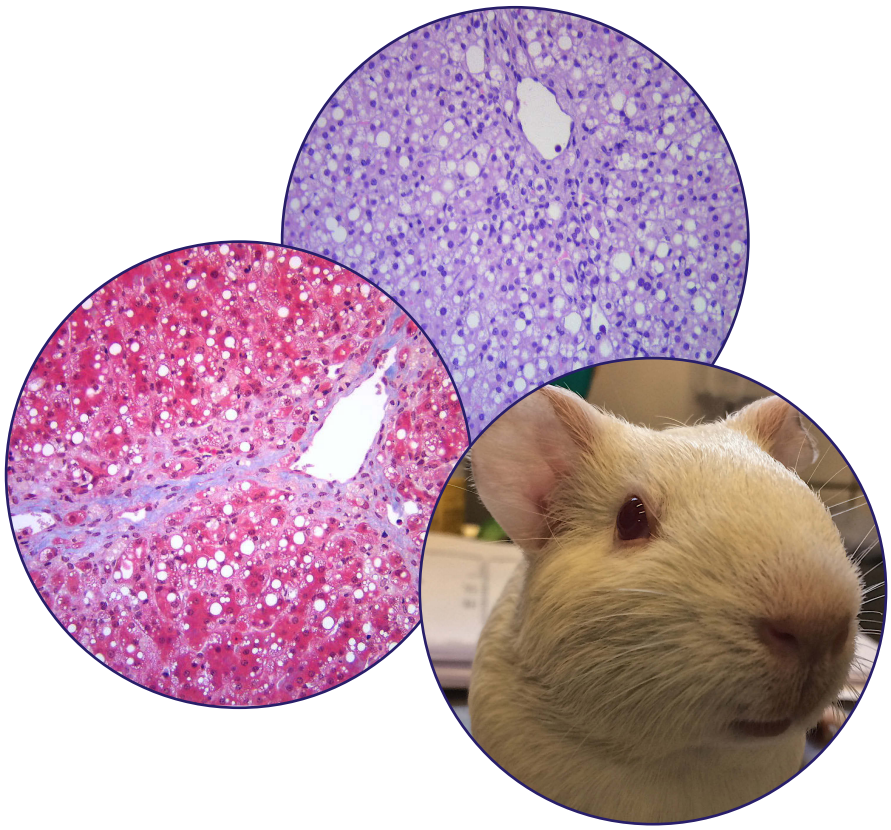
UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

The guinea pig as a model for dyslipidemia and non-alcoholic fatty liver disease

PhD THESIS 2017 · David Højland Ipsen



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DAVID HØJLAND IPSEN

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PhD thesis 2017 • David Højland Ipsen

IN VIVO PHARMACOLOGY

GRADUATE PROGRAMME FOR IN VIVO PHARMACOLOGY & EXPERIMENTAL ANIMALS

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Preface

This PhD was conducted from January 1st 2014 to December 31st 2016 and was funded by the Lifepharm Centre for In Vivo Pharmacology and has been carried out under the supervision of Jens Lykkesfeldt (principal supervisor), Pernille Tveden-Nyborg, Bidda Rolin and Günaj Rakipovski (co-supervisors). The work was conducted at the University of Copenhagen and at Novo Nordisk and included a three month stay at the Department of Public Health, University of Copenhagen.

All animal experiments were approved by the Danish Animal Experiments Inspectorate under the Ministry of Food, Agriculture and Fisheries and in accordance with European Union directive 2010/63/EU.

Papers

The following papers are included in this thesis and are referred to by their Roman numerals:

Paper I Diet-induced dyslipidemia leads to nonalcoholic fatty liver disease and oxidative stress in guinea pigs. Tveden-Nyborg P, Birck MM, Ipsen DH, Thiessen T, Feldmann L de B, Lindblad MM, Jensen HE, Lykkesfeldt J. Transl Res. 2016 Feb;168:146-60

Paper II High-fat but not sucrose intake is essential for induction of dyslipidemia and non-alcoholic steatohepatitis in guinea pigs. Ipsen DH, Tveden-Nyborg P, Rolin B, Rakipovski G, Beck M, Mortensen LW, Færk L, Heegaard PMH, Møller P and Lykkesfeldt J. Nutrition & Metabolism. 2016 Aug;13(1):1-10.

Paper III Liraglutide decreases hepatic inflammation and injury in advanced nonalcoholic steatohepatitis. Ipsen DH, Rolin B, Rakipovski G, Skovsted GF, Madsen A, Kolstrup S, Schou-Pedersen AM, Lykkesfeldt J and Tveden-Nyborg P (*Ready for submission to EBioMedicine*)

Paper IV Normal weight dyslipidemia: Is it all about the liver? Ipsen DH, Tveden-Nyborg P, Lykkesfeldt J. Obesity (Silver Spring). 2016 Mar;24(3):556-67.

Paper V Dyslipidemia: Obese or not obese, that is not the question. Ipsen DH, Tveden-Nyborg P and Lykkesfeldt J. Curr Obes Rep. 2016 Dec;5(4):405-412.

Paper VI Does Vitamin C Deficiency Promote Fatty Liver Disease Development? Ipsen DH, Tveden-Nyborg P, Lykkesfeldt J. Nutrients. 2014 Dec;6(12):5473-99.

Abbreviations

ACAT: acyl-coenzyme A cholesterol acyltransferase.

ADMA: asymmetric dimethylarginine.

ALT: alanine aminotransferase

AST: aspartate aminotransferase

BW: body weight.

CETP: cholesteryl ester transfer protein.

FFA: free fatty acids.

GLP-1: glucagon-like peptide 1.

GLUT2: glucose transporter 2.

HDL: high density lipoprotein.

IL1β: interleukin 1β.

Ip: intraperitoneal

LCAT: lecithin-cholesterol acyl-transferase.

LDL: low density lipoprotein.

MDA: malondialdehyde

NAFLD: non-alcoholic fatty liver disease.

NASH: non-alcoholic steatohepatitis.

OGTT: oral glucose tolerance test.

PUFA: polyunsaturated fatty acids.

SFA: saturated fatty acids

TNFα: tumor necrosis factor α.

VLDL: very low density lipoprotein.

Summary

An unhealthy diet and the consistent intake of high amounts of fat, cholesterol and non-complex carbohydrates is recognized as a key facilitator of many of today's most common life-style associated diseases, e.g. dyslipidemia, non-alcoholic fatty liver disease (NAFLD), cardiovascular disease and diabetes. However, the complex interaction between different dietary components and disease development is not fully elucidated. The current work aimed to establish a guinea pig model and use it to investigate the relationship between specific macronutrients and the development of dyslipidemia, NAFLD, cardiovascular disease and diabetes and to assess the effect different treatments options on these diseases. Study I characterized the model and found that guinea pigs develop human-like dyslipidemia, NAFLD and oxidative stress when fed a high fat diet. Progression of NAFLD towards non-alcoholic steatohepatitis (NASH) and fibrosis is associated with increased mortality in patients, but the relative importance of specific dietary factors is still not clear. Study II, therefore, investigated the effect of sucrose and fat/cholesterol on the development of dyslipidemia and NAFLD and the progression towards NASH. NAFLD progressed to NASH with severe fibrosis, but sucrose did not promote disease development nor did it exacerbate the effect of the high fat/cholesterol diets, suggesting fat and cholesterol as the primary factors driving dyslipidemia and NASH. The first two studies did not find evidence of cardiovascular disease, adipose tissue dysfunction or compromised glucose tolerance and as streptozotocin-induced diabetes proved unfeasible, further investigations focused on NASH and dyslipidemia. Although it is the most common chronic liver disease in the world, there is currently no approved for treatment for NAFLD/NASH. High intakes of saturated fat and cholesterol have been reported for NAFLD patients and the high fat diets consistently induced dyslipidemia and NASH in the guinea pig model. Thus, dietary intervention could be a treatment option. Not all patients are able to adhere to life-style changes and may benefit from pharmacological treatment. The literature suggested that the glucagon-like peptide 1 analogue liraglutide ameliorated NASH and the effect of liraglutide on NASH was, therefore, examined further. Liraglutide reduced hepatic inflammation and hepatocyte ballooning – both key histological features of NASH – while dietary intervention improved dyslipidemia and, alongside liraglutide, mediated a minor improvement in liver redox-balance. Combination therapy resulted in rapid weight-loss necessitating periodically interruption of the liraglutide treatment, weakening results from this group.

Collectively the results of this thesis suggest that the guinea pig may be used as a model for dyslipidemia and advanced NASH with fat and cholesterol, but not sucrose, fueling disease development. Liraglutide

seems promising for the treatment of NASH, but dietary intervention may benefit from longer treatment periods.

Summery in Danish – Resumé på dansk

En usund diæt med høje niveauer af fedt, kolesterol og ikke-komplekse kulhydrater menes at være en af de grundlæggende årsager til mange af hvor dags mest almindelige livsstilsygedomme, så som dyslipidæmi, ikke-alkoholisk fedt lever (NAFLD), karsygdomme og diabetes. Det komplekse sammenspil mellem forskellige næringsstoffer og sygdomsudvikling er dog endnu ikke fuldt belyst. Formålet med dette arbejde var at etablere en marsvinemodel og bruge denne til at anskueliggøre forholdet mellem specifikke næringsstoffer og udviklingen af dyslipidæmi, NAFLD, karsygdomme og diabetes, samt at undersøge effekten af forskellige behandlinger på disse sygdomme. Studie I karakteriserede modellen og viste at marsvinet, som følge af fodring med en høj fedt diæt, udvikler "menneskelig" dyslipidæmi, NAFLD og oxidativt stress. NAFLD kan udvikle sig til ikke-alkoholisk leverinflammation (NASH) med fibrose hvilket er forbundet med øget mortalitet, men indflydelsen af specifikke diætkomponenter er stadig uklar. Derfor undersøgte Studie II hvorledes sukrose og fedt/kolesterol påvirkede udviklingen af dyslipidæmi og NAFLD og progressionen mod NASH. NAFLD progrederede til NASH med samtidig udvikling af betragtelig fibrose, men sukrose påvirkede ikke denne udvikling eller forværrede effekten af høj fedt/kolesterol diæterne, hvilket indikerer at fedt og kolesterol er de primære drivkræfter bag dyslipidæmi og NASH. Der blev i de to første studier ikke fundet tegn på karsygdomme, dysfunktionelt fedtvæv eller kompromitteret glukose tolerance, og da streptozotocin-induceret diabetes viste sig ikke at være muligt, fokuserede de efterfølgende studier på dyslipidæmi og NASH. Til trods for at være den mest udbredte kroniske leversygdom i verden, findes der ingen godkendt behandling af NAFLD/NASH. NAFLD patienter er blevet beskrevet som havende et højt indtag af fedt og kolesterol og høj fedt diæterne inducerede konsekvent dyslipidæmi og NASH i marsvinemodellen. Det var derfor tænkeligt, at intervention med diæt kunne bruges som behandling. Ikke alle patienter kan vedholde livsstilsændringer og disse vil muligvis have gavn af en farmakologisk behandlingsmulighed. Litteraturen viste at liraglutide, som er en såkaldt *glucagon-like peptide 1* analog, kunne have gavnlig virkning på NASH og denne effekt blev efterfølgende undersøgt nærmere. Liraglutide reducerede graden af inflammation og *ballooning* hepatocytter i leveren – hvilke begge er vigtige histologiske kendetegn for NASH – mens diæt-interventionen sænkede graden af dyslipidæmi og ligesom liraglutide forbedrede redox-balancen en smule. Kombinationsbehandling ledte til et hurtigt vægttab som nødvendiggjorde at behandlingen med liraglutide i perioder måtte ophøre, hvilket svækker resultaterne fra denne gruppe. På baggrund af resultaterne fra denne afhandling kan det sammenfattende konkluderes at marsvinet kan bruges som model for dyslipidæmi og fremskreden NASH, hvor fedt og kolesterol, men ikke sukrose, driver

sygdomsudviklingen. Liraglutide virker lovende som en potentiel behandlingsmulighed for NASH, hvorimens intervention med diæt muligvis kan have gavn af øget behandlingsvarighed.

1. Introduction

Although essential for life, diet is one of the most important risk factor for death and disease (1). During the past years, diets have changed worldwide and unhealthy dietary patterns are becoming more prominent than the healthy dietary patterns (2). An unhealthy 'Western' diet, rich in fat, cholesterol and non-complex carbohydrates, promotes the development of dyslipidemia, leading to ectopic lipid deposition and lipotoxicity in various organs and tissues (V). As a result, dyslipidemia serves as a key mediator of metabolic diseases like non-alcoholic fatty liver disease (NAFLD), cardiovascular disease, adipose tissue dysfunction, insulin resistance and diabetes (3, 4)(IV, V). The prevalence of NAFLD has been increasing worldwide in parallel with dyslipidemia, obesity and type 2 diabetes (5). Affecting up to 25% of the adult population, NAFLD is now the most common liver disease in the world (6). The term covers a range of hepatic disorders, ranging from steatosis to non-alcoholic steatohepatitis (NASH) and advancement to cirrhosis. Although the disease is initially reversible, disease progression imposes irreversible damage to the liver and NASH is now the third most common cause of liver transplantations in the United States (7). Importantly, NAFLD is associated with changes in the hepatic and systemic lipid metabolism, which further propagates the dyslipidemic state, causing a self-perpetuating, vicious cycle (IV). The Western diet is believed to be important with regard to disease development, however, the complex relationship between dietary components and the development of dyslipidemia and NAFLD is not yet completely understood (1, 4, 8)(IV). Furthermore, there are currently no approved pharmacological treatment or established treatment guidelines for NAFLD (9). As much of the research conducted within the field of nutrition and metabolic disease rely on experimental animal models, there is a need for well-validated animal models: These models should reflect the human pathophysiology, enabling a better understanding of the relationship between dietary components and the development of disease and the ability to treat these diseases.

2. Objectives and hypotheses

This PhD investigated the impact of the specific components of a Western diet on the development of four major disease complexes: dyslipidemia, NAFLD, cardiovascular disease and insulin resistance/diabetes and assessed the effect of potential treatment options on these disease complexes.

The guinea pig was chosen as a model of dyslipidemia and NAFLD based on the results of Study I. Study II then examined the impact of specific dietary components on the development and progression of disease over time. Having identified the time-frame for the progression of NAFLD towards NASH, it was possible to design an intervention study and assess the effect of different treatment options on NASH in Study III.

The hypotheses for this PhD thesis are given below:

1. A high fat diet induces dyslipidemia and NAFLD, which progress to NASH with fibrosis.
2. Increasing amounts of dietary sucrose exacerbates the metabolic and hepatic consequences of a high fat diet.
3. Liraglutide and dietary intervention (removal of excess dietary fat, cholesterol and sucrose) ameliorates NASH in guinea pigs fed a high fat diet, and combined the treatments exert an additive or synergistic effect on NASH.

3. Background

3.1 Dyslipidemia and life-style diseases

Diet-related health problems, like NAFLD, cardiovascular disease, and diabetes represent some of the most important threats to public health (8). Dyslipidemia - characterized by increased triglycerides, cholesterol, very low density lipoprotein cholesterol (VLDL-C), low density lipoprotein cholesterol (LDL-C) and/or decreased high density lipoprotein cholesterol (HDL-C) – is seemingly a common denominator for a series of diseases and is often a direct result of a poor diet containing disproportionately high levels of fat, cholesterol and sugar (4, 10). Impaired lipid metabolism, especially in the liver, has been suggested as a central contributor to the development of dyslipidemia and its associated co-morbidities like cardiovascular disease. However, the pathophysiological mechanisms underlying the development of diet-induced dyslipidemia are currently not fully elucidated. Three reviews were conducted during this PhD in order to assess and better understand these complex mechanisms and identify relevant areas of interest, which would warrant further investigation.

Investigating the role of the liver and its relation to dyslipidemia suggested that excessive intake of saturated fat (SFA) and cholesterol may promote dyslipidemia and NAFLD, regardless of obesity status (IV). In addition to this, adipose tissue dysfunction may contribute to disease progression in both lean and obese individuals (V). Lastly, the effect of the antioxidant vitamin C on the development of NAFLD was examined (VI). Humans and guinea pigs are unable to synthesize this essential vitamin and a diet low in vitamin C may accelerate the development of NAFLD (VI). However, time limitations prohibited further investigations into the relationship between vitamin C deficiency and NAFLD during this PhD.

3.2 The guinea pig as a model for human dyslipidemia and NAFLD

As mentioned above, dyslipidemia seems to be a cornerstone in the development of multiple diseases. In order to ensure translatability, suitable animal models should be applied to investigate and understand the effect of different diets and interventions on the development of these diseases. The lipoprotein profile and enzymes involved in the metabolism of lipids are, therefore, likely to be important. In humans, the major lipoprotein is LDL, but the majority of animal models display lipoprotein profiles dominated by HDL and it remains questionable whether they reflect human dyslipidemia (11). Contrary to this, the guinea pig mimics the human lipoprotein profile, having LDL as the predominant lipoprotein (12) (Figure 1).

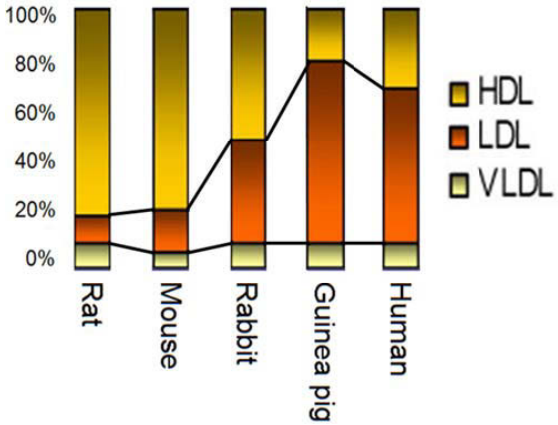

Figure 1 The lipoprotein profile of different species

The rat, mouse and rabbit display a HDL dominant lipoprotein profile that is significantly different from humans and guinea pigs, which are characterized by utilizing LDL as their major lipoprotein.

*HDL: high density lipoprotein. LDL: low density lipoprotein. VLDL: very low density lipoprotein. Modified with permission from (12). The original article is an open access article distributed under the terms of the Creative Commons Attribution License (*http://creativecommons.org/licenses/by/2.0*), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.*

Both humans and guinea pigs require dietary vitamin C and, in both species, females have higher concentrations of HDL-C than males (13, 14). Guinea pigs display cholesteryl ester transfer protein (CETP) (in contrast to other rodents), lecithin-cholesterol acyl-transferase (LCAT) and acyl-coenzyme A cholesterol acyltransferase (ACAT) activity and these enzymes are central to the lipid metabolism (12). In the liver, moderate rates of cholesterol synthesis and catabolism corresponds to humans (12). Furthermore, guinea pigs have previously been found to develop dyslipidemia and NAFLD when fed a high fat diet and both guinea pigs and humans respond similarly to cholesterol lowering drugs, including statins (15-19). Collectively, this suggests the guinea pig as a suitable model for the study of diet-induced dyslipidemia and related co-morbidities. To gain a better understanding of the physiological changes in guinea pigs fed a Western diet, Study I validated and characterized the guinea pig model with regards to dyslipidemia, NAFLD, atherosclerosis and diabetes (I).

3.3 Dyslipidemia, NAFLD and the influence of diet

Although common in obese individuals, NAFLD may also be present in lean individuals (IV, V). Excessive caloric intake is associated with obesity, but does not always distinguish between individuals with normal liver histology, fatty liver or NASH (20). This suggests that factors besides caloric intake and

obesity are also important for the development of NAFLD and, in this regard, dietary composition has been implicated as an important factor (21). NAFLD patients have been reported to ingest an unhealthy diet, containing high amounts of SFA, cholesterol and sugar, which may actively contribute to disease development (22)(IV). However, it remains unclear if certain types of macronutrients are more likely to cause dyslipidemia and NAFLD than others (23). Hepatic lipids may originate from intestinal uptake or from circulating free fatty acids (FFA) that are transported to the liver and stored as triglycerides (24).To mitigate the increasing fat stores, the liver increases the export of VLDL-triglycerides, thus promoting the development of dyslipidemia (25). In the liver, the fatty acids can activate sterol regulatory binding protein 1c, a lipogenic gene, which promotes *de novo* lipogenesis and further increases hepatic fat content (Figure 2) (26). In healthy subjects, *de novo* lipogenesis accounts for approximately 5% of the hepatic lipids, whereas it may contribute with up to 25% in NAFLD patients (27). Comparing isocaloric high fat/low carbohydrate or low fat/high carbohydrate diets, high amounts of dietary fat has been found to increase hepatic lipid accumulation in humans, even in the absence of weight-gain (28, 29). Diets with high amounts of SFA and low polyunsaturated fatty acids (PUFA) seem to increase liver fat content compared to diets with low amounts of SFA and high PUFA in subjects with abdominal obesity, indicating the importance of dietary fat quality (30). SFA and cholesterol may also reduce hepatic LDL-uptake by reducing the expression and activity of the LDL-receptor, resulting in increased circulating levels of LDL (4, 31). Additionally, cholesterol may induce steatosis by enhancing *de novo* lipogenesis and facilitate progression towards NASH through elevated oxidative stress and cellular apoptosis (Figure 2) (32, 33). It should, however, be noted that hypercaloric diets seem to increase liver fat while hypocaloric diets decrease liver fat, regardless of dietary fat content and type (34). Consumption of simple and total carbohydrates has been found to be higher in patients with NASH compared patients with “simple” hepatic steatosis (35). Both human and animal studies have implicated non-complex carbohydrates, such as sucrose and fructose, in the development of both dyslipidemia and NASH: Non-complex carbohydrates have been found to increase hepatic *de novo* lipogenesis, leading to steatosis and increased export of VLDL-triglycerides, thereby, causing dyslipidemia (Figure 2) (36). However, the evidence in a recent meta-analysis was not sufficient to draw any conclusion regarding the effect of sucrose on NAFLD development, weakening the putative link between sucrose and NAFLD (37). In addition to this, most studies utilizes very high (and likely physiologically irrelevant) concentrations of sucrose and/or hypercaloric diets making it difficult to dissociate the effect of sucrose from excessive energy ingestion (36). To better understand the relative importance of specific macronutrients, Study II investigated the effect of dietary sucrose and fat/cholesterol on the development dyslipidemia and NAFLD (II). The length of the study was also prolonged compared to Study I, in order to assess the progression of NAFLD towards the more advance stages of NASH with fibrosis (II).

