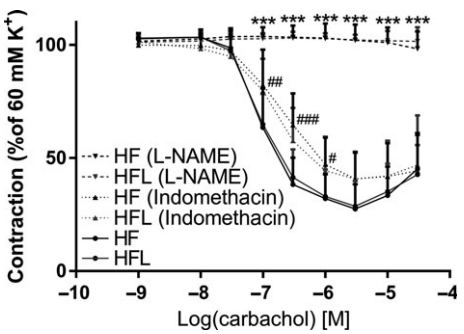

Fig. 4. Endothelial function by myography. Carbachol induced vasodilation in HF- and HFL-treated animals, which could be inhibited by L-NAME. Indomethacin tended to reduce carbachol-induced vasodilation, but this was only significant for HF at carbachol concentrations of 10^{-7}, 3×10^{-7} and 10^{-6} M. ***$p < 0.001$ HF(L-NAME) and HFL(L-NAME) *versus* HF/HFL or HF/HFL(Indomethacin). $^{\#}p < 0.05$ $^{\#\#}p < 0.01$ $^{\#\#\#}p < 0.001$ HF(Indomethacin) *versus* HF. Means with S.D. n = 8 (HF, HF L-NAME,) n = 7 (HFL, HF Indomethacin and HFL Indomethacin) n = 6 (HFL L-NAME) (variable group sizes due to exclusion of segments with a contraction <1 mN/mm). L-NAME: N^{ω}-nitro-L-arginine methyl ester hydrochloride.

C levels. We have previously reported that high-fat diets decrease hepatic vitamin C content in guinea pigs [16,51] suggesting that inadequate vitamin C status may contribute to the development of NASH [52]. As absolute food intake was not increased in HFC compared to HF (data not shown), improvements in hepatic vitamin C status are likely to reflect an augmentation of the antioxidant system and not just increased dietary intake. Liver vitamin C levels were not different in HFC and HFL/HFCL when adjusting for food intake, suggesting that lower vitamin C levels in these groups were caused by lower food intake. Conversely, plasma vitamin C adjusted for food intake did not differ between groups (data not shown), again implying decreased food intake as the cause for lower vitamin C and not liraglutide *per se*.

In a large Asian cohort study, non-obese individuals with NAFLD were found to be at significantly increased risk of cardiovascular disease especially if the NAFLD had progressed to NASH [53]. Consequently, we investigated the endothelial function in coronary arteries from the HF and HFL group, but did not find an effect on liraglutide on vasodilation. This is in agreement with circulating markers of oxidative stress and endothelial function, as levels of 8-isoprostanes and BH_2/BH_4 and L-arginine/ADMA ratios were not different between groups. We have previously reported the absence of atherosclerotic lesions in the aorta of guinea pigs fed a high-fat diet for 16 weeks. It is therefore possible that the current experimental set-up does not induce vascular dysfunction [16]. Alternatively, the speculated effect of liraglutide may not be associated with carbachol-mediated vasodilation in guinea pigs within the duration of the intervention period.

Limitations of the present study include the adjustments made in liraglutide dose in the HFCL group, necessary to comply with the humane end-point of 20% weight loss. The decision not to include a chow-fed control could be argued to be a limitation of the experimental design. However, we have previously and repeatedly reported that high-fat diets comparable to the one applied in the current study, in a set-up closely resembling the current study, do not enhance weight gain or body fat deposition in guinea pigs when compared to a chow-fed control, confirming the lean phenotype of model [16,17]. It should be noted that high-fat-fed guinea pigs do not exhibit any form of growth retardation, nor display any clinical signs of malnutrition or disease. In addition, the 4-week duration of the intervention period may have been too short to detect changes in steatosis and fibrosis in the current model. We have previously demonstrated that long-term (25 weeks) high-fat feeding in guinea pigs induces NASH with concomitant fibrosis [17]. As similar hepatic changes were confirmed in the HF group of the current study, this clearly suggests that the observed histological improvement in HFL animals was due to liraglutide. However, as pre-intervention biopsies were not obtained, it cannot be ruled out that liraglutide delays the development of, rather than reversing NASH.

In conclusion, this pre-clinical study in non-obese guinea pigs with advanced NASH found dietary intervention to improve dyslipidaemia and reduce liver cholesterol and weight, although potential effects related to NASH histology may require a longer intervention period. Liraglutide improved key histological features of NASH and hepatic markers of oxidative stress, even in the setting of severe fibrosis and a persistent intake of high-fat and cholesterol. These results suggest GLP-1 analogues as a potential treatment option in non-

obese NASH should the observed effects translate to human beings.

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Conflict of Interest

This study was supported in part by the LifePharm Centre for In Vivo Pharmacology at University of Copenhagen and Novo Nordisk A/S. BR and GR are employed by Novo Nordisk A/S that produces liraglutide. The remaining authors declare no competing interests that could influence this work.

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Supporting Information

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

Table S1. Exact dietary composition.

Table S2. Liver weight and markers of plasma oxidative stress.