3.3.1 Oxidative stress as an underlying mechanism of NAFLD

Oxidative stress is caused by an imbalance in the generation and removal of oxidants and is thought to be a key mechanism underlying NAFLD and the progression towards NASH (33, 38, 39). Markers of oxidative stress are often increased in patients with NAFLD and oxidative stress has been reported to be independently associated with NAFLD (40-42). Mitochondrial dysfunction generates reactive oxygen species and compromises β-oxidation of fatty acids, thereby, enhancing hepatic lipid accumulation (43). The degree of lipid peroxidation has been found to correlate with the availability of fatty acids (38) and in a lipid-rich environment such as NAFLD, lipid peroxidation may create reactive aldehyde products such as malondialdehyde (MDA), which can compromise mitochondrial function even further (39, 43). Following diffusion into the extracellular space, MDA might damage adjacent cells, thereby, amplifying the effect of the intracellular reactive oxygen species (43). Cellular damage can then lead to increased infiltration of inflammatory cells, which is a key feature of NASH (38). Inflammation further fuels disease progression by enhancing hepatic steatosis and insulin resistance (33, 44). Ultimately, the augmented production of inflammatory cytokines and reactive oxygen species may activate the hepatic stellate cells, resulting in the deposition of collagen and the development of fibrosis (45) (Figure 2).

The body contains multiple anti-oxidant systems, designed to maintain redox-balance: Glutathione, glutathione peroxidase and superoxide dismutase are among the primary defense mechanisms against oxidative stress, but this defense system seems impaired in patients with NAFLD/NASH (46, 47). Vitamins like vitamin C and E can function as free-radical scavengers and decrease oxidative stress (38). Vitamin E denotes a group of tocopherols and tocotrienols, of which α-tocopherol has the highest biological activity (48). α-Tocopherol protects against lipid peroxidation and can be regenerated by vitamin C (49). In addition to this, vitamin C has been suggested to protect against NAFLD by decreasing the amount reactive oxygen species and inflammation and by increasing the activity of superoxide dismutase and glutathion peroixidase (VI).

Based on the above, oxidative stress seems to contribute significantly to the development of NAFLD and the progression towards NASH. Therefore, Study I examined the extent of oxidative stress in guinea pigs with dyslipidemia and NAFLD by measuring the levels of some of the key anti-oxidant enzymes and vitamins alongside the lipid peroxidation end-product MDA.

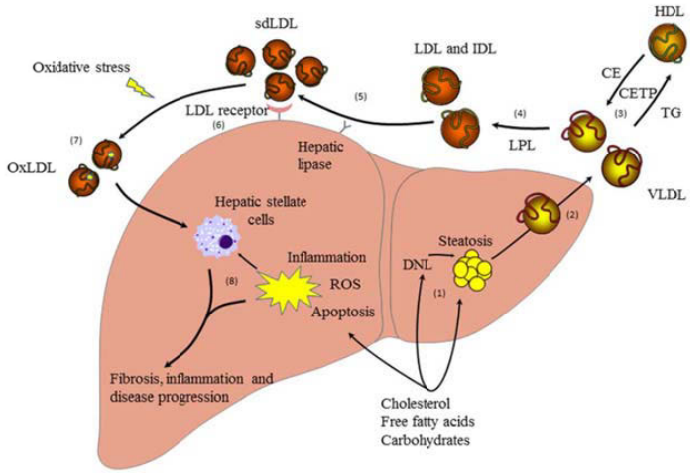

Figure 2 Pathophysiological relationship between NAFLD and dyslipidemia

(1) Dietary carbohydrates, free fatty acids, and cholesterol may all contribute to the development of NAFLD by inducing steatosis. Despite the hepatic fat accumulation, carbohydrates and cholesterol activate ChREBP and SREBP1c, respectively, enhancing de novo lipogenesis. (2) The liver not only stores excess lipids as triglycerides but also increases export of cholesterol and triglycerides through elevated synthesis and secretion of VLDL particles. (3) However, enhanced CETP activity in the face of NAFLD increases the exchange of cholesteryl esters and triglycerides between HDL and VLDL. Thus, VLDL-cholesterol concentrations are raised, whereas HDL concentrations are decreased, promoting dyslipidemia and an atherogenic lipoprotein profile. (4) In the periphery, lipoprotein lipase converts circulating VLDL into LDL. (5) LDL may then be converted to small, dense LDL particles by hepatic lipase, which exhibits enhanced expression in NAFLD. (6) Normally, LDL is cleared from the circulation through the LDL receptor. However, NAFLD is also characterized by decreased expression of the LDL receptor, ultimately increasing circulating levels of LDL. (7) Oxidative stress facilitates generation of oxLDL that may be taken up by the liver through CD36 and subsequently activate hepatic stellate cells. (8) Furthermore, cholesterol and free fatty acids induce macrophage and hepatocyte apoptosis alongside inflammation and ROS, all leading to activation of hepatic stellate cells. Subsequently, the activated hepatic stellate cells deposit collagen and α-smooth muscle actin, resulting in hepatic fibrosis and disease progression. CE: cholesteryl ester. CETP: cholesteryl ester transfer protein. CD36: cluster of differentiation 36. ChREBP: carbohydrate regulatory element binding protein. DNL: de novo lipogenesis. HDL: high density lipoprotein. IDL: intermediate density lipoprotein. LDL: low density lipoprotein. LPL: lipoprotein lipase. NAFLD: nonalcoholic fatty liver disease. OxLDL: oxidized low density lipoprotein. ROS: reactive oxygen species. sdLDL: small, dense low density lipoprotein. SREBP1c: sterol regulatory element binding protein 1c. TG: triglyceride. VLDL: very low density lipoprotein. Figure adapted from (IV).

3.3.2 Dyslipidemia, NAFLD and adipose tissue dysfunction

As discussed in (V), adipose tissue dysfunction could play an important role in the progression of dyslipidemia and NAFLD. Adipose tissue dysfunction is associated with excessive intake of dietary fat and cholesterol, causing adipocyte hypertrophy and inflammation, regardless of obesity status (V). Adipocyte hypertrophy facilitates macrophage infiltration and increases the release of pro-inflammatory

cytokines. Adipocyte death occurs alongside adipose tissue dysfunction and further promotes infiltrations of inflammatory cells (50). As the dying/dead adipocytes are surrounded by macrophages, they form so-called crown-like structures, a hallmark in dysfunctional adipose tissue (50). Macrophages also promote adipocyte hypertrophy, leading to insulin resistance and increased release of FFA, which may be pro-inflammatory in the adipose tissue (51-53). Thus, a vicious cycle is established, augmenting adipose tissue dysfunction. The release of FFA also impacts other tissues: They may be taken up by the liver where they are deposited and stored as triglycerides or exerts lipotoxic effects, resulting in NAFLD and dyslipidemia by increasing the secretion of VLDL-triglycerides (V). High levels of circulating lipids may then again promote adipose tissue dysfunction by supplying additional cholesterol and triglycerides to the adipocytes (54). However, while inflamed dysfunctional adipose tissue is often found to develop alongside dyslipidemia and NAFLD, it remains unclear which tissues are the first to be affected and initiate the detrimental crosstalk between tissues (54). To address this, Study II examined the extend of adipose tissue dysfunction in the visceral adipose tissue of guinea pigs with NASH by measuring levels of inflammatory cytokines and the degree of adipocyte hypertrophy (II).

3.3.3 Treatment options for NAFLD

Diet is a cornerstone in the management of life-style diseases such as obesity, but its effect on NAFLD is not yet fully elucidated (55). Improvement of NASH has been reported by both randomized clinical trials and prospective studies utilizing lifestyle intervention (diet and exercise) aimed at weight-loss (56, 57). The studies found that a higher degree of weight-loss was associated with a greater reduction of almost all histological features of NASH and that weight-loss above 7% was necessary to induce improvements in liver histology (56, 57). Unfortunately, a considerable number of patients are not able to adhere to long-term life-style changes and would instead be likely to benefit from a pharmacological treatment option (58). A recent investigator initiated randomized controlled trial has reported promising results regarding the therapeutic effects of the glucagon-like peptide 1 (GLP-1) analogue liraglutide (Victoza®, Novo Nordisk A/S), which is normally used to treat patients with type 2 diabetes (59). Sharing 97% amino acid sequence homology with human GLP-1, liraglutide is a full agonist of the GLP-1 receptor. A C16 fatty acid attached to lysine in position 26 facilitates binding to circulating albumin, prolonging the half-life and enabling once daily administration, leading to improved glycemic control, weight-loss and preservation of β-cells (60). Liraglutide ameliorated NASH by decreasing hepatic steatosis and hepatocyte ballooning, while also preventing the progression of fibrosis (59). These results are supported by pre-clinical studies, suggesting a beneficial effect of liraglutide on NAFLD (Table 1).

However, histological evaluation of the liver (besides steatosis) was usually not included in the pre-clinical trials, thereby, hindering assessment of the potential effects on NASH and fibrosis (61-68).

The mechanisms underlying the beneficial effect of liraglutide in NAFLD are not yet fully elucidated. GLP-1 has been suggested to reduce hepatic *de novo* lipogenesis, increase hepatic oxidation of fatty acids, reduce levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and improve adipose tissue dysfunction, thereby, decreasing systemic inflammation (69, 70). Infusion of GLP-1 through the jugular vein in rats limits intestinal absorption of triglycerides, without affecting cholesterol absorption (71). Reduced lipid absorption could potentially be of importance with regards to preventing the development of NAFLD and dyslipidemia. Interestingly, the hepatic effects of GLP-1 treatment have been found to, at least partially, be weight-independent (72). GLP-1 reduced body weight, hepatic lipid content and liver enzymes, whereas weight-loss induced by caloric restriction did not significantly reduce hepatic lipids (72). Although GLP-1 exerts its effect on a plethora of tissues, which indirectly affects the development of NAFLD, it remains controversial whether a direct hepatic effect – mediated through the GLP-1 receptor – exists. While the GLP-1 receptor has been reported in primary human and rodent hepatocytes and is apparently down-regulated in patients with NASH (73-75), recent studies have questioned these results and have not found GLP-1 receptor expression in the liver of primates or $ApoE^{-/-}$ mice (76, 77). Importantly, when employing extensive controls, three commonly used commercial antibodies were found to produce nonspecific bonds, which were similar in size to that of the GLP-1 receptor and this could have confounded the results of studies not employing proper controls (77).

Based on the current literature, intervention with both liraglutide and diet seems to hold promise as treatments for NAFLD. This also creates the interesting prospect of utilizing life-style intervention in combination with pharmacotherapy in patients with NAFLD, which could combine the benefits from both treatment options, potentially resulting in an additive or synergistic effect. Study III, therefore, investigated the impact of dietary intervention and liraglutide, alone or in combination, on NASH. Given the involvement of oxidative stress in NASH (section 3.3.1), Study III also investigated if the effects of dietary intervention and liraglutide were mediated by improvements in redox-balance (III).

Table 1 Pre-clinical studies investigating the effect of liraglutide on NAFLD and dyslipidemia.

Species	Liraglutide	Duration	Outcome	Ref
Sprague Dawley rats	13.5-54 nmol/kg 2x daily	12 wk. HFD → 4 wk. treat.	NASH↓ Liver enzymes ↓ Dyslipidemia ↓	(61)
CETP transgenic mice	54 nmol/kg 1x daily	20 wk. HFD → 4 wk. treat.	Hepatic triglycerides ↓ Hepatic cholesterol ↔ Dyslipidemia ↔	(62)
Wistar rats	6.75-27 nmol/kg 1x daily	3 wks. HFD → 1 wk. treat.	Hepatic steatosis ↓	(63)
C57BL/J6 mice	54 nmol/kg 1x daily	8 wk. HFD → 4 wk. treat.	Hepatic steatosis ↓ Dyslipidemia ↓	(64)
C57BL/J6 mice	54 nmol/kg 1x daily	8 wk. HFD → 4 wk. treat.	Hepatic steatosis ↓	(65)
Sprague Dawley rats	162 nmol/kg 1x daily	12 wk. HFD → 4 wk. treat.	Hepatic steatosis ↓ Hepatic oxidative stress ↓ Liver enzymes ↓ Dyslipidemia ↓	(66)
C57BL/J6 mice	40.5 nmol/kg 1x daily	16 wk. HFD → 4 wk. treat.	Hepatic steatosis ↓ Hepatic oxidative stress ↓	(67)
C57BL/J6 mice	54 nmol/kg 1x daily	12 wk. HFD → 4 wk. treat.	NAS↓ Liver enzymes ↓	(68)

CETP: cholesteryl ester transfer protein. HFD: high fat diet. NAS: Non-alcoholic fatty liver disease activity score (78). NASH: non-alcoholic steatohepatitis. Treat: treatment. Wk: week(s). ↓ decreased ↔ unchanged

3.4 Streptozotocin-induced diabetes in guinea pigs

The prevalence of NAFLD appears to be higher in diabetic compared to non-diabetic patients, and NAFLD has been found to be associated with an increased risk of developing type 2 diabetes (79, 80). Therefore, it was relevant examine the effects of diabetes in connection with dyslipidemia and NAFLD.

Chemically-induced diabetes offers a rapid and cost-effect choice for the study of diabetes and streptozotocin and alloxan can be used to induce insulin-deficiency with concurrent hyperglycemia (81). As the guinea pig is seemingly resistant to the effect of alloxan, streptozotocin was used to induce hyperglycemia in subsequent experiments (82, 83). Initially isolated from *Streptomyces acromogenes* in 1959, streptozotocin was used as a treatment against pancreatic cancer. However, owing to its diabetogenic effects, it soon became the drug of choice for experimentally induced diabetes (84). Streptozotocin is a glucose analogue which is selectively taken up by the glucose transporter 2 (GLUT2) (85). This selective uptake accounts for the specificity of streptozotocin-toxicity towards pancreatic β-cells, as they express GLUT2 (86). Uptake of streptozotocin results in the death of the β-cells, rendering the pancreas unable to produce and secrete insulin, resulting in hyperglycemia.

Generally, streptozotocin is administered either as multiple low doses or a single high dose. Off-target toxicity in the liver, kidney and other organs may be more pronounced with single high does injections, compared to multiple low doses (84). Thus, a multiple low dose protocol was initially used to induce hyperglycemia (Pilot study I and II). The dose of streptozotocin was selected based on the available literature describing streptozotocin-induced hyperglycemia in guinea pigs (87-95). Although these studies almost exclusively utilize a single high dose protocol, the doses were used to conservatively estimate the total amount of streptozotocin in the pilot studies. The total dose of streptozotocin was then administered as multiple smaller doses, aiming to induce a partial reduction of the β-cell mass and mimicking a state of type 2 diabetes.

4. Study overview

4.1 Study I

The aim of this study was to establish a model of dyslipidemia and NAFLD and to examine the development of cardiovascular disease and glucose intolerance in guinea pigs fed two different high fat diets. Thus, animals were fed a chow (control, 4% fat, 0% sucrose, 0% cholesterol), a high fat sucrose (HF, 20% fat, 15% sucrose, 0.35% cholesterol) or a high fat high sucrose diet (HFS, 20% fat, 20% sucrose, 0.35% cholesterol) for 16 weeks (Figure 3).

4.2 Study II

This study evaluated the effects of dietary sucrose and fat/cholesterol on the development of dyslipidemia and NAFLD as well as the progression towards NASH and hepatic fibrosis. Guinea pigs were fed five different diets for 16 or 25 weeks: chow (control, 4% fat, 0% sucrose, 0% cholesterol), very high sucrose (vHS, 4% fat, 25% sucrose, 0% cholesterol), high fat (HF[1], 20% fat, 0% sucrose, 0.35% cholesterol), high fat high sucrose (HFHS[2], 20% fat, 15% sucrose, 0.35% cholesterol) and high fat very high sucrose (HFvHS, 20% fat, 25% sucrose, 0.35% cholesterol) (Figure 3).

4.3 Study III

The last study investigated the therapeutic effect of liraglutide and dietary intervention, alone or in combination, on NASH. Guinea pigs were fed a high fat diet (20% fat, 15% sucrose, 0.35% cholesterol) for 20 weeks and block-randomized based on weight into four groups: A control group continuing on the diet (HF), a liraglutide treated group continuing on the diet (HFL), a group changed to chow diet (HFC, 4% fat, 0% sucrose, 0% cholesterol) and a group changed to chow diet and treated with liraglutide (HFCL) (Figure 3).

4.4 Pilot studies

Three pilot studies were conducted aiming to establish a protocol for streptozotocin-induced diabetes (Pilot study I and II) and to determine the dose of liraglutide for Study III (Pilot study III). The pilot studies are described in detail in section 5.3 and 5.4.

[1] Please note that the HF group of Study II is not the same as the HF group of Study I.

[2] Please note that the dietary composition of the HFHS group corresponds to that of the HF group in Study I.

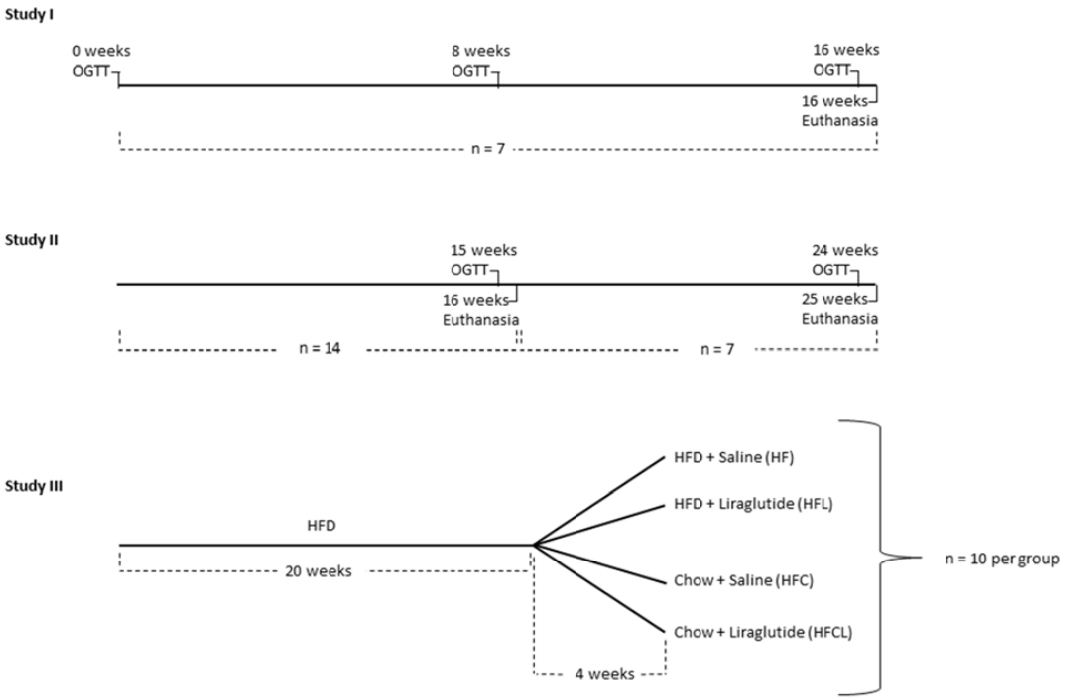

Figure 3 Schematic representation of the study design of Study I, Study II and Study III.

HFD: high fat diet. OGTT: oral glucose tolerance test

5. Results and discussion

5.1 The guinea pig as a model for diet-induced metabolic disease

Animal models of NAFLD and NASH should provide knowledge regarding both the pathophysiology of the disease and the therapeutic potential of different treatments. The ideal model of NASH encompasses hepatic steatosis, lobular inflammation, ballooning hepatocytes and fibrosis alongside metabolic abnormalities, e.g. dyslipidemia and insulin resistance (5). Despite the many different animal models currently available, they seldom reflect the complete histopathological and pathophysiological spectrum of human NAFLD (5). The models may broadly be divided into dietary, genetic or a combination of the two, but while genetic modifiers may contribute to NAFLD, outright genetic defects are not often the cause of NAFLD in humans (96)(IV). Thus, diet-induced NAFLD may be more relevant with regards to human disease. Furthermore, NAFLD is closely associated with dyslipidemia and the ability of the model to mimic the human lipoprotein profile may be advantageous (9). With this in mind, the guinea pig was investigated as a dietary model of human dyslipidemia and NAFLD (I).

5.1.1 The guinea pig develop dyslipidemia, NAFLD and hepatic oxidative stress, but does not become obese when fed a high fat diet

High fat diets induced dyslipidemia with elevated plasma cholesterol, which was mainly located in the LDL-particles, similar to the human lipoprotein profile (I). This dyslipidemic pattern may be relevant for a large part of patients with dyslipidemia, since high LDL-C was estimated to be present in approximately 27% (53.5 million) of all patients with lipid abnormalities in the United States (97). In addition, increased hepatic triglycerides, cholesterol and macro- and microvesicular steatosis (grade 2-3) were induced by high fat feeding compared to control. Hepatic inflammation seemed more prominent in the high fat fed groups; reaching statistical significant in HFS compared to control.

Redox balance was compromised in the liver following high fat feeding; markers of oxidative stress (MDA and asymmetric dimethylarginine (ADMA)) was increased and anti-oxidants (superoxide dismutase and vitamin C) decreased (I), which has been suggested to be important factors in NAFLD (section 3.3.1) (33, 38). Elevated levels of ALT and AST in the high fat groups were in agreement with hepatic damage, while lower levels of alkaline phosphatase suggested that biliary obstruction was not present in this model (I). Ballooning (degenerative) hepatocytes were not evaluated; consequently, a diagnosis of NASH could not be made (98). Hepatic fibrosis was only investigated in a subset of animals

(n=2) and although this indicated that the high fat diets induced fibrosis, the sample size was too small for any definitive conclusion.

In Study I and II, the high fat diets did not induce obesity compared to a chow diet (I, II), in accordance with other studies (15, 99, 100). Coconut oil — constituted mainly of medium-chain fatty acids — was the major source of fat in the high fat diets. Medium-chain fatty acids might be less obesogenic compared to long-chain fatty acids as they do not require transportation by the carnitine transporter to enter the mitochondria prior to oxidation (101). They are, therefore, oxidized more readily and deposited less in the adipose tissue, which could have contributed to the absence of obesity (101). Hepatic lipid retention may also have decreased storage of triglycerides in the adipose tissue. In support of this, plasma triglycerides were lower in the high fat fed animals and hepatic triglyceride export has been found to decrease in humans, as hepatic steatosis progresses towards NASH (102, 103)(I, II). Similarly, plasma triglycerides were higher in guinea pigs fed a low fat (2.5%) starch-based diet compared to guinea pigs fed a low fat sucrose-based diet or a high fat sucrose/starch-based diet (99). Lipoprotein lipase activity was increased in the high fat diet groups, which may contribute to the reduced levels of circulating triglyceride (99). This is in contrast with other results, suggesting that sucrose elevated plasma triglycerides compared to starch (104). As caloric contribution from carbohydrates was lower and the fat source contained less SFA, this potentially accounts for study differences (104). Circulating levels of FFA were not increased in Study I or II. However, as obesity has been associated with an increase in FFA, this may reflect the lack of obesity in the guinea pigs, as also reported for a non-obese rabbit model of NASH (105, 106). Importantly, the prevalence of NAFLD among lean individuals has been reported to be 3-25%, suggesting that the disease may develop independent of obesity and that this actually occurs in a relatively large number of individuals (IV). The guinea pig may then be of particular use when investigating dyslipidemia and NAFLD in non-obese individuals.

5.1.2 Guinea pigs does not become glucose intolerant when fed a high fat diet

Oral glucose tolerance tests (OGTT) did not reveal glucose intolerance in guinea pigs following high fat feeding (I). This is in contrast to a study of male guinea pigs fed a high fat high sucrose (21% fat, 27% sucrose) or high fat high fructose diet (21% fat, 27% fructose) in which glucose intolerance was evident after 150 days (≈21 weeks) (107). These guinea pigs spend considerably longer time on the high fat diets than the animals of Study I, and it could be speculated that, given more time, the guinea pigs of Study I would have developed glucose intolerance. However, in Study II, glucose tolerance was maintained even

after 24 weeks of high fat feeding (II). Previous studies have utilized male guinea pigs, which were obese relative to the chow fed control group, suggesting that obesity status might be important for the development of glucose intolerance (107). In contrast to this, a low fat carbohydrate-rich diet induced insulin resistance in male guinea pigs, while a high fat carbohydrate-restricted diet did not, indicating that weight gain alone may not promote insulin resistance, but might require concomitant intake of high amounts of carbohydrates (108). This is in accordance with Study II and may, at least partially, explain the absence of glucose intolerance in the high fat fed groups as the proportion of dietary carbohydrates was higher in control and vHS. Gender might also influence the development of glucose intolerance as female rats fed a high fat diet maintained glucose tolerance better than male rats albeit being more obese (109). Accordingly, estrogen (even at endogenous levels) may protect against high fat diet-induced glucose intolerance and insulin resistance (110). Despite not being glucose intolerant, the guinea pigs in Study I and II may still have been insulin resistant, maintaining glucose levels by a compensatory overproduction of insulin and it could be speculated that a state of dysfunctional insulin secretion and loss of functional pancreatic β-cells (resulting in hyperglycemia) had not yet been reached (111). Insulin tolerance tests and/or measurements of plasma insulin levels could address the possibility of insulin resistance. However, the amino acid sequence of guinea pig insulin differs from that of other species, including humans, rats and mice (112). This complicated the analysis and although an array of different commercial kits was tested, insulin levels could not be determined in the plasma of guinea pig. Despite of this, the results of Study I and II do not support the notion that a high fat diet induces glucose intolerance and female guinea pigs.

5.1.3 Atherosclerotic lesions do not develop in the aorta of guinea pigs fed a high fat diet

NAFLD increases the risk and prevalence of cardiovascular disease, even in non-obese subjects (113, 114). Plasma levels of levels of ADMA and oxidized LDL particles were increased in guinea pigs with NAFLD after 16 weeks (I). ADMA, an inhibitor of nitric oxide synthase, is, alongside oxidized LDL particles, believed to play an important role in the progression of atherosclerosis (115, 116). Increased levels of these markers indicated that high fat feeding might have induced cardiovascular disease, which was subsequently investigated. However, histological examination of the aorta and aortic arch did not reveal lipid accumulation or morphological signs of atherosclerosis in the high fat fed groups compared to control (Figure 4) (I). L-Arginine is the substrate for nitric oxide synthase and may oppose the negative effects of ADMA and the L-Arginine to ADMA ratio could be a more sensitive indicator of atherosclerosis (117). Although reduced in the HF group, the L-Arginine to ADMA ratio was not significantly different between groups, which is in agreement with the absence of atherosclerosis (I).

Figure 4 Histological assessment of lipid depositions in the aorta

Lipids (red/brownish color) in reprehensive aorta segments stained with Sudan IV from control, HF and HFS. The percentage of the lumen area covered by lipids did not differ between groups (Birck MM unpublished data from Study I).

Atherosclerosis is a chronic disease, which develops over long periods of time (118) and the endothelial function of the mesenteric arteries was, therefore, examined in HFvHS and control animals at the 25 week time-point of Study II. Vasodilation was compromised in the HFvHS compared to control (Figure 5). Although statistically significant, the change in endothelial function seemed to be relatively small and arteries were not examined histologically, preventing a comparison to potential morphological changes. Other authors have reported cholesterol accumulation, increased expression of inflammatory genes and small lipid droplets in the aorta guinea pigs fed high cholesterol (0.25-1.25%) diets, but no definitive signs of atherogenesis (17, 119-122). Plaques have been observed in the aorta of guinea pigs fed egg yolks for nine months, suggesting study duration as an important factor in atherosclerosis development (123). All of these studies utilized male guinea pigs, which may account for study discrepancies as female guinea pigs seem to develop atherosclerosis to a lesser degree than males (124). Regardless, none of the reported lesions were characterized as advanced and may, at best, reflect early atherosclerotic development. This suggest that the female guinea pig might not be the optimal model for the examination of advanced cardiovascular disease, or that longer time periods may be needed to induce disease development. Thus, results from Study I and II do not support the development of atherosclerosis in the high fat fed guinea pig.

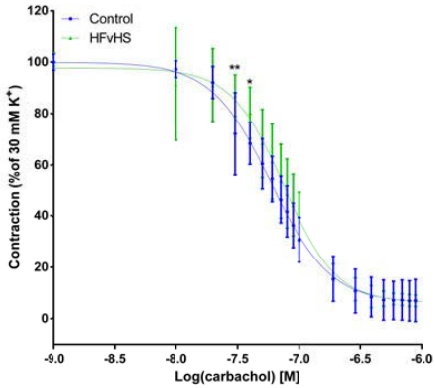

Figure 5 Endothelial function of mesenteric arteries

*Carbachol (muscarinic acetylcholine receptor agonist) induced vasodilation in mesenteric arteries isolated from guinea pigs fed a control or HFvHS diet for 25 weeks. Vasodilation was compromised in HFvHS compared to control at carbachol concentrations of $10^{-7.5}$ and $10^{-7.40}$ M. * p<0.05 ** p< 0.01. Means with SD. Analysed by a 2-way repeated measure ANOVA with Tukeys post-hoc test. n=6. (Skovsted GF unpublished data from Study II).*

In summary, a guinea pig model of dyslipidemia and NAFLD, with particular relevance to the non-obese patient, was established. Glucose intolerance and atherosclerosis was not observed in this model, although increased study duration may be required before these metabolic abnormalities develop.

5.2 The importance of diet on the development of dyslipidemia and NAFLD

Having established an animal model, it was feasible to investigate the effect of specific dietary components on the development of NAFLD and dyslipidemia. Study I indicated that a small (5%) difference in dietary sucrose content did not induce notable metabolic or hepatic alterations. However, three questions remained; would a more significant difference in dietary sucrose content affect the development of dyslipidemia and NAFLD, would a high fat diet without sucrose also induce disease and could sucrose alone promote disease development? Study II utilized diets in which the sucrose content differed with 10-25% and included a high fat group without sucrose and a chow group with sucrose (II). Based on the results from Study I, it also remained unclear to which degree disease progression would occur if the high fat feeding period were prolonged and if the guinea pigs would develop advanced NASH. Consequently, histological assessment of ballooning hepatocytes and fibrosis was included in all animals and study duration prolonged to 25 weeks.

5.2.1 NAFLD progresses towards NASH with fibrosis in high fat fed guinea pigs, but sucrose does not affect the development of dyslipidemia and NASH

Hepatic steatosis and ballooning hepatocytes was present after 16 weeks of high fat feeding. At week 25, the disease progressed to NASH as inflammation became significantly more pronounced in guinea pigs fed the high fat diets. There were no differences between groups in which the high fat diet contained sucrose (HFHS and HFvHS) and the high fat group in which carbohydrates mainly originated from starch (HF). Likewise, a chow diet with high amounts of sucrose (vHS) did not induce hepatic steatosis or NASH (II). High fat diets induced progressive perisinusoidal/pericellular (chicken wire) fibrosis, culminating in bridging fibrosis (grade 3) at week 25 – closely resembling human fibrosis. Advanced fibrosis is not commonly reported in animal models utilizing high fat diets and the guinea pig may be an advantageous model in this regard (96). In accordance with its impact on NAFLD/NASH, sucrose alone or added to a high fat diet did not affect the development of hepatic fibrosis (II). The effect of dietary carbohydrates on the development of NAFLD is still being debated: A recent meta-analysis suggested that isocaloric diets, in which fructose was exchanged for other carbohydrates did not induce hepatic fat accumulation in healthy individuals, but addition of fructose, creating a hypercaloric diet, may promote fat accumulation (125). Although the trials included in the meta-analysis lacked histological confirmation of NAFLD, this indicates that a certain caloric excess may be important, at least with regards carbohydrates. Energy intake was higher in the vHS group compared to control and high fat fed groups, but despite of this, dyslipidemia and NASH was not present, further implicating fat and cholesterol as key macronutrients (II). Study II is unable to differentiate the effect(s) of dietary fat and cholesterol, but other studies have suggested important roles of both macronutrients with regards to disease development: Fat alone promotes dyslipidemia and hepatic fat accumulation in guinea pigs (100). Omitting cholesterol from a high fat diet reduced hepatic inflammation in LDL-receptor knock-out or apolipoprotein E2 knock-in mice (126). Likewise, addition of cholesterol to a high fat diet exacerbated the degree of hepatic inflammation and fibrosis in LDL-receptor knock-out mice (127). Cholesterol may promote apoptosis of macrophages and hepatocytes, increase oxidative stress and activate hepatic stellate cells, which cause fibrosis (128)(IV). Activated hepatic stellate cells subsequently enhance their accumulation of cholesterol, establishing a vicious cycle that may contribute to the adverse hepatic effects of cholesterol (128). It could be speculated that cholesterol and fat – particularly SFA – work in concert to promote NAFLD and this may be of relevance since increased dietary intake of both cholesterol and SFA has been reported for patients with NAFLD (IV). However, further studies are needed to verify this.

Hepatic histology and dyslipidemia with LDL as the major lipoprotein is in accordance with human disease pathology. These changes were induced by a diet resembling the human Western diet and without extremely high amounts of fat (≥60% of total energy) and cholesterol (1-2%) or reduced/deficient levels of essential nutrients like methionine and choline, commonly used in other animal models (5, 96, 129). Collectively, Study I and II indicate the guinea pig as an advantageous model of NASH and dyslipidemia with a high degree of similarity to humans. The model was, therefore, used to elucidate some of the underlying mechanisms of advanced NASH by assessing the degree of genomic instability and DNA damage. Reduced telomere length have been reported in patients with type 2 diabetes who developed NAFLD over a six year follow-up period compared to patients who did not (130). However, DNA strand breaks and telomere length was not different between the groups in Study II. Longer study durations may have been required to observe an effect on telomere length and/or — as discussed in (II) — progression towards cirrhosis may be necessary before telomere-shortening occurs. Alternatively, telomere-shortening and DNA strand breaks may not be underlying mechanisms in this guinea pig model of NASH.

In agreement with Study I, plasma levels of cholesterol, VLDL-C and LDL-C were increased in all high fat groups compared to the control and vHS group after 16 weeks. These lipids remained elevated, but did not increase further after 25 weeks on the diets, suggesting that a plateau is encountered and levels of circulating cholesterol do not increase further. Sucrose did not induce dyslipidemia nor did it exacerbate the dyslipidemia induced by a high fat diet, thus, the effect of the diets on circulating lipids corresponded to their effect on the liver (II). The addition of sucrose to a high fat diet has previously been found to increase LDL-C compared to a starch-based high fat diet, while exchanging sucrose and starch in a low fat diet did not influence LDL-C levels (99). Likewise, sucrose increased circulating levels of triglycerides compared to starch in both high and low fat diets (131). Accounting for approximately 70% of the total energy content in the low fat high sucrose diet (131), the sucrose content was higher compared to Study II, potentially explaining why sucrose did not increase plasma triglyceride in the chow-based high sucrose diet (vHS) compared to control. In both studies (99, 131), palm oil and butterfat were used as the fat source and the high content of long-chained saturated and mono-unsaturated fatty acids may interact differently with sucrose compared to the coconut oil (medium-chained SFA) utilized in Study II. SFA seems to increase LDL-C in male and female guinea pigs (132), and given their adverse effects and high abundance in the diets, they may have confounded any potential effect(s) of sucrose. Dietary fats may also have a more pronounced effect on plasma LDL-C in guinea pigs, while the effect of dietary carbohydrates may be greater on VLDL apoB secretion rates and removal from plasma (131). Compromised VLDL assembly and export (as discussed in (I), (II) and section

5.1.1) could then also confound the effect of sucrose in Study II. However, the chow-based high sucrose diet (vHS) did not induce any metabolic abnormalities, reiterating the idea of fat and cholesterol as primary driving factors in disease development.

A fixed fatty acid composition-ratio was not utilized in Study II, which may, inadvertently, have decreased the relative amount of certain types of fatty acids believed to be beneficial for NAFLD, e.g. PUFA (IV), and this could have impacted the outcome of the study. Nevertheless, guinea pigs developed dyslipidemia and NASH with advanced fibrosis when fed a high fat diet and hypothesis 1 was accepted, but since sucrose did not promote disease development or exacerbate the effects of the high fat diets, hypothesis 2 was rejected.

5.2.2 Dyslipidemia and NAFLD develop in the absence of adipose tissue dysfunction

Reviewing the literature indicated that adipose tissue dysfunction is not exclusive to obese individuals, but may also be present in metabolically unhealthy lean individuals (V). Consequently, the extent of adipose tissue dysfunction was investigated in the established non-obese guinea pig model of dyslipidemia and NAFLD (Study II, unpublished data).

Sucrose did not affect levels of circulating lipids or NAFLD (II). Therefore, adipose tissue function was examined in a subset of the animals (i.e. control, HF and HFHS) at week 25 of Study II (see Table 2 for methodology). The percentage of large and small adipocytes and the number of crown-like structures in the visceral adipose tissue did not differ between groups. Levels of tumor necrosis factor α (TNFα), interleukin 1β (IL1β) and adiponectin were assessed in the adipose tissue of the control and HFHS group, but were not significantly different (Table 2 and Figure 6). Adiponectin levels correspond to those reported in Study I and are consistent with the absence of adipose tissue dysfunction (I). Taken together, this indicates that NAFLD and dyslipidemia may develop in the absence of and/or prior to adipose tissue dysfunction in female guinea pigs.

Table 2 Adipocyte size and levels of inflammatory cytokines in the adipose tissue

	Control	HF	HFHS
Percentage of large adipocytes (>40 µm)	30.2±16.1	16.8±16.1	12.8±11.4
Percentage of small adipocytes (≤40 µm)	69.9±16.1	83.2±16.1	87.2±11.4
Number of crown-like structures ‡	3 (2-6)	3 (1-7)	2 (1-12)
TNFα (pg/mg total protein)	884±255	-	721±259
IL1β (pg/mg total protein)	124±52.8	-	85.4±29.9
Adiponectin (pg/mg total protein)	7042±3222	-	7834±1975

Paraffin-embedded visceral adipose tissue from the left kidney was stained with haematoxylin and eosin and the number of crown-like structures was quantified by light microscopy in 30 fields (300x225 µm per field) at x20 magnification. Additionally, 200 adipocytes were evaluated by size and assigned as large (>40 µm) or small (≤40 µm) based on the broadest cross-section. Levels of TNFα, IL1β (LSBio, Seattle, Washington, USA) and adiponectin (RayBiotech, Norcross, Georgia, USA) were measured in tissue homogenates using commercially available ELISA kits according to the manufactures specifications. Means with standard deviations. ‡ Medians with range. n=7. Analyzed by Kruskal-Wallis test or t-test.

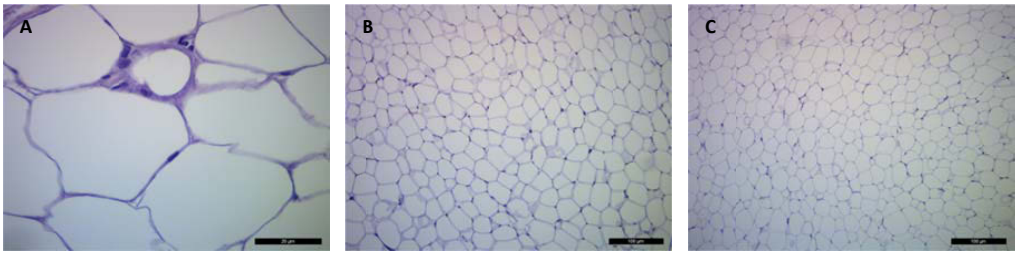

Figure 6 Histological assessment of adipose tissue dysfunction

(A) Crown-like structure and representative visceral adipose tissue from control (B) and HF/HFHS (C) guinea pigs after 25 weeks. Scale bar 20 µm (A) and 100 µm (B and C).

Non-obese male guinea pigs fed a high fat diet (0.25% cholesterol, 15% fat) for six weeks had smaller adipocytes, macrophage infiltration and increased levels of cholesterol and inflammatory cytokines in the epididymal adipose tissue (133). Study I and II examined inguinal fat and visceral adipose tissue located around the kidney in female guinea pigs, respectively, and did not find evidence of adipocyte dysfunction. The anatomical location of the fat tissue and the gender of the animals may affect the morphology, function, inflammatory profile and gene expression of specific adipose depots (134). Similar to its effect on glucose tolerance, estrogen might protect the female sex from adipose tissue dysfunction and this could account for differences between studies (135, 136). Measuring adipocyte cholesterol content may have provided valuable information as cholesterol-overload seems to constitute an important mechanism underlying adipocyte dysfunction in both the obese and non-obese

(54). The results of Study II may reflect the increased hepatic lipid retention and low levels of circulating triglycerides. Unable to reach the adipose tissue for storage, excess triglycerides would not be able to promote adipocyte hypertrophy and initiate adipose tissue dysfunction. Importantly, the guinea pigs still had a considerable amount of body fat (approximately 23% and 28% after 16 weeks of high fat and chow diet, respectively (I)), demonstrating that lipid transport to and storage in the adipose tissue were still functioning, albeit potentially impaired. Certain limitations are associated with the current investigation: Only a single adipose depot was sampled and differences may have been more apparent at other locations. Furthermore, assessment of adipocyte size could have been improved by using more precise techniques, such as flow cytometry.

In summary, high fat diets induced both dyslipidemia and NAFLD with increased levels of oxidative stress, which progressed to NASH with advanced fibrosis (I, II). Hypothesis 1 was, therefore, accepted. High fat diets with or without sucrose did not differ with regards to circulating lipids or development and progression of NAFLD and fibrosis. In addition to this, a high sucrose diet alone did not induce dyslipidemia or NAFLD either, hypothesis 2 was therefore rejected.

5.3 Establishing the streptozotocin-induced diabetic guinea pig model

Guinea pigs did not develop glucose intolerance even after prolonged (25 weeks) high fat feeding (I, II). The potential of streptozotocin to induce partial β-cell destruction with resulting hyperglycemia was, therefore, evaluated in two pilot studies (Pilot study I and II). Insulin resistance (a fundamental component of type 2 diabetes) is not inherently present in the streptozotocin-model, but may be induced by high fat feeding prior to streptozotocin-treatment (84). However, dietary composition — e.g. a chow diet compared to a high fat diet — may affect the sensitivity of the animals to streptozotocin (81). Consequently, an initial high fat feeding period was included in both pilot studies.

In Pilot study I (for methodological details see Figure 7), blood glucose was not elevated following multiple low dose injection of streptozotocin (Figure 7A, group a-c). Given the dose-dependent relationship between streptozotocin and the development of hyperglycemia, it followed that higher doses might be needed to induce hyperglycemia (137). In Pilot study II, streptozotocin was administered in increasing amounts, as a single high dose, to the guinea pigs previously treated with multiple low doses (group a-c) and another multiple low dose group receiving a much larger dose of streptozotocin (group d) was also included. Hyperglycemia was not induced by any of the dosing-protocols (Figure 7B). Instead, three animals – one from each single high dose group (a, b and c) – became severely

hypoglycemic and died following the streptozotocin treatment in Pilot study II. This suggests a considerable inter-individual variation in the response to streptozotocin. The death of the animals was likely caused by the rapid release of large amounts of insulin from destroyed β-cells (84).

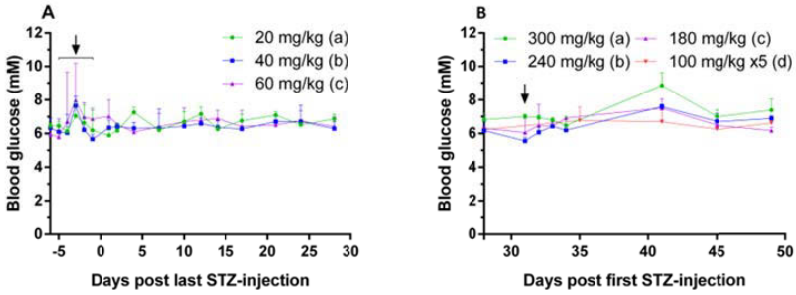

Figure 7 Non-fasted blood glucose levels following streptozotocin treatment

(A) Blood glucose in guinea pigs injected intraperitoneal (ip) with 20-60 mg/kg streptozotocin (freshly dissolved in 0.05M citrate buffer, pH = 4.5) each day for five continuous days (Pilot study I). (B) Four weeks after the initial streptozotocin treatment, a single high ip dose of streptozotocin of 180, 240 or 300 mg/kg body weight (BW) was administered to the same guinea pigs who previously received 60, 40 or 20 mg/kg BW, respectively. Simultaneously, guinea pigs who had been fed the high fat diet for ten weeks (and were not previously treated with streptozotocin) were injected ip with 100 mg/kg streptozotocin each day for five continuous days (Pilot study II). n = 2-3. Means with standard deviations. Arrows indicate streptozotocin injection(s).

It remains unclear why streptozotocin failed to induce hyperglycemia. Unfortunately, the inability to measure plasma levels of insulin (section 5.1.2) prohibited insight into the relationship between the dose of streptozotocin and insulin production. As mentioned above, streptozotocin-induced hyperglycemia has been reported for guinea pigs, although these were mainly males (section 3.4). Female mice have been found to be less sensitive, or even non-responsive, to streptozotocin-induce diabetes compared to male mice, emphasizing the potential importance of gender differences (81). Higher doses of streptozotocin or different dosing protocols may be needed to induce hyperglycemia in female guinea pigs, but would require additional pilot studies. Since the development of a streptozotocin-induced diabetes model was not the primary aim of this thesis, no further attempts were made at this.

5.4 Liraglutide and dietary intervention for the treatment of NASH

A high dietary intake of SFA and cholesterol has been reported for individuals with NAFLD compared to healthy controls (IV). Taken together with studies suggesting a beneficial effect of life-style intervention on NAFLD (section 3.3.3) and the results from Study I and II, in which different high fat diets consistently induced NASH, it followed that dietary intervention might be employed as a treatment option for NASH. The available literature also indicated that liraglutide may be used to mediate the unmet need for pharmacological treatment (section 3.3.3). Using the time-frame established in Study II, Study III was planned as an intervention study and examined the effect of dietary intervention and liraglutide, alone or in combination, on NASH. This was done to enhance translation and mimic the clinical situation in which therapy are more likely to be initiated once NAFLD/NASH has developed instead of prophylactically (138).

Liraglutide treatment has not been reported previously in guinea pigs, necessitating an initial dose-finding study (Pilot study III). In humans, liraglutide are dosed once daily due to its half-life of approximately 12 hours, but the shorter half-life in rats, mice, rabbits and monkeys of about 4-8 hours requires twice daily administration (139, 140). Assuming a similar half-life in guinea pigs, Pilot study III was designed to determine the twice daily dose of liraglutide which induced a 10-15% weight-loss and a dose of 30 nmol/kg BW was judged to be sufficient to induce and maintain weight-loss in guinea pigs. The dose had to be escalated slowly during approximately a week as the weight-loss occurred too rapidly otherwise (Figure 8).

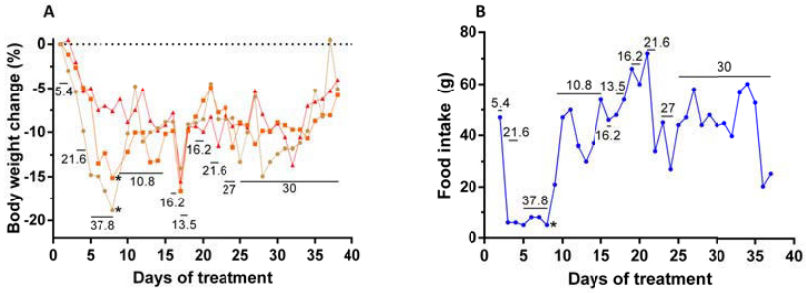

Figure 8 Body weight and food intake following liraglutide treatment

*Guinea pigs were fed a high fat diet for seven weeks and then injected subcutaneously with liraglutide (Novo Nordisk A/S) twice daily (Pilot study III). Liraglutide induced a rapid weight-loss (A) by suppressing food intake (B — measured as the total food intake of all three animals), requiring a three day dose-holiday in two of the animals (onset indicated by *). A stable weight-loss of 10-15% of the initial body weight prior to treatment could be maintained by slowly increasing the dose of liraglutide to 30 nmol/kg BW twice daily. Doses and duration are indicated by bars; doses are given in nmol/kg BW. n = 3.*

5.5 Liraglutide and dietary intervention as treatment options for NASH

Although expected, the weight-loss in the liraglutide and dietary intervention group (HFCL) occurred more rapidly than initially anticipated. This was due to an almost complete cessation of food intake, resulting in a weight-loss approaching the predefined humane endpoint of 20%. Dietary intervention was maintained, but liraglutide was discontinued and not resumed until the animal had regained weight corresponding to a weight-loss of <10% of the initial body weight. It is, therefore, important to recognize that continuous liraglutide treatment was not possible in the HFCL group and this should be considered when interpreting the results of Study III. Food preferences are established early in guinea pigs and sudden dietary changes may not always be feasible as this might reduce normal feeding behavior (141). In combination with the appetite suppressing effect of liraglutide, this could explain the abrupt decrease in food intake. Thus, in future studies it may be necessary to have an even slower dose escalation of liraglutide or any other appetite reducing compounds and/or gradually introduction the new dietary source before initiating liraglutide treatment.

5.5.1 Liraglutide reduced hepatic inflammation and hepatocyte degeneration

Liraglutide alone (HFL) and in combination with dietary intervention (HFCL) reduced hepatic inflammation compared to control (HF). HFL decreased hepatocyte ballooning compared to HF and a tendency towards reduced portal inflammation was also observed. Dietary intervention (HFC) seemed to reduce hepatic steatosis, hepatocyte ballooning and inflammation, but this was not statistically significant to HF. Similar trends were seen within the HFCL group with regards to steatosis and hepatocellular degeneration. ALT and AST were elevated approximately two-fold and ten-fold, respectively, in the HF group compared to the control group in Study II, but none of the treatments decreased liver enzyme levels (III). Hepatocyte ballooning and inflammation are distinguishing features of NASH and play key roles in the disease (98). Ballooning degeneration has been reported to be independently associated with disease progression and fibrosis, suggesting the importance of continuous hepatocyte injury/damage in NASH (142). Inflammation may promote cellular stress and is a prominent finding in many major metabolic disease, e.g. NAFLD, diabetes and cardiovascular disease (143). In NAFLD, inflammation may induce hepatic steatosis, insulin resistance and disease progression (33, 143). Histological lesions can be ranked in order from higher risk of mortality to lower risk of mortality as: Fibrosis, followed by the diagnosis of NASH, portal inflammation and ballooning, respectively (144). Amelioration of NASH features by liraglutide, especially hepatocyte ballooning and portal inflammation, could then be important in improving NASH-related mortality.

The histopathological effects of liraglutide in Study III are similar to previous studies in humans: Hepatic steatosis, measured by different imaging techniques, was unaffected by 12 weeks of liraglutide treatment (145, 146). Fibrosis assessed by formulae (i.e. NAFLD fibrosis score, fibrosis-4 score and aspartate aminotransferase to platelet ratio index) (145) and liver enzymes were unaltered as well (145, 146). Armstrong et al. investigated the effect of liraglutide in a randomized clinical trial where liver biopsies were obtained before and after treatment (59). Steatosis, hepatocyte ballooning and inflammation grade were not significantly different following 48 weeks of intervention, but a larger percentage of patients in the liraglutide group showed improvement of steatosis, hepatocyte ballooning and resolution of NASH compared to the placebo group (59). The fibrosis grade and the number of patients with improvements in fibrosis were similar between the groups, however, liraglutide prevented worsening of fibrosis compared to placebo and the authors speculated that longer treatment periods might be necessary to reduce/reverse fibrosis. Additionally, univariate analysis suggested that patients with severe fibrosis (grade 3-4) at baseline were less likely to respond to liraglutide treatment (59). Thus, the timing of the intervention and the severity of the disease seem important, when treating NASH, which has also been suggested by studies utilizing antioxidants against NASH (147). In Study III, bridging fibrosis (grade 3) was observed in almost all animals of the HF group, but liraglutide still improved NASH, indicating the beneficial effects of using liraglutide even in the face of advanced disease. Furthermore, it may be speculated that the effect of liraglutide could be more pronounced if initiated earlier, i.e. prior to the development of grade ≥3 fibrosis.

While inflammation and ballooning were decreased, liraglutide did not reduce hepatic steatosis or fibrosis. Liraglutide has previously been found to decrease hepatic steatosis in obese glucose intolerant/insulin resistant murine models (61-64, 66-68), but the underlying mechanisms of liraglutide might be different in guinea pigs: Liraglutide normalizes blood glucose levels and increases insulin sensitivity, which could reduce glucose-induced *de novo* lipogenesis in the liver and enhance insulin-suppression of lipolysis in the adipose tissue, leading to decreased levels of circulating free fatty acids and, thereby, decreased hepatic lipid accumulation (69). Such effects may not be relevant to the lean glucose tolerant guinea pigs used in Study III and circulating free fatty acids were even increased following liraglutide treatment, likely attributable to weight-loss. This could then account for study discrepancies. Only one of the pre-clinical studies reporting an effect of liraglutide on steatosis investigated the extend of hepatic fibrosis (68). Animals in that study had mild sinusoidal fibrosis (68), suggesting that NASH with less advanced fibrosis may allow reversal of steatosis by liraglutide and reiterating the importance of intervention timing. The apparent absence of hepatic GLP-1 receptors

suggests an indirect effect of liraglutide on NASH (section 3.3.3) (76, 77). Adipose tissue inflammation was found to be decreased by GLP-1 in *ob/ob* mice, potentially limiting the flux of inflammatory cytokines to the liver, which is believed to contribute to NAFLD (148). Study II did not find any evidence of adipose tissue dysfunction and inflammation, indicating that this is not a target of liraglutide in guinea pigs or that fat depots other than visceral fat surrounding the kidney are affected. In agreement with reduced hepatocyte ballooning in the HFL group, liraglutide has been found decrease endoplasmic reticulum stress and promote hepatocyte survival in high fat fed mice with NAFLD (65). The macrophage activation marker sCD163 has been found to be elevated in patients with NAFLD and was associated with the severity of the liver histology (149). This association was independent of anthropomorphic, inflammatory and metabolic measures, prompting the authors to suggest a direct association between macrophage activation and liver injury (149). Liraglutide reduced sCD163 in diabetic patients independent of body weight changes and HbA1c, suggesting that liraglutide directly reduces macrophage activation (150). It could then be speculated that decreased activation of immune cells may mediate some of the anti-inflammatory effects seen in the liver of guinea pigs treated with liraglutide.

In contrast to liraglutide, dietary intervention reduced hepatic cholesterol and ameliorated dyslipidemia by decreasing circulating levels of total cholesterol, LDL-C and VLDL-C (III). Diet alone decreased hepatic steatosis compared to HFL with a similar trend being observed compared to HF. Inflammation and hepatocellular ballooning was not significantly affected, but considering the tendency towards improvements in the overall histopathology, it could be speculated that longer study duration may be necessary to effectively treat NASH with dietary intervention (III). Accordingly, pre-clinical studies have reported an effect of dietary changes after 7 and 12 weeks, while clinical studies utilizing life-style intervention to treat NASH lasted 48-52 weeks (56, 57, 151, 152). In *foz/foz* mice, dietary intervention decreased hepatic steatosis by suppressing hepatic fatty acid uptake and partitioning lipids towards the adipose tissue instead (152). Inflammation was not reduced, but liver macrophages were shifted toward the more anti-inflammatory M2 type and it is possible that such mechanisms may underlie the beneficial effects of dietary intervention (152). These findings also substantiates the idea of combined pharmacological and dietary therapy, which could potentially target NASH and the dyslipidemia which affects the majority of the patients with fatty liver disease (6).

5.5.2 Dietary intervention and liraglutide induces slight improvements in hepatic oxidative stress

Study I found that oxidative stress developed concurrent to NAFLD (I). In addition, liraglutide has been suggested to reduce redox-imbalance in the livers of mice with NAFLD (66, 67). Study III, therefore,

investigated if the effect of the treatments on NASH was mediated through reduction of hepatic oxidative stress.

High fat feeding for 24 weeks induced oxidative stress in the liver of guinea pigs (HF) compared with the control group of Study I — again consolidating the reproducibility of the model. Liraglutide increased levels of the fat-soluble anti-oxidant α-tocopherol (vitamin E). Randomized clinical trials have reported that the number of subjects with histological improved NASH was higher in the vitamin E treated group compared to placebo in both adult and children (153, 154). Thus increased α-tocopherol levels may contribute to the improved hepatic inflammation and hepatocyte ballooning observed in the HFL group (III). Previous studies have reported a more pronounced effect on hepatic redox-balance following treatment with liraglutide (66, 67). As discussed in (III) differences between the previous studies and Study III, such as gender, species and liraglutide-dose may account for these discrepancies. The relatively small change in redox-status could also be due to the unaffected levels of hepatic cholesterol as free cholesterol has been suggested to induce oxidative stress by depleting mitochondrial glutathione and by sensitizing hepatocytes to TNFα-mediated NASH (155, 156). Dietary intervention reduced hepatic cholesterol and improved the hepatic antioxidant system by increasing levels of vitamin C, which may play a role in the development of NAFLD (VI). Hepatic cholesterol levels were still approximately two-fold higher compared to guinea pigs fed a chow diet for 25 weeks (II). In accordance with the cytotoxic effect of cholesterol, a greater impact on the hepatic redox-balance may require hepatic cholesterol levels to be reduced even further (155, 156). The combination of dietary intervention and liraglutide increased hepatic levels of MDA and did not facilitate the same improvement of oxidative stress as liraglutide or dietary intervention alone. The HFCL group may be considered severely food restricted, at least in the initial intervention-period, which has been found to increase hepatic MDA levels in rats (157). Overall, oxidative stress was only slightly decreased in the liver following dietary intervention or liraglutide, suggesting that improved liver histology is not mediated solely by reduction of oxidative stress and/or that longer treatment duration might be needed before redox-balance is affected further.

5.5.3 Liraglutide did not affect endothelial function in coronary arteries

The decline in endothelial function following high fat feeding (Figure 5, Study II) alongside elevated biochemical markers linked to endothelial dysfunction (I), prompted the investigation of the effect of liraglutide on endothelial dysfunction in Study III. This investigation was performed in the HF and HFL group only.

The L-Arginine to ADMA ratio and the BH_2 to BH_4 ratio, both markers of endothelial dysfunction, did not differ between HF and HFL (158, 159)(III). Accordingly, carbachol-mediated vasodilation was not affected by liraglutide (III). Liraglutide has previously been reported to inhibit plaque progression and improve plaque stability in early onset, low-burden atherosclerotic $ApoE^{-/-}$ mice fed a high fat diet for four weeks (160). However, endothelial function was not affected by the high fat feeding or by liraglutide (160). Feeding $ApoE^{-/-}$ mice a high fat diet for 12 weeks induced late onset atherosclerosis with a heavy plaque burden (160), and liraglutide has been reported to improve vascular function in $ApoE^{-/-}$ mice after 12 weeks on a high fat diet (161). The guinea pigs of Study III may not yet have developed substantial endothelial dysfunction, supported by the apparent absence of atherosclerotic plaques in Study I and the only slightly compromised vasodilation in Study II. This could hamper investigation into potential beneficial effects of liraglutide on endothelial function.

In summary, a differential effect of liraglutide and dietary intervention was observed: Liraglutide improved histological features of NASH (hepatocyte ballooning and inflammation) even in the presence of severe fibrosis. Dietary intervention tended to improve liver histology and significantly reduced hepatic and circulating cholesterol levels. Small improvements of hepatic redox-balance were observed in both groups and decreased oxidative stress may, therefore, not constitute the only/main mechanism underlying histological improvement of NASH. This differential effect suggested that combination of diet and liraglutide might be relevant to the treatment of NASH, but Study III was not able to fully assess the effected of the combined therapy and, thus, hypothesis 3 remain unanswered.

6. Conclusion and future perspectives

NAFLD and hepatic oxidative stress develops following high fat feeding and prolonging the feeding period leads to the development of advanced NASH and severe hepatic fibrosis, with close resemblance to human histopathology (I, II). This suggests the guinea pig as an excellent model for human dyslipidemia and NASH. Excessive weight-gain was not associated with the high fat diets and the guinea pig thus offers a model for non-obese dyslipidemia and NASH (I, II), which may affect a considerable number of individuals (IV). Glucose intolerance and adipose tissue dysfunction were not evident following high fat feeding and could imply that dyslipidemia and NASH develop prior to other metabolic abnormalities, potentially serving as cornerstones in the development of metabolic disease, at least in this model (I, II). Detailed investigating of several different arteries did not reveal atherosclerosis or pronounced endothelial dysfunction and, despite of the favorable lipoprotein profile, the female guinea pig may not be the optimal model for atherosclerosis research. The use of male or ovariectomized female guinea pigs may minimize the protective effect of estrogen, allowing for a more marked decline of metabolic function in other tissues, which could be relevant for future studies.

According to Study II, dietary fat and cholesterol seems to be the primary drivers of dyslipidemia and NASH, while dietary sucrose does not impact disease development (II). Thus, controlling intake of SFA and cholesterol may be beneficial in preventing NAFLD. The extremely complex interplay between diet and disease development should be kept in mind, and while Study II proposes the relative importance of fat/cholesterol it does not refute a potential effect of sucrose in other models or when ingested in combination with a different dietary composition.

Liraglutide improved NASH histology even in the face of severe fibrosis, while dietary intervention reduced dyslipidemia, suggesting that combined treatment may be effective in treating both NASH and the closely associated dyslipidemia (III). Decreased hepatic oxidative stress may contribute to the histological improvements, but it is likely that other mechanisms also underlie the effect of the therapies (III). The unmet need for treatments of NASH warrants further investigations of liraglutide both alone and in combination with dietary intervention, as well as the elucidation of the underlying mechanisms. Furthermore, fibrosis is emerging as a cardinal feature associated with increased mortality, liver-transplantation and liver-related events in patients with NAFLD (162). Although the evidence is not yet conclusive, fibrosis seems reversible in NASH at least up to a certain point (163). Regression of fibrosis could then be regarded as an important endpoint when treating NASH and the guinea pig model may also prove valuable in this regard due to the development of advanced fibrosis. Lastly, intervention timing and treatment duration will likely be important factors to consider when designing future studies.

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Papers I-VI

Paper I

Diet-induced dyslipidemia leads to nonalcoholic fatty liver disease and oxidative stress in guinea pigs.
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Diet-induced dyslipidemia leads to nonalcoholic fatty liver disease and oxidative stress in guinea pigs

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Chronic dyslipidemia imposed by a high-fat and high-caloric dietary regime leads to debilitating disorders such as obesity, nonalcoholic fatty liver disease (NAFLD), and insulin resistance. As disease rates surge, so does the need for high validity animal models to effectively study the causal relationship between diet and disease progression. The dyslipidemic guinea pig displays a high similarity with the human lipoprotein profile and may in this aspect be superior to other rodent models. This study investigated the effects of 2 long-term Westernized diets (0.35% cholesterol, 18.5% vegetable oil and either 15% or 20% sucrose) compared with isocaloric standard chow in adult guinea pigs. Biochemical markers confirmed dyslipidemia in agreement with dietary regimens; however, both high-fat groups displayed a decreased tissue fat percentage compared with controls. Macroscopic appearance, histopathologic evaluation, and plasma markers of liver function confirmed NAFLD in high-fat groups, supported by liver redox imbalance and markers suggesting hepatic endothelial dysfunction. Plasma markers indicated endothelial dysfunction in response to a high-fat diet, although atherosclerotic lesions were not evident. Evaluation of glucose tolerance showed no indication of insulin resistance. The 5% increase in sucrose between the 2 high-fat diets did not lead to significant differences between groups. In conclusion, we find the dyslipidemic guinea pig to be a valid model of diet imposed dyslipidemia, particularly with regards to hepatic steatosis and endothelial dysfunction. Furthermore, the absence of obesity supports the present study setup as targeting NAFLD in nonobese individuals. (Translational Research 2016;168:146–160)

Abbreviations: ADMA = asymmetric dimethylarginine; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; BH_2 = dihydrobiopterin; BH_4 = tetrahydrobiopterin; BW = body weight; CTRL = isocaloric control standard chow diet; DEXA = dual-energy x-ray absorptiometry; eNOS = endothelial nitric oxide synthase; GSH = glutathione; GSSG = oxidized glutathione; H&E = Mayer's hematoxylin and eosin; HDL = high-density lipoprotein; HF = high fat + 15% sucrose; HFS = high fat + 20% sucrose; HPLC = high-performance liquid chromatography; L-Arg = L-arginine; LDL = low-density lipoprotein; MDA = malondialdehyde; NAFLD = nonalcoholic fatty liver disease; NASH = nonalcoholic steatohepatitis; NO = nitric oxide; OGTT = oral glucose tolerance test; Ox-LDL = oxidized low-density lipoprotein; SOD = superoxide dismutase; TC = total cholesterol; TG = triglyceride; VitC = vitamin C; VLDL = very low-density lipoprotein

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AT A GLANCE COMMENTARY

Tveden-Nyborg P, et al.

Background

As the obesity pandemic continues to expand, the need for valid animal models is increasing. Owing to a high similarity with the human lipoprotein profile, the guinea pig may prove to be superior compared with mice and rat models of diet-induced dyslipidemia and subsequent debilitating disease.

Translational Significance

This study shows extensive liver damage and oxidative stress as well as indicates endothelial dysfunction imposed by a diet-induced dyslipidemia. Our findings support the applicability of the guinea pig model in future in vivo studies, particularly targeting the nonobese form of nonalcoholic liver disease.

INTRODUCTION

The obesity pandemic and increase in debilitating chronic diseases such as cardiovascular disease, type 2 diabetes, and metabolic disorders, as a consequence of a high-fat—"Westernized"—diet, have highlighted the need for accurate animal models. In this aspect, the guinea pig (*Cavia porcellus*) may be a natural and superior model because of a unique similarity with the human lipoprotein profile and the enzymatic processing of lipids in vivo. On the contrary, both mice and rats display a large proportion of high-density lipoprotein (HDL) and comparatively low levels of low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL), thus showing the almost inverse pattern compared with humans and guinea pigs in response to a fatty meal.[1]

Deposition of lipids (in the form of triglycerides [TGs]) in the liver and subsequent development of nonalcoholic fatty liver disease (NAFLD) is linked to the intake of excess calories and is regarded as a hepatic consequence of, but not limited to, obesity and the metabolic syndrome.[2,3] In humans, NAFLD currently constitutes the most prevalent liver disease in the industrialized world, surveys estimating as much as 20% of the population to be affected and encompassing both obese and nonobese individuals.[4,5] Although early stages of NAFLD comprise the reversible changes of simple steatosis, the disease may progress to include nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma imposing irreversible damage and ultimately liver failure.[4,6]

The mechanisms governing the accumulation of TGs in hepatic cells include changes in hepatocellular metabolism,[7-10] propagating an imbalance between uptake and de novo fatty acid synthesis, VLDL formation and subsequent export, and oxidation capacity.[3,11] The resulting hepatic TG accumulation leads to the imbalance in redox homeostasis and increased free radicals with negative consequences for hepatocytes including mitochondrial dysfunction.[12,13] Hepatocellular distress in turn induces the release of inflammatory cytokines and eventually also fibrosis. A reduced insulin response disturbs hepatic autoregulation increasing endogenous glucose production from fatty acids and depleting glycogen reserves.[14,15] In addition, insulin resistance in adipocytes may increase free fatty acid influx to the liver enhancing NAFLD and subsequent hepatocellular damage potentially developing into NASH.[11,16] Hyperplasia and hypertrophy of adipocytes are followed by macrophage activation and infiltration, reprogramming of adipocytes (adipocyte dysfunction) leading to a deviated secretion of anti-inflammatory cytokines and adiponectin, as well as systemic redox imbalance and low-grade inflammation.[5,17,18] This creates a self-propagating viscous circle in which cellular metabolism is distorted and fueled by an excess caloric intake.[16,19] Apart from the association between dyslipidemia and NAFLD induced in experimental models by high-fat meals,[20-22] the composition of dietary fatty acids and the intake of refined carbohydrates (such as fructose) have been suggested as comorbidity in NAFLD disease development.[11,23,24]

Using guinea pigs as animal models of diet-induced dyslipidemia, we investigated the effects of 2 high-fat Westernized diets (0.35% cholesterol, 18.5% vegetable oil and either 15% sucrose [HF diet] or 20% sucrose [HFS diet]) compared with an isocaloric control standard chow diet (CTRL). We hypothesize that high-fat feeding would induce NAFLD in guinea pigs and that a 5% increase in sucrose would accelerate disease progression.

MATERIALS AND METHODS

Animals. Animals were treated in accordance with the Animal Experimentation Act of Denmark, which is in accordance with the Council of Europe Convention ETS 123. The study was approved by the National Animal Experimentation Board.

Twenty-one female Hartley guinea pigs (aged 12 weeks; Charles River Laboratories, Kisslegg, Germany) were tagged with a 12-mm microchip subcutaneously in the neck for identification (Pet ID, West

Sussex, United Kingdom) on arrival. Animals were randomized into 3 weight-stratified groups and allowed 2 weeks of acclimatization on a standard guinea pig chow diet (ssniff Spezialdiäten, Soest, Germany). The guinea pigs were group housed in floor pens with wood shavings, straw and hay, and ad libitum access to food pellets and water. All pens were equipped with a heating lamp, shelters, and environmental enrichment. The room temperature was kept constant between 20°C and 24°C, and a 12:12-hour dark–light cycle was maintained. From the age of 14 weeks, the groups (n = 7 per group) were placed on 1 of the 3 experimental diets, CTRL, HF, and HFS (ssniff Spezialdiäten; Table I), for a total of 16 weeks, animals reaching the age of 30 weeks at euthanasia. Diets were kept at −20°C for storage and freshly thawed twice a week. To evaluate potential differences in lipid oxidation between diets, the malondialdehyde (MDA) content in samples from all 3 diets was compared at study termination (protocol as described subsequently for tissue samples).

Animals were inspected daily by animal caretakers and body weight (BW) recorded weekly. At no time did any of the animals display clinical signs of disease or malnutrition. One animal (HF group) was found dead in the morning after dual-energy x-ray absorptiometry (DEXA) scan (Lunar Prodigy DEXA scanner; GE Healthcare, Brondby, Denmark) and associated blood sampling from the jugular vein at T = 16 weeks. Postmortem necropsy revealed perivenous bleeding around the right jugular vein and pericardial blood effusion. The death was consequently attributed as iatrogenic in connection to blood sampling.

Measurement of body composition by DEXA. DEXA scans (Lunar Prodigy DEXA scanner [GE Healthcare] and associated software enCORE version 14.10) were performed on all animals immediately before study start and shortly before study termination. Animals were anesthetized by 0.08 mL/kg BW of "Zoletil mixture" (125 mg of tiletamine [0.93 mg/kg] and 125 mg of zolazepam [0.93 mg/kg, Zoletil 50; Virbac Laboratories, Carros, France] + 200 mg of xylazine [1.49 mg/kg, Narcoxyl vet 20 mg/mL; Intervet International, Boxmeer, Holland, the Netherlands] + 7.5 mg of butorphanol [0.06 mg/kg; Torbugesic vet 10 mg/mL; ScanVet, Fredensborg, Denmark]) diluted 1:10 in isotonic saline and injected subcutaneously. After 15 minutes rest until anesthetic effect, guinea pigs were placed in sternal recumbency, limbs stretched away from the body. The scan was performed in a cranial to caudal direction including the entire body. Animals were scanned in a randomized order, and the scanner was calibrated according to the manufacturer's instructions and quality control software before each scanning session.

Table I. Diet composition

Contents	CTRL	HF	HFS
Alfalfa (%)	22.0	22.0	22.0
Wheat (%)	28.27	10.25	8.25
Barley (%)	18.00	—	—
Sucrose/fructose (%)	—	15.02	20.0
Cellulose (lignocellulose; %)	4.6	4.6	5.6
Sunflower meal (%)	3.0	3.0	3.0
Soybean meal (%)	12.0	6.0	4.2
Soybeans (full fat; %)	2.6	2.6	2.7
Soybean concentrate (%)	2.0	12.0	—
Soybean isolate (90% protein; %)	—	—	9.77
Amino acids (%)	0.5	0.5	0.55
Vitamins and trace element (%)	1.0	1.0	1.0
Vitamin C (stay-C; %)	0.15	0.15	0.15
Minerals (%)	2.49	2.74	2.64
Choline Cl (%)	0.3	0.3	0.3
Sugar beet pulp (%)	1.0	1.0	1.0
Cholesterol (%)	—	0.35	0.35
Coconut oil, hydrogenated (%)	—	18.0	18.0
Soybean oil (%)	2.1	0.5	0.5
Crude protein (%)	16.8	16.7	16.8
Crude fat (%)	4.2	20.0	19.9
Crude fiber (%)	12.6	11.4	11.5
Crude ash (%)	6.5	6.6	6.5
Starch (%)	27.9	7.7	6.0
Sugar (%)	3.8	17.6	22.0
Carbohydrates (%)	47.1	37.9	38.9

Abbreviations: CTRL, control; *HF,* high fat; *HFS,* high fat + 5% sucrose.

Oral glucose tolerance testing. The oral glucose tolerance tests (OGTTs) were carried out at T = 0, 8, and 16 weeks. The OGTT was performed at 8:00 AM in semifasted guinea pigs, that is, test diet (chow) was removed from the pens 12 hours before the OGTT, but animals had free access to hay and water. An oral glucose load of 1 g/kg BW (T = 0) and 3 g/kg BW (T = 8 and 16 weeks; glucose monohydrate 500 mg/mL; Trekroner Apotek, Valby, Denmark) was given by syringe into the mouth of each guinea pig. Blood samples for glucose measurement were collected from the saphenous vein or by puncturing the ear vein at T = −5, 15, 30, 60, 120, and 180 minutes and analyzed using an Accu-Chek Glucometer (Roche A/S Diagnostics, Hvidovre, Denmark) immediately after sampling.[25]

Blood sampling and analysis. Venous blood was collected from the jugular vein in semifasted anesthetized (0.08 mL/kg BW of Zoletil mixture as previously specified) guinea pigs at T = 0, 8, and 16 weeks for measurement of lipids, liver enzymes, and systemic oxidative stress markers.[25] A sample of 300 μL of heparin-stabilized blood was immediately transferred to an Eppendorf tube with 12 μL of cold 0.1% dithioerythritol for tetrahydrobiopterin (BH_4) and

dihydrobiopterin (BH_2) measurement and centrifuged (16.000 × *g* for 1 minute at 4°C). Centrifugation of the remaining heparin-stabilized blood was continued (3.500 × *g* for 5 minutes at 4°C) after which plasma was collected for MDA, lipid, and liver enzyme analysis. Plasma was stored at −80°C until further analysis.

Euthanasia and tissue collection. At study termination, guinea pigs were anesthetized with 0.08 mL/kg BW of Zoletil mixture as previously described. After 15–20 minutes, animals were placed on inhalation anesthesia (4%–5% isoflurane [Isoba vet 100%; Intervet International]). At the disappearance of reflexes, the thoracic cavity was opened and intracardial blood sampling was performed through the left ventricle by an 18G needle and 5-mL syringe previously flushed with 5% EDTA. Once blood sampling was completed, the right ventricle was incised and manual transcardiac perfusion was achieved by injecting cold physiological saline (5 × 10 mL) into the left ventricle, hereby perfusing the still-beating heart and aorta and euthanizing the animal by exsanguination. Organs were rapidly collected and stored in either fixative (10% formalin) or at −80°C until further analysis. Subcutaneous fat pad in the neck region was measured using a ruler, and sections of the left inguinal fat pad were collected and stored at −80°C until analysis. The heart and aorta were sprinkled with ice-cold saline and carefully dissected before the removal and subsequent fixation or staining.

Analysis of plasma samples. Analysis of repeated (baseline, 8 and 16 weeks on diet) total cholesterol (TC) and TG was performed on a Horiba ABX Pentra 400 Chemistry Analyzer (HORIBA, Irvine, Calif) with ABX Pentra 400 reagents (Triolab A/S, Brondby, Denmark). Quantification of TC and plasma lipid protein fractions (VLDL, LDL, and HDL) at euthanasia was achieved by enzymatic assay followed by chromatography as described previously.[26]

Oxidized low-density lipoprotein (ox-LDL) was analyzed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Oxidized LDL Competitive ELISA #10-1158-01; Mercodia AB, Uppsala, Sweden). Analysis of liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) was performed using ADVIA Chemistry systems (Siemens Healthcare Diagnostics Inc, NY). Biochemical analysis of BH_2 to BH_4 ratio and MDA was performed by high-performance liquid chromatography (HPLC) with colorimetric detection as previously described.[27-29] Level of 8-F2-isoprostanes was assessed by ELISA according to the manufacturer's instructions (cat no. 516351; Cayman Chemicals, Ann Arbor, Mich). Analysis of asymmetric dimethylarginine (ADMA) and L-arginine (L-Arg) was performed by HPLC as previously outlined,[30,31] in short 50 μL of plasma was mixed with acetonitrile + 0.01 M of perchloric acid (100% vol/vol) before centrifuging (16.000 × *g* for 10 minutes at 4°C). Finally, 10 mg of Isolute C18 (E)[a] was added to the extracted supernatant before HPLC analysis.

Unless otherwise stated, samples were run in a blinded randomized fashion, in duplicates or more, together with standardized controls.

Analysis of tissue samples. Tissue sections were derived from frozen (−80°C) segments to meet sample size requirements, before homogenization in phosphate buffered saline (Dulbeccos, pH 7.4, 1:10) at 4°C. Samples for HPLC analyses of vitamin C (vitC) and dehydroascorbic acid were stabilized in metaphosphoric acid and subsequently processed as previously described.[32-34] Analysis by HPLC of MDA (including assessment of MDA in feed), glutathione (GSH), oxidized glutathione (GSSG), ADMA, and L-Arg was performed as previously described.[27,28,31,32,34,35] Superoxide dismutase (SOD) levels were measured by the Ransod colorimetric assay (SD125; Randox Laboratories Ltd, Crumlin, United Kingdom) according to the manufacturer's instructions.

Quantification of TC, TG, free glucose, and glycogen in the liver was achieved by an analysis of homogenate on Cobas c 501 (Roche Diagnostic Systems, Bern, Switzerland), in short approximately 30 mg of liver tissue was homogenized by steel-bead tissue lysing (TissueLyser II; Qiagen, Hilden, Germany) in 1.0 mL of extraction buffer (0.15 M of sodium acetate supplemented with 0.75% Triton X). Samples were subsequently placed in 100°C water bath for 2 minutes and placed on ice before 0.5 mL of extraction buffer was added. For measurements of lipids, cholesterol, and free glucose, 500 μL of the sample was centrifuged for 10 minutes (9000 RPM) and 100 μL of the supernatant transferred to Cobas cups before analysis in the Cobas system. For measurement of total glucose, 20 μL of amyloglucosidase was added to 400 μL of homogenate sample and left at room temperature overnight. Sample was then centrifuged (9000 RPM, 10 minutes) and 100 μL of the supernatant transferred to Cobas cups. Analysis was performed on single samples. Glycogen concentration was calculated by subtracting free glucose levels from total glucose levels.

Adiponectin expression was assessed in inguinal fat samples. Samples were briefly thawed and approximately 50 mg was sectioned. Protein extraction was performed as previously described.[36] Total protein

content was determined by bicinchoninic acid Protein Assay kit (number 71285-3) according to the manufacturer's instructions (Novagen, San Diego, Calif). Protein samples were subsequently analyzed in triplicates for adiponectin expression by ELISA (Human Adiponectin/Acrp30 ELISA kit #ELH-Adiponectin; RayBiotech Inc, Norcross, Geor) according to the manufacturer's instructions.

Unless otherwise stated, tissue samples were analyzed in a blinded randomized fashion in duplicates or triplicates and together with standardized controls.

Histology (liver and spleen). After 48 hours in 10% formalin, the tissue samples were processed conventionally and embedded in paraffin. Samples were cut into 2–4 μm sections for routine histology (Mayer's hematoxylin and eosin[H&E]). Tissue sections from left lateral liver lobe (lobus hepatis sinister lateralis) and right medial liver lobe (lobus hepatis dexter medialis) from all guinea pigs (n = 20) were used to assess and describe hepatic morphology. Scoring of hepatic steatosis and intralobular inflammation was assessed using a semiquantitative scoring system (0 = none; 1 = mild; 2 = moderate; and 3 = severe) and according to the previously published guidelines.[37,38] As no difference between scorings of segments from the left lateral and right medial liver lobe could be detected, only data from the left lateral liver lobe are included in the Results section.

To confirm the presence of intracellular lipid deposition in hepatocytes, oil red O staining was performed on cryosections of formalin-fixed tissue (left lateral liver lobe), from 2 randomly selected animals from each group. Sections were incubated 15 minutes in oil red O solution (1% oil red O in 99% isopropanol, supplemented with 1% dextrin solution), rinsed in 70% ethanol and water, followed by H&E staining before mounting on glycerol-coated coverslips.

To confirm that the findings from H&E-stained sections were indeed fibrosis, Masson's trichrome staining was performed on sections from the left lateral liver lobe of 2 randomly chosen animals from each group. Sections were deparaffinized before postfixation in Bouin's solution (Bie & Berntsen/VWR, Søborg, Denmark), washed in water, stained for 10 minutes in Weigert's iron hematoxylin before another wash step followed by 2 minutes staining in Biebrich Scarlet–acid fuchsin, then washed and incubated 10–15 minutes in phosphorus molybdenum wolfram acid, followed by 5 minutes staining in Masson's methyl blue. Sections were washed in water and acetic acid before dehydration in ethanol and mounting with coverslips by xylene.

To evaluate the findings of splenomegaly in HF animals further, 2 transverse sections (2–4 μm) from the spleen from the CTRL (n = 7) and HF (n = 6) were included for morphologic evaluation (H&E staining). Furthermore, staining with Perl solution for confirmation of hemosiderin deposition was performed on a single section, initial deparaffinization, followed by 5 minutes incubation in potassium ferrocyanide, after which a 10% HCl solution was added and left for 20 minutes of incubation. The section was washed in distilled water and counterstained with neutral red before dehydration in ethanol and mounting on coverslips with xylene.

All histologic evaluations were performed on sections blinded to the observer during data collection. The weighted kappa coefficients for the included scorings were all >0.90, confirming scoring integrity and reproducibility.

Assessment of aortic atherosclerotic plaques. The entire length of the aorta was dissected until the iliac bifurcation. The aorta was trimmed of adventitial fat under a dissecting microscope, opened and pinned in a normal anatomic position on a plastic plate, and placed in 4% formalin. After 4–6 days in fixative, the tissue was stained with filtered Sudan IV for 5 minutes, followed by a 90-second rinse in 96% ethanol. Sudan IV was prepared the day before by dissolving 5 g of Sudan IV (number A12181.22, VWR, Søborg, Denmark) into 1 L of 96% ethanol overnight. Directly after staining, the aorta was scanned using an Epson Perfection V600 Photoscanner. Images were imported as TIFF format, and % luminal surface area covered by lipid-filled Sudan IV–stained deposits was evaluated.

After scanning, the tissue was returned to formalin fixative. The next day, 2 animals from each group (n = 2) were randomly selected, and sections from 4 different regions of the thoracic aorta were embedded in paraffin, sectioned, and H&E stained before histologic evaluation.

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 5.01 and version 6.03 (GraphPad software, San Diego, Calif). One-way analysis of variance (ANOVA) and Tukey's post hoc test were used to test differences between groups. In the event of inhomogeneity of variances, data were transformed to the natural logarithm. If inhomogeneity in variances persisted, the Kruskal-Wallis test with the Dunn multiple comparisons test was used. For samples taken over time, 2-way ANOVA for repeated samples with Bonferroni's post hoc test for multiple comparisons was calculated. Histologic scorings were evaluated by the Kruskal-Wallis test with the Dunn multiple comparisons test. Results are presented as the mean ± standard deviation. P values <0.05 were considered statistically significant.

Table II. Body composition and adipose tissue at study termination

Body fat	CTRL (n = 7)	HF (n = 6–7)	HFS (n = 7)
Body weight (g)	944 ± 77.8	890.4 ± 30.6	920.3 ± 73.4
Tissue fat percentage	28.10 ± 1.68	22.40 ± 1.43*	23.03 ± 2.43*
Fat (g)	255.4 ± 31.61	190.3 ± 16.75*	200.3 ± 28.18†
Fat free (g)	674.1 ± 38.68	678.1 ± 24.38	688.6 ± 51.61
Neck fat pad (cm)	1.37 ± 0.20	1.23 ± 0.12	1.23 ± 0.05
Adiponectin (pg/mL)	6643 ± 2194	8006 ± 1550	13,658 ± 6185‡

Abbreviations: CTRL, control; *DEXA*, dual-energy x-ray absorptiometry; *HF*, high fat; *HFS*, high fat + 5% sucrose.
Data are presented as the mean ± standard deviation. Tissue fat percentage, fat, and fat-free content were assessed by DEXA scan. Adiponectin expression was measured in inguinal fat pad.
*$P < 0.001$ compared with CTRL.
†$P < 0.01$ compared with CTRL.
‡$P < 0.05$ compared with CTRL.

RESULTS

Body composition. Baseline values of BW, fat-free mass, total fat, and tissue fat percentage (%) assessed by DEXA scanning were comparable between groups at study start. BWs were not different between groups during the study or at euthanasia (see Table II, Fig 1); however, a significantly reduced tissue fat % in HF and HFS groups compared with CTRL was observed ($P < 0.001$). Nonfat tissues were equal between groups. Measurement of the subcutaneous neck fat pad at euthanasia indicated an increase in CTRL animals compared with HF and HFS albeit this did not reach significance (Table II). Adiponectin levels in inguinal fat were increased in HFS animals compared with CTRL and HF counterparts ($P < 0.05$; correcting for total protein did not affect the recorded significance, data not shown).

Plasma and vascular system. All groups were comparable at baseline with regards to TC and TG. After 8 weeks on the diet, TG decreased in HF and HFS compared with CTRL ($P < 0.01$ and $P < 0.05$, respectively), but this significance was only maintained for HFS animals ($P < 0.05$) at study termination (CTRL, 0.90 ± 0.37 mmol/L; HF, 0.59 ± 0.25 mmol/L; and HFS, 0.52 ± 0.07 mmol/L). All groups displayed a decrease in TG from 8 weeks compared with 16-week values (Fig 2) confirming an effect of both diet and time on TG plasma levels ($P < 0.001$).

In samples from fasted animals taken at the 8- and 16-week time points, TC was consistently higher in HF and HFS compared with CTRL ($P < 0.005$ [HF] and $P < 0.001$ [HFS]). However, both groups displayed a peak at the age of 22 weeks (8 weeks on diet) and then declined significantly at the age of 30 weeks ($P < 0.001$ in both cases).

At euthanasia, plasma samples (nonfasted) showed an increase in TC in HF and HFS animals compared with CTRL ($P < 0.01$). In addition, VLDL, LDL, and HDL were increased in HF and HFS animals ($P < 0.001$, $P < 0.01$, and $P < 0.001$, respectively; Table III) compared with CTRLs. Reviewing the distribution of the measured lipoproteins at euthanasia as a percentage of TC in plasma, the LDL fraction was significantly increased in both HF and HFS ($P < 0.001$), whereas VLDL and HDL were decreased ($P < 0.05$ and $P < 0.001$, respectively) compared with CTRL animals (Fig 2).

Response to OGTT was uniform between groups before study start, and additional OGTT trials at the age of 22 and 30 weeks did not reveal differences between groups (1-way ANOVA of area under the curve; data not shown).

Euthanasia samples showed an increase in ox-LDL in HFS animals ($P < 0.001$) and an increase in MDA in HFS compared with CTRL and HF ($P < 0.001$ and 0.05, respectively; Table III). Analysis of the MDA content of the feed revealed a higher amount in the control diet compared with high-fat diets ($P < 0.001$). To account for this difference, values normalized to the MDA content of the feed are also presented and resulted in significant higher plasma MDA of both HF and HFS groups compared with CTRLs ($P < 0.001$). Isoprostanes (8-F_2-isoprostanes) were increased in HF and HFS groups, although levels only reached significance in HF animals ($P < 0.001$). To assess imbalances in nitric oxide (NO)-mediated control of vasodilation, BH_4 and its oxidized counterpart BH_2 and also ADMA and L-Arg were measured. No difference was found in the BH_2 to BH_4 ratio between groups (Table III); however, both BH_4 and BH_2 were increased in HF and HFS animals compared with CTRLs ($P < 0.05$ or less; data not shown). ADMA was significantly increased in HF ($P < 0.05$ compared with CTRL and HFS), as was L-Arg ($P < 0.05$ and $P < 0.01$ compared with CTRL and HFS, respectively; Table III). Albeit reduced in the HF group (mean ± standard deviation, 0.358 ± 0.60) compared with CTRL and HFS (0.48 ± 0.15, 0.48 ± 0.06, respectively), the L-Arg to ADMA ratios were not statistically different.

Morphometric evaluation by Sudan IV en face[39] detection of excess lipid deposition within the thoracoabdominal aortic wall did not reveal differences between groups. Likewise, no atherosclerotic lesions were observed by light microscopy of H&E-stained sections derived from 3 segments of the aortic arch from a subset of animals from each group (n = 2). As

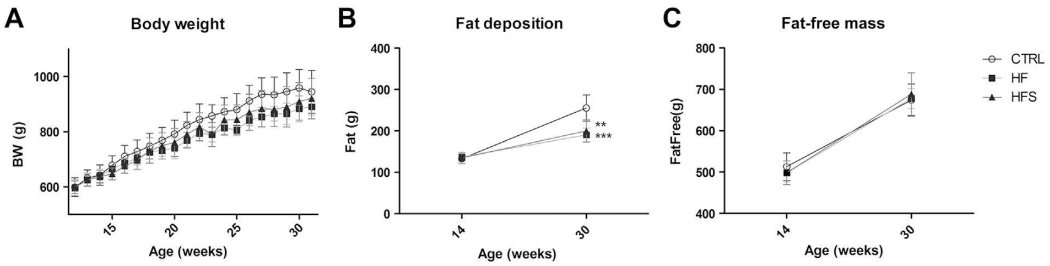

Fig 1. Body composition over time. (**A**) Body weights between groups were not different during the 16 weeks of dietary regimens. (**B** and **C**) In vivo DEXA scanning revealed no differences between groups in either fat content or nonfat tissue at study start. At study termination (at the age of 30 weeks; 16 weeks on the diet), control animals (CTRL) displayed increased fat content compared to high fat (HF) and high fat + 5% sucrose (HFS) groups. No difference was detected in nonfat tissue content between groups at any of the investigated time points. Data are presented as the mean ± standard deviation. $^{**}P < 0.01$; $^{***}P < 0.001$. BW, body weight; DEXA, dual-energy x-ray absorptiometry.

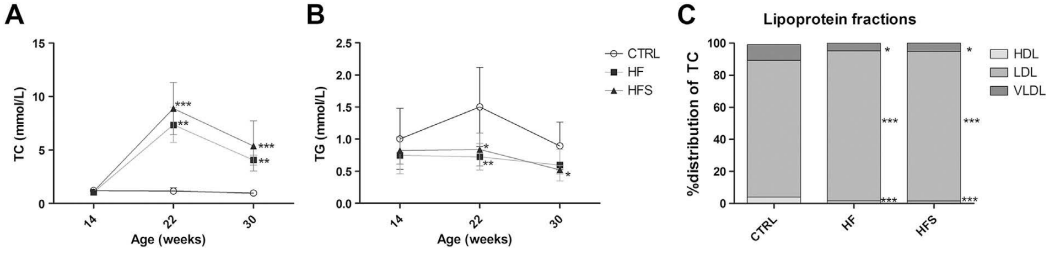

Fig 2. Progression of plasma dyslipidemia. No differences were recorded in TC and TG plasma concentrations between groups at study start. (**A**) A peak in TC was observed in HF and HFS after 8 weeks on the diet followed by a decline but maintaining statistically significant increase compared with control (CTRL) counterparts. (**B**) CTRL animals displayed an increase in TG after 8 weeks of diet; however, this declined over time and only reached significance in HFS animals at the end of the study period (at the age of 30 weeks, 16 weeks on the diet). (**C**) Lipoprotein fractions of TC in plasma at euthanasia showed an increase in LDL together with decreases in VLDL and HDL for HF and HFS groups. Data in (**A**) and (**B**) are presented as the mean ± standard deviation. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. HDL, high-density lipoprotein; HF, high fat; HFS, high fat + 5% sucrose; LDL, low-density lipoprotein; TC, total cholesterol; TG, triglyceride; VLDL, very low-density lipoprotein.

both histologic methods were in agreement and consistent in not showing lesions in either of the dietary groups, no further morphologic evaluation of aortic samples was performed.

Liver. Liver weight was significantly increased ($P < 0.001$) in HF and HFS compared with CTRL in accordance with the macroscopic appearance of hepatomegaly. Hepatic TC and TG were increased 5- to10-fold, respectively, ($P < 0.001$) in HF and HFS animals. Glycogen content was unaffected by the dietary regime.

Lipid oxidation was higher in CTRL livers compared with those of high-fat fed animals, but when normalized to MDA content in feed, increased MDA in HF and HFS groups vs CTRL was observed ($P < 0.001$). SOD activity was decreased in HF and HFS groups compared with CTRL ($P < 0.001$). The concentration of vitC was decreased by about 40% in HF and HFS ($P < 0.001$), whereas the proportion of the oxidized form, dehydroascorbic acid, was increased 4- to 5-fold in high-fat fed animals compared with CTRLs ($P < 0.05$). However, no effect on GSH, GSSG, or the GSSG to GSH ratio was recorded. As summarized in Table IV, the findings collectively indicate the presence of cellular redox imbalance and oxidative stress. ADMA levels were increased in both HF and HFS groups ($P < 0.01$ and $P < 0.05$, respectively), accompanied by an increase in L-Arg in HF animals ($P < 0.05$; Table IV). As for plasma, the L-Arg to ADMA ratio did not differ between groups.

Plasma markers of liver function AST and ALT were increased in HF and HFS groups during the study period

Table III. Plasma values at study termination

Plasma	CTRL (n = 6–7)	HF (n = 5–6)	HFS (n = 6–7)
TG (mmol/L)	0.90 ± 0.37	0.59 ± 0.25	0.52 ± 0.07*
TC (mmol/L)	0.99 ± 0.26	4.06 ± 0.47*	5.38 ± 2.36†
HDL (mg/dL)	1.29 ± 0.20	5.08 ± 1.09‡	4.81 ± 1.10‡
LDL (mg/dL)	28.84 ± 7.33	286.2 ± 17.07†	304.3 ± 90.30†
VLDL (mg/dL)	3.40 ± 1.89	14.97 ± 4.63‡	18.33 ± 16.04‡
Ox-LDL (U/L)	0.51 ± 0.07	0.65 ± 0.09	0.89 ± 0.13‡
MDA (μM)	0.26 ± 0.10[a]	0.31 ± 0.03[a]	0.36 ± 0.10‡,b
MDAnorm	0.015 ± 0.006	0.059 ± 0.005‡	0.081 ± 0.023‡
BH_2 to BH_4 ratio	0.14 ± 0.06	0.14 ± 0.03	0.15 ± 0.03
8-F_2-Isoprostanes (pg/mL)	58.94 ± 17.13	184.66 ± 112.22‡	111.90 ± 23.41
ADMA (μM)	16.48 ± 7.16	26.33 ± 9.82*,b	14.67 ± 2.85[a]
L-Arg (μM)	1.96 ± 0.08[a]	2,19 ± 0.22*,bb	1.93 ± 0.08[a]

Abbreviations: ADMA, asymmetric dimethylarginine; *BH_2*, dihydrobiopterin; *BH_4*, tetrahydrobiopterin; *CTRL*, control; *HDL*, high-density lipoprotein; *HF*, high fat; *HFS*, high fat + 5% sucrose; *L-Arg*, L-arginine; *LDL*, low-density lipoprotein; *MDA*, malondialdehyde; *MDAnorm*, MDA normalized to MDA content in feed; *TC*, total cholesterol; *TG*, triglyceride; *VLDL*, very low-density lipoprotein.
Data are presented as the mean ± standard deviation.
Different superscript letters (a and b) refer to differences between groups.
*$P < 0.05$ compared with CTRL.
†$P < 0.01$ compared with CTRL.
‡$P < 0.001$ compared with CTRL.

Table IV. Markers of liver status at study termination

Liver	CTRL (n = 6–7)	HF (n = 5–6)	HFS (n = 6–7)
Weight (g)	24.93 ± 3.60	43.50 ± 5.50*	47.0 ± 5.10*
TG (μmol/g)	4.70 ± 1.47	49.71 ± 6.78*	48.41 ± 6.23*
TC (μmol/g)	6.05 ± 0.68	32.11 ± 5.90*	30.04 ± 3.86*
Glycogen (μmol/g)	197.3 ± 139.2	142.4 ± 76.88	167.2 ± 63.29
MDAnorm†	6.19 ± 1.27	11.94 ± 2.46*	13.18 ± 2.66*
SOD (μmol/mg)†	4455.05 ± 702.60	2564.89 ± 420.80*	2609.91 ± 236.37*
GSSG/GSH	0.074 ± 0.009	0.072 ± 0.014	0.078 ± 0.017
VitC (nmol/g)	1238.81 ± 186.50	667.91 ± 79.77*	767.75 ± 108.50*
%DHA	0.83 ± 2.50	3.85 ± 2.17‡	4.45 ± 1.36‡
L-Arg (nmol/mg)†	0.51 ± 0.04	1.02 ± 0.20§	0.81 ± 0.19
ADMA (nmol/mg)†	0.92 ± 0.37	1.58 ± 0.1.90§	1.40 ± 0.36‡
ALT (U/L)	41.1 ± 7.69	106.9 ± 42.94*	82.14 ± 16.82‡
AST (U/L)	71.14 ± 44.68	471.0 ± 247.10*	314.3 ± 114.70*

Abbreviations: ADMA, asymmetric dimethylarginine; *ALT*, alanine aminotransferase; *AST*, aspartate aminotransferase; *CTRL*, control; *%DHA*, Per cent dehydroascorbate of total VitC; *GSH*, glutathione; *GSSG*, oxidized glutathione; *HF*, high fat; *HFS*, high fat + 5% sucrose; *L-Arg*, L-arginine; *MDA*, malondialdehyde; *MDAnorm*, MDA concentration normalized to MDA in feed; *SOD*, superoxide dismutase; *TC*, total cholesterol; *TG*, triglyceride; *VitC*, vitamin C.
Data are presented as the mean ± standard deviation.
*$P < 0.001$ compared with CTRL.
†Corrected to total protein.
‡$P < 0.05$ compared with CTRL.
§$P < 0.01$ compared with CTRL.

(Fig 3, Table IV); AST to ALT ratios increased accordingly in HF and HFS (4.43 ± 1.04 and 3.78 ± 0.89, respectively; $P < 0.001$ compared with CTRL at euthanasia). Liver weight was correlated to AST and ALT values at euthanasia (AST, Pearson's coefficient [rho] = 0.72 [$P < 0.001$] and ALT, rho = 0.77 [$P < 0.0001$]). ALP displayed a time-dependent decline for all groups ($P < 0.001$); however, this decrease was larger in HF ($P < 0.005$) and HFS ($P < 0.05$) compared with CTRL. ALP values at euthanasia were weakly inversely correlated to liver weight (rho = −0.62; $P < 0.01$).

Blinded macroscopic inspection of livers revealed CTRL livers of normal color and appearance, whereas both HF and HFS animals displayed enlarged livers much lighter in color with rounded edges and enlarged lobules consistent with a disseminated lipid deposition (Fig 3). Oil red O staining confirmed excessive lipid

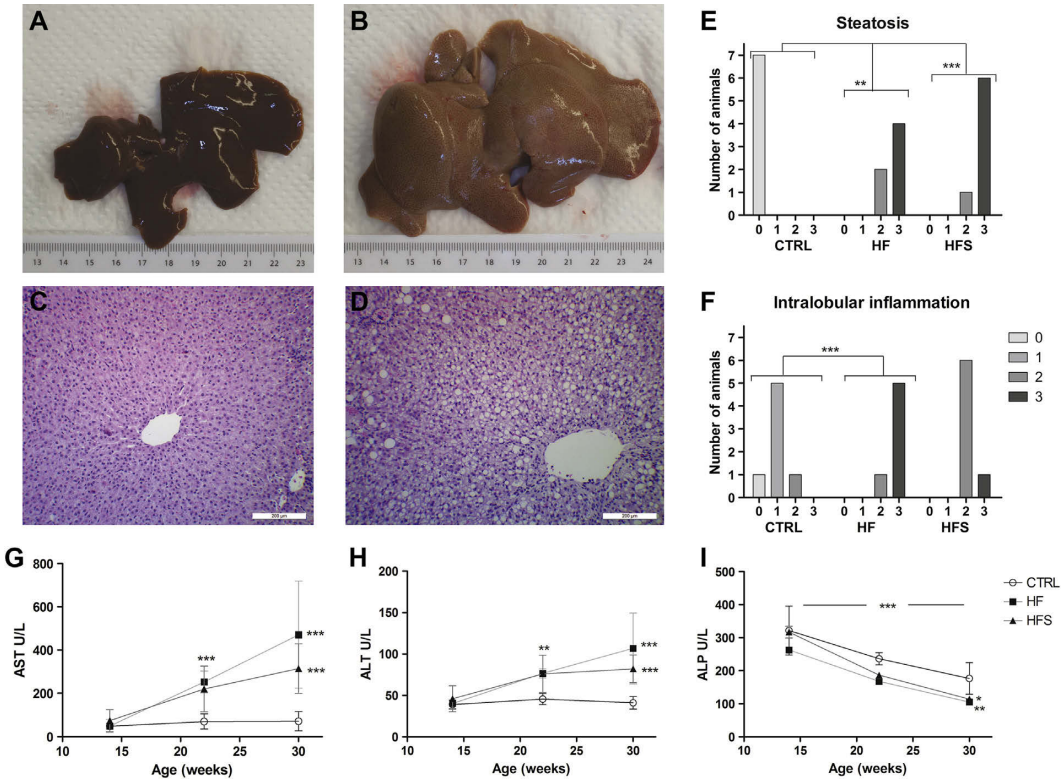

Fig 3. Evaluation of liver damage between dietary groups. Macroscopic appearance of livers representative of control (CTRL) (**A**) and high-fat fed (**B**) animals. The increase in size in both high fat (HF) and high fat + 5% sucrose (HFS) combined with alterations in color and texture indicated disseminated hepatic lipid deposition. Steatosis was confirmed by histologic evaluation of liver sections (**C** and **D**) showing extensive hepatocellular accumulation of lipid vesicles in HF and HFS groups. Semiquantitative scorings of liver sections (**E** and **F**) revealed steatosis and increased frequency of intralobular inflammation in agreement with NAFLD development in HF and HFS animals and indicating propagation toward NASH. (**G** and **H**) AST levels and ALT in plasma reflected a reduced liver function in HF and HFS groups, whereas ALP (**I**) displayed a time-dependent decrease but no effect of diet. Data in (**G**), (**H**), and (**I**) are presented as the mean ± standard deviation. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

deposition in hepatocytes from HF and HFS groups compared with negative findings in CTRL animals. Semiquantitative scoring according to the guidelines of the NASH clinical network scoring system[37,38] revealed steatosis in both high-fat fed groups, HF animals (n = 6) with 33% presenting grade 2 (>33%–66% lipid infiltration) and 66% presenting grade 3 (>66% lipid infiltration), whereas in HFS animals (n = 7) 14% presented grade 2 and 86% presented grade 3 (Fig 3). The lipid accumulation displayed a lobular pattern with macro- and microvesicular steatosis present in zones 2 and 3, whereas zone 1 displayed fewer macrovesicular vacuoles. Scoring of intralobular inflammation disclosed the CTRL group as displaying 14% grade 0 (no foci), 72% grade 1 (<2 foci), and 14% grade 2 (2–4 foci). HF displayed 17% grade 2 and 83% grade 3 (>4 foci), whereas in the HFS group 86% showed grade 2 and the remaining 14% grade 3 (Fig 3). Fibrosis was assessed in a subset (n = 2 per group), revealing none or mild collagen deposition surrounding only a few central or portal veins in CTRL, whereas collagen depositions surrounding the central vein or pericentral area and extending perisinusoidally were observed in HF and HFS groups (formation of

bridging fibrosis was observed in 1 HF and both HFS animals) indicating progression from NAFLD toward NASH and cirrhosis.

Spleen. Enlarged spleen was recorded for HF animals (mean, 2.20 ± 0.65 g) compared with HFS (1.46 ± 0.39 g) and CTRL (1.02 ± 0.27 g; $P < 0.001$ and $P < 0.05$, respectively). Macroscopic appearance did not reflect signs of pathologic irregularities besides enlargement, the spleen appearing of normal color and texture. Histologic findings in CTRL and HF animals revealed normal anatomic architecture of CTRL, whereas increased cellular density with central accumulations of erythrocytes was recorded for the HF group. The presence of disseminated hemosiderin depositions within the spleen was recorded for both groups.

DISCUSSION

The present study demonstrates the development of NAFLD in the dyslipidemic guinea pig model, affecting macroscopic, histopathologic, and biochemical parameters. Increased overall fat deposition and obesity was not present, and although biochemical markers suggest systemic alterations in redox balance and NO homeostasis, progression of vascular disease was not supported by histologic finding in the aortic wall. Albeit increasing levels of plasma ox-LDL and adiponectin expression in adipose tissue, the 5% increase in sucrose of the HFS diet could not be attributed to lead to a significant change compared with the HF diet.

The macroscopic appearance of excess lipid deposition in the liver was confirmed by histopathologic findings of steatosis in HF and HFS animals compared with CTRL, hall-marking NAFLD, and with the emergence of inflammation and fibrosis also the progression to NASH.[3,5,40] The excess hepatocellular storage of lipids and increased lipolysis results in the diffusion of lipid-peroxidation products to the extracellular space promoting oxidative damage to liver cells, including Kupffer and stellate cells subsequently initiating the inflammatory response and developing fibrosis.[41,42] The enlarged livers in HF and HFS animals correlated with increases in AST and ALT demonstrating a negative effect on liver function, despite the absence of clinical symptoms. The reduction in ALP in all 3 groups illustrates a significant time dependency and an effect of diet, as levels in HF and HFS groups were lower compared with CTRLs. The ALP decrease in coherence with increases in ALT and AST indicates primary damage to parenchyma liver cells, although an intact biliary system was maintained, as has been reported for experimental models of NAFLD/NASH and in human patients.[43-45] The development of splenomegaly as a result of high-fat diet and NAFLD has been reported in humans, rats, and guinea pigs in agreement with our observations of splenomegaly in HF animals.[40,46-48] Portal hypertension and stasis have been suggested to cause the observed spleen enlargement, supported by our observation of increased erythrocytes in the spleen of HF animals.

The development and progression of NAFLD is associated with increased oxidative stress.[41] This is supported by the recorded vitC decrease and increased ascorbate oxidation ratio in HF and HFS animals and is in agreement with previous findings of high cholesterol–induced reduction of vitC levels in guinea pig tissues.[49] Moreover, low vitC status has been shown to decrease mitochondrial oxidation of fatty acids in hepatocytes by impairing carnitine synthesis, hereby promoting TG deposition.[50,51] We have previously reported decreased levels of GSH in the liver of guinea pigs subjected to severe diet-induced vitC deficiency.[52,53] In the present study, GSH levels and GSSG to GSH ratios did not differ between groups, suggesting that the redox imbalance induced by high-fat feeding does not exceed the capacity of the GSH antioxidant system.[52,53] It should be noted that the decreased vitC status may not exclusively be a result of increased oxidative stress but may also be attributed to additional factors including a reduced feed intake in HF and HFS groups vs CTRLs[40] and feed composition–induced differences in vitC bioavailability. Animals were group housed and it was not possible to monitor individual food intake. However, as BWs did not differ between groups, a decreased intake of the isocaloric diets in HF and HFS animals would be relatively small. Likewise, the equal levels of hepatic glycogen between groups do not support a state of generalized undernutrition in the high-fat fed groups. A disturbed redox balance is supported by increased MDA indicating damage to polyunsaturated lipids including in the mitochondrial membrane. Reduced mitochondrial DNA transcription and damage to the mitochondrial DNA as well as ultrastructural alterations and decreased function of the respiratory chain have been shown in liver steatosis,[54-57] linking mitochondrial dysfunction to NAFLD and NASH.[12,13] In murine models of SOD-deficiency (*Sod1* knock out and *Sod1+2* double knock out), increased hepatic lipid oxidation was observed alongside increased TG accumulation, degradation of apolipoprotein B, and reduced TG secretion.[58] The recorded decrease in SOD in HF and HFS groups could therefore be associated with a reduced export of TGs and subsequent accumulation in hepatocytes, directly promoting steatosis. Furthermore, decreases in SOD levels are likely to enhance the deleterious effects of cellular redox imbalance by impairing the cellular defense against superoxide

adding further insult to the stressed cell and mitochondria. Excess superoxide reacts efficiently with NO forming the deleterious species peroxynitrite, which may further exacerbate the negative consequences on hepatic circulation.[59,60]

The viscous circle accelerating oxidative stress is associated with hepatic endothelial dysfunction and compromised microcirculation.[61,62] ADMA, an N-methylated derivative of L-Arg formed during cellular protein degradation, decreases the bioavailability of NO by reducing the activity of NO synthase enzymes hereby fueling the progression of endothelial dysfunction.[63-65] The ADMA-metabolizing enzyme dimethylarginine dimethylaminohydrolase is decreased in hypertensive hepatocytes leading to increased ADMA levels.[59,66] The increased ADMA in liver and plasma of HF and HFS animals suggests a diet-induced detrimental effect on liver endothelial function as has been reported in humans and in experimental models.[59,61,65,67-69] Paradoxically, inflammation, NASH, and cirrhosis increase hepatic inducible nitric oxide synthase expression.[70-72] However, the synthesized NO may increase peroxynitrite formation in turn promoting nitration of cellular lipids and proteins.[73,74] Hepatic oxidative stress may further propagate endothelial dysfunction by oxidizing the endothelial nitric oxide synthase (eNOS) cofactor BH_4, a situation directly associated with eNOS uncoupling.[75,76] In agreement, supplemen-tation with BH_4 has been shown to improve liver function and reduce cellular lipid peroxidation in rats.[77,78] Several studies have shown that vitC is the primary reductant responsible for recycling BH_4 from BH_2,[79] and consequently, a reduction in vitC status could enhance hepatic endothelial dysfunction.

A consistent increase in TC and LDL in HF and HFS groups reflected dyslipidemia, with lipoprotein distribution showing higher LDL fraction and lower VLDL and HDL fractions in HF and HFS animals compared with CTRLs. Concentrations of TGs were below or equal to CTRLs in the high-fat diet groups. This corresponds to the increased liver levels in HF and HFS groups, supporting that TGs are accumulated in hepatocytes rather than entering the systemic circulation. When exposed to dietary cholesterol levels superseding endogenous cholesterol synthesis, guinea pigs—like humans—respond by decreasing cholesterol synthesis and increasing cholesterol metabolism hereby increasing hepatic TG deposition.[80-83] An increased plasma TC reduces LDL receptors hereby increasing plasma LDL concentrations,[82,84] supported by our findings of increases in TC and LDL. Absolute levels of HDL and VLDL were increased in HF and HFS, whereas the fractions relative to TC were reduced. The mechanisms governing alterations in the dyslipidemic lipoprotein profile and NAFLD are poorly characterized but have been suggested to be because of an overall dysregulation in the excess of dietary cholesterol levels.[84,85] The present study shows a diet-induced increase in plasma 8-F_2-isoprostanes and MDA indicating lipid oxidation as a result of systemic redox imbalance. The observed ADMA increase in HF animals may in part originate from the liver. However, as this is not apparent in HFS animals, it seems unlikely that the increase in HF plasma is exclusively because of liver levels. Increased plasma ADMA concentration has been associated with progressing cardiovascular disease,[86,87] thus our finding may reflect early endothelial dysfunction in the vascular wall. Furthermore, ox-LDL was increased albeit only reaching significance in HFS animals. Whether this relatively subtle difference in sugar content may directly influence the size and subsequent oxidation of LDL particles, as has been proposed for high-carbohydrate diets in guinea pigs,[88] or if the recorded levels of ox-LDL represent a general trend for both high-fat groups, remains to be elaborated. An increase in systemic ox-LDL is associated with redox imbalance and endothelial dysfunction supporting a propagation of deleterious vascular effects. Previously, diets containing 0.25% cholesterol have been shown to induce the expression of inflammatory cytokines and increases in ox-LDL within the aortic wall in guinea pigs, indicating early stage atherosclerosis albeit not manifested by histologically evident plaque formation.[1,89] Fatty streaks in the aortic wall have also been reported for diets containing 0.25% cholesterol and to increase with 0.33% cholesterol during a 12-week feeding period.[90] Despite the slight increase to 0.35% cholesterol in the present study, histologic signs of atherosclerotic lesions were absent. Lesions were also reported to be scarce in guinea pigs subjected to a 0.33% cholesterol diet for a period of 6 months[91]; however, it is tempting to speculate that a prolonged study period could have resulted in histologically evident atherosclerotic lesions in the aortic wall. The HFS diet displayed only a few significant differences compared with HF animals. The increase in ox-LDL could suggest that the HFS diet imposed an increased risk of endothelial dysfunction; however, a progression is not supported by L-Arg and ADMA levels not reaching significance, whereas this was the case for the HF group. Another marker in which HFS animals differed was plasma MDA concentration; however, once normalized to MDA in feed both HF and HFS were significantly increased compared with CTRL.

An altered response to cholesterol depending on high and low levels of dietary carbohydrate has been shown in guinea pigs[89,92]; however, the used diets differed to

a much larger extend (eg,10% carbohydrate compared with 55%). In our study, total carbohydrate content only differed slightly between groups, and this may be a main contributing factor in the relatively absence of detectable differences associated with the increased sugar content of the HFS diet. However, group sizes were relatively small and in most cases where HF and HFS groups differ, it seems as if both groups follow a similar trend compared with CTRL animals. It is therefore likely that the findings of HFS animals differing from HF are attributable to biological variation and would be eliminated with increased n values.

The dietary regimes were reflected in plasma lipid and lipoprotein profiles and in the presence of NAFLD. However, excess deposition of overall body fat did not occur in either HF or HFS groups. DEXA scans revealed an increase in tissue fat % in CTRLs compared with much lower values achieved for animals in HF and HFS groups. Thus, our high-fat diet animals could be perceived as leaner than CTRLs in agreement with findings by others reporting a tendency of reduced BW over time in guinea pigs fed high-fat diet.[91] A contributing factor could be the higher starch content in the CTRL diet (27.9% in CTRL vs 7.7 and 6% in HF and HFS, respectively). As excess carbohydrate is processed to glucose and stored as fat, this could explain some of the differences in fat deposition. Furthermore, HF and HFS animals displayed high TG levels in the liver together with low plasma TG concentration, indicating that the dietary fat is accumulated in the increasingly dysfunctional liver and not released to the blood stream. Hence, high-fat diet groups acquired a lower tissue fat % but suffered hepatic consequences for the dietary lipid overload. The combined finding of no significant differences in BW and fat-free mass between groups does not support the observed difference in fat, but is likely because of the DEXA scan not allowing for a more specific detection of differences in fat accumulation, for example, in internal organs. The increase in HF and HFS liver mass constitute around 40% of the scanned difference in fat (grams) compared with CTRLs and may be attributed to both increased lipid content and hepatocyte proliferation, which has been reported in guinea pigs fed high-cholesterol diet.[40,92] The remaining gap between decreased fat and equal BW could be because of additional distribution of lipids, possibly differences in gastrointestinal feed content and even increases in specific organ size, as was observed in the spleen (other organs were not measured in the present study).

The plasma glucose elimination curves in the performed OGTTs and intact hepatic glycogen levels between groups indicate an absence of type 2 diabetes. However, as direct effects on insulin within the dietary groups could not be evaluated, an approaching insulin resistance cannot be entirely ruled out. Although OGTT is an indirect marker of insulin resistance, our findings are in agreement with reports from rabbits subjected to high dietary cholesterol (1%) presenting dyslipidemia and hepatic steatosis, without developing obesity and insulin resistance.[93] The progression of insulin resistance and NAFLD in humans is associated with reprogramming of adipocyte metabolism, including a decreased adiponectin production. In view of the recorded measures of body fat composition and glucose tolerance, this is in agreement with the absence of obesity and insulin resistance. The increase in adiponectin expression in HFS animals may reflect a compensatory upregulation before a switch toward a stage of dysfunctional adipocyte metabolism. Together, our findings may represent an early stage of disease progression rather than a consistent absence of insulin resistance and adipocyte dysfunction. It may, however, also be that the adiponectin levels recorded for CTRL animals are, in fact, decreased in response to the higher fat deposition compared with the HFS (and HF) animals. This does not seem to be likely as CTRL animals did not display dyslipidemia; however, studies on adiponectin expression patterns in guinea pigs are scarce and remain to be further investigated.

The limitations of the present study include various aspects. Owing to the progressive nature of obesity and associated disease development, the results might have shown increased effects, for example, on atherosclerosis and glucose tolerance had a larger time frame been selected. In addition, had high-caloric and high-fat diets rather than isocaloric diets been used, fat deposition might have increased in HF and HFS groups. The assessment of tissue fat % by DEXA scanning is relatively crude; for future studies, a more accurate determination of regional fat deposition enabling a distinction between visceral, abdominal, and subcutaneous fat would be beneficial. With regards to accurately monitoring the development of insulin resistance, the insulin response during the OGTT should be measured in future studies. In addition, a gender-associated effect on susceptibility has been reported, showing an enhanced response to dietary-induced hypercholesterolemia and LDL particle size in females, but that male guinea pigs are more prone to exhibit atherosclerotic fatty streaks.[90,94,95] Regarding gender-specific differences, the present study thus targets adult premenopausal females. The age of the animals at inclusion and during the study period may also have influenced the response, as age-related differences in the development of dietary imposed metabolic syndrome[96] and adipose cellularity[97] have been shown in rodent models. Finally,

group sizes are relatively small. Although this appears sufficient to detect differences in the examined biochemical markers and in the histologic evaluation of liver disease, it is possible that an increased number of animals could have disclosed atherosclerotic alterations.

CONCLUSIONS

We found the dyslipidemic guinea pig to constitute a valid and natural model for human diet-induced NAFLD, enabling studies of disease progression to NASH. The current model did not display obesity, thus targets the nonobese NAFLD at the current stage not displaying decreased glucose tolerance. Dyslipidemia induced systemic oxidative stress and increased markers of endothelial dysfunction supporting the applicability of the model in preatherosclerotic studies.

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Conflicts of Interest: All authors declare no conflicts of interest that could influence the present work, in coherence with the instructions stated for the journal. All authors have read the journal guidelines for publication.

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All authors have read and approved the manuscript.

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Paper II

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High-fat but not sucrose intake is essential for induction of dyslipidemia and non-alcoholic steatohepatitis in guinea pigs

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Abstract

Background: Non-alcoholic fatty liver disease (NAFLD) and dyslipidemia are closely related. Diet plays an important role in the progression of these diseases, but the role of specific dietary components is not completely understood. Therefore, we investigated the role of dietary sucrose and fat/cholesterol on the development of dyslipidemia and NAFLD.

Methods: Seventy female guinea pigs were block-randomized (based on weight) into five groups and fed a normal chow diet (control: 4 % fat), a very high-sucrose diet (vHS: 4 % fat, 25 % sucrose), a high-fat diet (HF: 20 % fat, 0. 35 % cholesterol), a high-fat/high-sucrose diet (HFHS: 20 % fat, 15 % sucrose, 0.35 % cholesterol) or a high-fat/very high-sucrose diet (HFvHS: 20 % fat, 25 % sucrose, 0.35 % cholesterol) for 16 and 25 weeks.

Results: All three high-fat diets induced dyslipidemia with increased concentrations of plasma cholesterol ($p < 0.0001$), LDL-C ($p < 0.0001$) and VLDL-C ($p < 0.05$) compared to control and vHS. Contrary to this, plasma triglycerides were increased in control and vHS compared to high-fat fed animals ($p < 0.01$), while circulating levels of free fatty acids were even between groups. Histological evaluation of liver sections revealed non-alcoholic steatohepatitis (NASH) with progressive inflammation and bridging fibrosis in high-fat fed animals. Accordingly, hepatic triglycerides ($p < 0.05$) and cholesterol ($p < 0.0001$) was increased alongside elevated levels of alanine and aspartate aminotransferase ($p < 0.01$) compared to control and vHS.

Conclusion: Collectively, our results suggest that intake of fat and cholesterol, but not sucrose, are the main factors driving the development and progression of dyslipidemia and NAFLD/NASH.

Keywords: Non-alcoholic fatty liver disease, Non-alcoholic steatohepatitis, Dyslipidemia, High-fat diet, Sucrose, Guinea pigs, Cholesterol

Background

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the Western world [1] and is closely associated with dyslipidemia [2, 3]. Affecting more than 30 % of the general adult population and with the potential to progress from simple steatosis to irreversible and life-threatening non-alcoholic steatohepatitis (NASH), it is an important public health concern [1, 4]. Disease progression is instigated by a series of parallel hits such as inflammation and oxidative stress, causing hepatocyte damage (e.g. metabolic dysfunction, DNA injury and apoptosis) and irreversible fibrosis, ultimately leading to cirrhosis and liver failure [5]. Although the etiology of NAFLD is not yet fully elucidated, changes in food composition are believed to play an essential role in disease progression [4]. Diets rich in saturated fat, cholesterol and non-complex carbohydrates (e.g. the disaccharide: sucrose) have been shown to induce dyslipidemia and hepatic lipid accumulation and are suggested to play a key role in the development of NASH in human

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patients [6–9]. However, potential interactions of different dietary components and whether certain components are more likely to cause NAFLD has not been determined [10]. Fat and cholesterol promotes oxidative stress, hepatocellular apoptosis, steatosis and NASH [5]. Furthermore, previous results from our group have indicated that sucrose may affect the development of NAFLD in guinea pigs [11]. Guinea pigs are one of the few species carrying the majority of their cholesterol in low density lipoprotein (LDL) particles; hence exhibiting a lipoprotein profile similar to that of humans [12–14]. Furthermore, we and others have reported that guinea pigs subjected to long-term feeding with diets high in fat, cholesterol and sucrose develop hepatic steatosis in accordance with NAFLD [11, 15, 16].

In the present study, we investigated the specific effects of dietary sucrose and fat/cholesterol, alone and in combination, on the development of dyslipidemia and NAFLD or NASH.

Methods

Animals and experimental design

Seventy female Hartley guinea pigs, 10 weeks old (Charles River Laboratories, Lyon, France), were block-randomized (based on body weight (BW)) into five homologous groups ($n = 14$) following one week of acclimation. The animals were group-housed in floor pens with wood shavings, hay, straw and environmental enrichment. Food and water was provided ad libitum and a 12 h light–dark cycle with temperatures between 20–24 °C was maintained. Groups were fed either chow (control), or chow-based diets of very high-sugar (vHS), high-fat (HF), high-fat/high-sugar (HFHS) or high-fat/very high-sugar (HFvHS) diets (Ssniff Spezialdiäten GmbH, Soest, Germany) (Table 1). The diets were stored at −20 °C and freshly thawed twice weekly (complete dietary compositions are shown Additional file 1).

Food intake in each group was estimated daily by weighing feed-remains prior to refill. After either 16 or 25 weeks, guinea pigs were semi-fasted over-night (no feed, but access to hay), pre-anaeshetized with 0.08 ml/kg BW Zoletil-mix, placed on isoflurane and euthanized by decapitation following an intra-cardial blood sample as previously described [11, 17]. Organs were rapidly collected, rinsed in phosphate buffered saline, weighed and stored at −80 °C or in paraformaldehyde for histological examinations.

Table 1 Composition of diets

Nutrient (g/kg diet)	Control	vHS	HF	HFHS	HFvHS
Protein	168	168	168	167	168
Fat	42	43	200	200	199
Carbohydrates (total)	471	535	363	379	411
Cholesterol	–	–	3.5	3.5	3.5
Sucrose (total amount added to the diet)	–	250	–	150	250
Metabolizable energy (MJ/kg)	12.3	13.4	16.4	16.7	17.2

Oral glucose tolerance test

An oral glucose tolerance test (OGTT) was performed after 15 and 24 weeks. Guinea pigs were semi-fasted overnight and dosed orally with a 50 % glucose solution (Amgros I/S, Copenhagen, Denmark) by syringe (2 g glucose/kg BW). Blood glucose was measured with an Accu-Chek Aviva glucometer (Roche A/S Diagnostics, Basel, Switzerland) in triplicate or duplicates at time points 0, 15, 30, 45, 90, 120 and 180 min post-glucose consumption.

Plasma samples

All samples obtained at euthanasia were collected intracardially, whereas samples taken during the study period (baseline triglyceride (TG) and total cholesterol (TC)) were collected from the *vena saphena* [18]. Samples for alkaline phosphatase (ALP) and free fatty acids (FFA) were collected in heparin and NaF-coated microvettes (Sarstedt, Nümbrecht, Germany), respectively. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), TG and TC were collected in K_3EDTA-coated microvettes (Sarstedt, Nümbrecht, Germany). Blood samples for all other analyses were collected in a K_3EDTA-flushed 10 ml syringe. The analyses of ALP, AST, ALT, FFA, TG and baseline TC were performed on a Cobas 6000 (Roche Diagnostic Systems, Berne, Switzerland) according to manufacturer's specifications. Lipoprotein fractions (very low density lipoprotein (VLDL), LDL and high density lipoprotein (HDL)) alongside TC at week 16 and 25 were analyzed by the *Lipoprotein Analysis Laboratory* (Wake Forest School of Medicine, Winston-Salem, North Carolina, USA) as described previously [19]. Serum amyloid A was determined by ELISA (Tridelta Development Ltd, Phase SAA Assay, Kildare, Ireland) and expressed as μg/ml porcine SAA equivalents as previously described [20].

Liver samples

TC and TG were analyzed on liver homogenates sampled from the left lateral (*lobus hepatis sinister lateralis*) and right medial (*lobus hepatis dexter medialis*) lobes on a Cobas 6000 according to manufacturer's specifications and as previously described [11].

Telomere length

The average telomere length was measured from total genomic DNA from liver tissue using real-time quantitative PCR as described previously [21, 22]. For measurement of telomere repeat copies (T), the primers were:

telg- 5′-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT-3′ and telc- 5′-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT-3′. Cycling conditions were: 2 min at 50 °C, 2 min of 95 °C, followed by 2 cycles of 95 °C for 15 s, 52 °C for 15 s and 36 cycles of 95 °C for 15 s, 62 °C for 15 s and 71 °C for 15 s. For measurement of single copy gene (S), the primers were: globin- 5′- ACT GGT CTA GGA CCC GAG AAG-3′ and globin- 5′- TCA ATG GTG CCT CTG GAG ATT-3′. The PCR was carried out in a 384-well 7900HT FAST Real-Time PCR System (Applied Biosystems, Slangerup, Denmark) using a reaction mix with 10–20 ng of genomic DNA in 1× SYBR® Green PCR Master Mix (Applied Biosystems, Slangerup, Denmark). The results are reported as the relative telomere length, i.e. the ratio of telomere repeat copy number (T) to single copy gene (S) copy number (T/S ratio).

Single cell gel electrophoresis (comet) assay

DNA strand breaks were measured as previously described [23]. Strand breaks were visually scored and assigned to one of five classes in a blinded fashion as described by [23]. Cells treated with Ro19-8022 (gift from F. Hoffmann-La Roche, Basel, Switzerland) and white light were used as controls. The level of DNA damage was expressed as a total score calculated as:

$$\begin{aligned}&\Big(Number\ of\ class\ I\cdot 1+Number\ of\ class\ II\cdot 2\\&\quad+\ Number\ of\ class\ III\cdot 3+Number\ of\ class\ IV\cdot 4\\&\quad+\ Number\ of\ class\ V\cdot 5\Big)-total\ number\ of\ scored\ comets\end{aligned}$$

Histology

Paraformaldehyde fixed sections of the left lateral liver lobe were imbedded in paraffin cut into 2–4 μm cross-sections and stained with Mayer's Haematoxylin and Eosin (H&E) or Masson's trichrome stain as previously described [11]. All histological evaluations were performed in a blinded fashion. Sections were evaluated by scoring three lobuli, defined by the presence of at least two portal areas surrounding a central vein, and in accordance with the semi-quantitative scoring scheme suggested by Kleiner et al. [24] as follows: Steatosis was graded from 0–3 reflecting the amount of lipids: 0: <5 %; 1: 5–33 %; 2: >33–66 %; and 3: >66 %. Lobular inflammation was evaluated as the number of inflammatory foci (defined as at least three inflammatory cells in close proximity of each other) in a ×200 field as 0: no foci; 1: <2 foci per field; 2: 2–4 foci per field; 3: >4 foci or diffuse infiltration of the entire field. Portal inflammation was scored as 0: none to minimal, 1: greater than minimal. The presence of ballooning hepatocytes were acknowledged as 0: none; 1: few (but definite ballooning hepatocytes); or 2: many ballooning hepatocytes. Fibrosis was evaluated on entire sections stained by Masson's trichrome. Fibrosis was graded as: 0: not present; 1: perisinusoidal *or* periportal; 1A: mild, zone 3 perisinusoidal; 1B: moderate, zone 3 perisinusoidal; 1C: portal/periportal; 2: perisinusoidal *and* portal/periportal; 3: bridging fibrosis; 4: cirrhosis.

Statistical analysis

All statistical analyses were performed in SAS Enterprice Guide 7.1 (SAS Institute Inc, Cary, North Carolina, USA) and graphs were made in GraphPad Prism 6.06 (GraphPad Software, La Jolla, California, USA). Weight, plasma TG and TC were analyzed by a generalized linear mixed model with random effect of animals. The rest of the data was analyzed using a generalized linear model and presented as means with standard deviations (SD). Data with inhomogeneous variance was logarithmically transformed and then analyzed. Subsequently, data was back-transformed and presented as geometric means with 95 % confidence intervals. Tukey's multiple comparisons test was used in all cases. Ordinal data (histopathological liver scores), DNA damage scores and telomere lengths were analyzed using non-parametric statistics followed by Bonferroni post-hoc test and are presented as medians with range. A p-value below 0.05 was considered statistically significant.

Results

Dietary regimes and OGTT

There were no differences in BW between groups at study start. Control and vHS gained more weight resulting in significantly higher BW compared to the high-fat diet (HFD) groups (HF, HFHS and HFvHS) after 16 weeks ($p < 0.01$) (Fig. 1a). After 25 weeks, BW only differed between control and HF ($p < 0.05$) and vHS and HFD ($p < 0.001$). Initially, average energy intake increased in all groups, after which they declined and then remained stable throughout the study period (Fig. 1b). Cumulative weekly energy intake was higher in vHS compared to HFvHS ($p < 0.01$), but similar among other groups, after 16 weeks (Fig. 1c). After 25 weeks, cumulative energy intake was higher in vHS ($p < 0.0001$), HFHS ($p < 0.001$) and HFvHS ($p < 0.01$) compared to control and increased in vHS compared to HF ($p < 0.01$) and HFvHS ($p < 0.05$) (Fig. 1d). No difference between dietary regimes and glucose tolerance was recorded at either time-point (Additional file 2).

Dyslipidemia and inflammation

Plasma TC and TG (Fig. 2a and b) did not differ between groups at baseline. At 16 and 25 weeks, plasma TC was increased in HF, HFHS and HFvHS compared to control

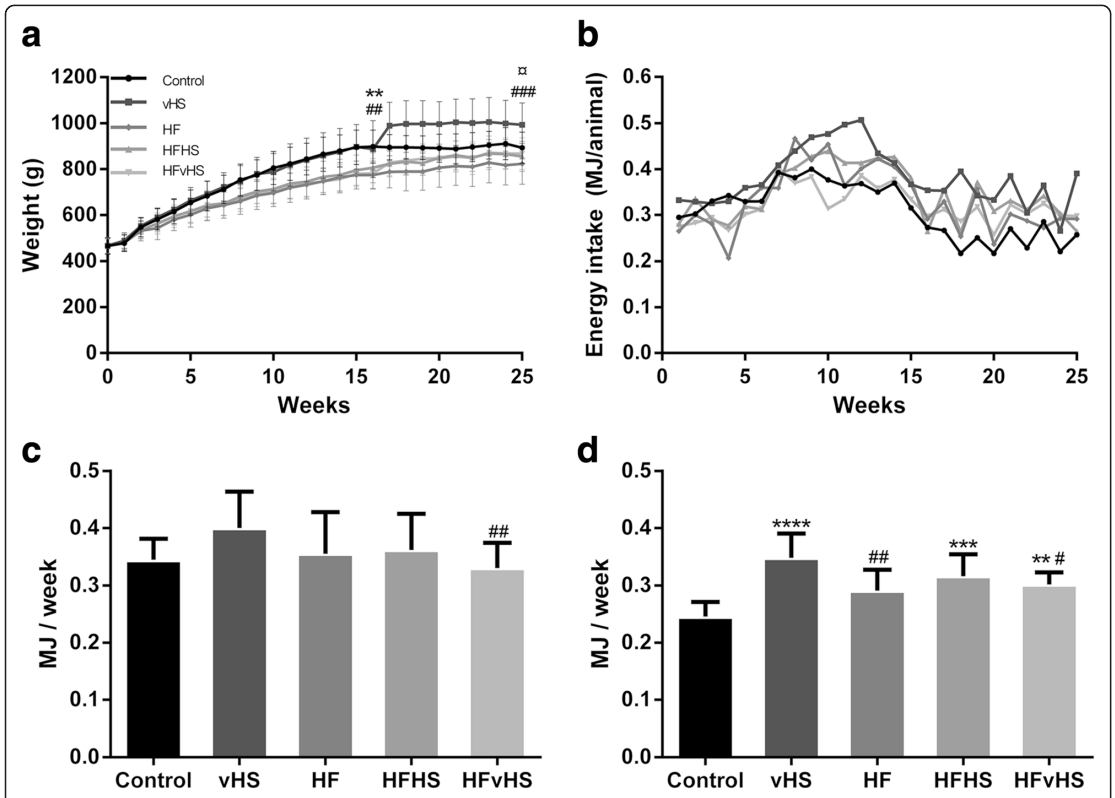

Fig. 1 Weight and energy intake during the study period. **a** Control and vHS animals weighed more than high-fat fed animals after 16 weeks. vHS continued to weigh more compared to high-fat fed animals after 25 weeks, while control only weighed more than HF. Average weekly energy intake (**b**) and weekly cumulative energy intake per group until week 16 (**c**) and 25 (**d**) suggest that vHS had greater energy intake compared to the other groups. Means with SD, $n = 7–14$. HFD vs. Control: **** $p < 0.0001$ *** $p < 0.001$ ** $p < 0.01$. HFD vs. vHS: ### $p < 0.001$ ## $p < 0.01$ # $p < 0.05$. HF vs. Control: ¤ $p < 0.05$

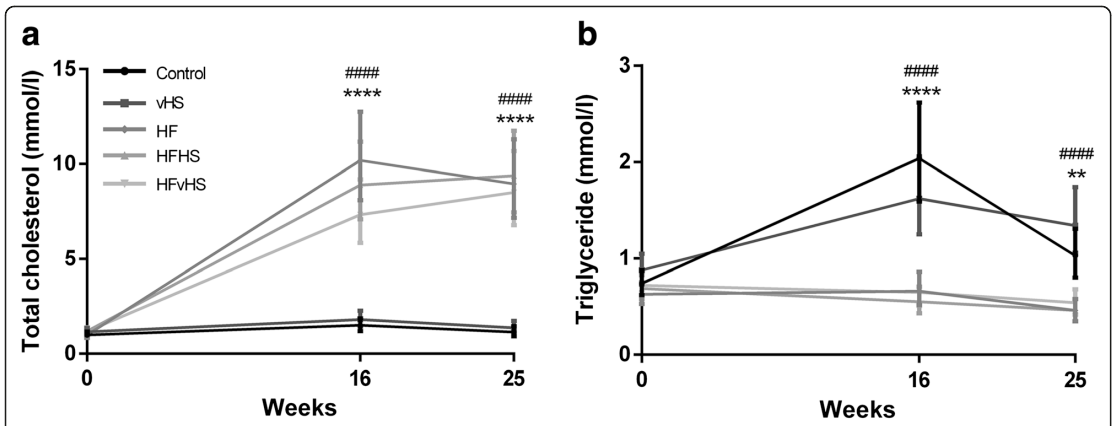

Fig. 2 Plasma total cholesterol and triglycerides. After 16 and 25 weeks, total cholesterol (**a**) was significantly increased by high-fat diets, while triglycerides (**b**) increased in control and vHS. Geometric means with 95 % confidence interval, $n = 7–14$. HFD vs. Control: **** $p < 0.0001$ ** $p < 0.01$. HFD vs. vHS: #### $p < 0.0001$

and vHS ($p < 0.0001$). Contrary to this, plasma TG was elevated after 16 weeks ($p < 0.0001$) and 25 weeks ($p < 0.01$) in control and vHS compared to HFD. FFA did not differ between any groups at any time point. After 16 weeks on the diets, VLDL-C ($p < 0.05$) and LDL-C ($p < 0.0001$) concentrations were increased in HFD groups compared to control and vHS (Table 2). The dyslipidemia persisted after 25 weeks, i.e. increased VLDL-C ($p < 0.001$) and LDL-C ($p < 0.0001$) in HFD groups compared to control and vHS (Table 3). While HDL-C increased upon high-fat feeding, this was not statistically significant relative to control and vHS after 16 or 25 weeks. SAA concentrations were lower in HF ($p < 0.01$) and HFvHS ($p < 0.001$) compared to control and lower in all HFD groups compared to vHS ($p < 0.01$) after 16 weeks (Table 2). At 25 weeks, only HFvHS displayed lower SAA compared to vHS ($p < 0.01$) (Table 3).

Liver biochemistry and genomic damage

Compared to control and vHS, liver weight relative to BW increased upon high-fat feeding after 16 and 25 weeks ($p < 0.0001$) (Table 2 and 3). Similar results were observed for absolute liver weights (Additional file 3), substantiating that increased relative liver weights were not caused by lower BW in HFD groups. Accordingly, lipids were increased in the liver of the high-fat fed animals: Hepatic TC was increased in the HF, HFHS and HFvHS compared to both control and vHS ($p < 0.0001$) on both time-points (Fig. 3a). After 16 weeks hepatic TG was increased in the HFD groups compared to vHS ($p < 0.05$). After 25 weeks, an increase in hepatic TG was seen compared to control ($p < 0.05$), but not when compared to vHS animals (Fig. 3b). Compared to controls and vHS, plasma ALT ($p < 0.001$) and AST ($p < 0.0001$) were increased in all HFD groups at 16 weeks (Table 2) and remained elevated after 25 weeks (ALT $p < 0.01$, AST $p < 0.0001$) (Table 3). Plasma ALP did not differ between any groups at any time point ($p > 0.05$). Genomic damage as assessed by the length of telomeres and level of DNA strand breaks did not differ between groups ($p > 0.05$) (Additional file 4).

Histology

Hepatic steatosis (grade 3), evident as micro- and macrovesicular lipid accumulation in zone 3, was found in most HFD animals, but not in control and vHS animals after 16 and 25 weeks ($p < 0.01$) (Fig. 3c, g and h). At week 16, lobular inflammation was not significantly higher in HFD groups compared to vHS. However, hepatic inflammation continued to progress in all HFD groups, resulting in severe inflammation (grade 3), which was significantly higher compared to control and vHS at week 25 ($p < 0.05$) (Fig. 3d). Concurrently, hepatocyte ballooning was more prominent in HFD groups at both time-points compared to control and vHS ($p < 0.05$) (Fig. 3e). Fibrosis (grade 1–3) was significantly increased in all HFD groups compared to control and vHS at week 16 ($p < 0.05$). At week 25, fibrosis, bridging between central veins (grade 3), was seen in almost all HFD animals, while fibrosis was absent in control and vHS ($p < 0.01$) (Fig. 3f, i and j). Portal inflammation was absent in all groups at both time points, apart from a single control animal at 16 weeks. The exact histological scoring is provided in Additional file 5.

Discussion

The present study shows that dietary fat and cholesterol, but not sucrose, are the main factors driving the progression of dyslipidemia and NAFLD to NASH in a guinea pig model. Additionally, adding sucrose to a high-fat diet does not exacerbate the metabolic or hepatic consequences of a high-fat diet *per se*.

Expectedly, addition of cholesterol to the dietary regime increased circulating levels of TC, similar to findings of other studies utilizing cholesterol-rich [25, 26] or high-fat diet [27]. Though Plasma TC and TG concentrations were not affected by addition of sucrose consistent

Table 2 Circulating lipids, relative liver weight and biochemical markers after 16 weeks on diets

	Control	vHS	HF	HFHS	HFvHS
VLDL-C (mM) [a]	0.19 (0.11–0.33)	0.18 (0.11–0.30)	1.01 (0.60–1.72) ** ###	0.89 (0.52–1.51) ** ##	0.68 (0.40–1.16) * #
LDL-C (mM) [a]	1.22 (0.92–1.62)	1.55 (1.17–2.06)	9.04 (6.81–12.0) **** ####	7.86 (5.90–10.4) **** ####	6.54 (4.93–8.68) **** ####
HDL-C (mM) [a]	0.04 (0.03–0.06)	0.04 (0.03–0.06)	0.07 (0.04–0.10)	0.10 (0.06–0.15)	0.09 (0.06–0.15)
FFA (mM)	0.77 ± 0.23	0.68 ± 0.16	0.50 ± 0.15	0.51 ± 0.09	0.52 ± 0.21
SAA (μg/mL) [a]	89.4 (47.5–168)	182 (91.8–359)	14.3 (7.22–28.3) ** ####	22.5 (10.7–47.5) ##	10.8 (5.77–20.4) *** ####
% Liver weight [a]	2.16 (1.92–2.43)	2.29 (2.03–2.57)	4.72 (4.19–5.31) **** ####	4.63 (4.11–5.21) **** ####	4.30 (3.82–4.84) **** ####
ALT (U/L) [a]	43.1 (34.1–54.7)	32.4 (25.6–41.1)	92.5 (73.0–117) *** ####	106 (83.8–128) ****####	98.1 (76.0–127)*** ####
AST (U/L) [a]	67.0 (47.3–95.2)	51.5 (36.3–73.1)	403 (284–572) **** ####	450 (317–639) **** ####	323 (221–471) **** ####
ALP (U/L)	70.3 ± 5.53	60.7 ± 18.5	59.0 ± 14.6	53.9 ± 9.19	51.9 ± 17.3

$n = 7$. Mean with SD. Compared to control **** $p < 0.0001$*** $p < 0.001$ ** $p < 0.01$ * $p < 0.05$. Compared to vHS #### $p < 0.0001$### $p < 0.001$ ## $p < 0.01$ # $p < 0.05$
[a] Data analysis performed on log10 transformed data, presented as geometric means with 95 % confidence interval

Table 3 Circulating lipids, relative liver weight and biochemical markers after 25 weeks on diets

	Control	vHS	HF	HFHS	HFvHS
VLDL-C (mM) [a]	0.12 (0.07–0.20)	0.13 (0.08–0.23)	1.02 (0.60–1.74) **** ####	1.38 (0.81–2.35) **** ####	0.89 (0.52–1.51) **** ###
LDL-C (mM) [a]	0.99 (0.74–1.31)	1.15 (0.87–1.52)	7.74 (5.83–10.3) **** ####	7.83 (5.90–10.4) **** ####	7.38 (5.56–9.79) **** ####
HDL-C (mM) [a]	0.03 (0.02–0.05)	0.04 (0.02–0.06)	0.06 (0.04–0.10)	0.08 (0.05–0.12)	0.07 (0.04–0.10)
FFA (mM)	0.48 ± 0.22	0.61 ± 0.12	0.58 ± 0.13	0.64 ± 0.13	0.43 ± 0.17
SAA (μg/mL) [a]	46.6 (24.8–87.7)	88.3 (46.9–166)	20.9 (10.6–41.3)	48.1 (24.3–95.2)	12.3 (6.56–23.2) ##
% Liver weight [a]	2.07 (1.84–2.32)	2.55 (2.27–2.87)	4.90 (4.35–5.51) **** ####	5.19 (4.61–5.84) **** ####	4.97 (4.42–5.59) **** ####
ALT (U/L) [a]	37.0 (29.2–46.9)	30.2 (23.8–38.2)	77.7 (61.3–98.3) ** ####	101 (79.7–128) **** ####	86.4 (68.3–109) *** ####
AST (U/L) [a]	43.6 (30.7–61.8)	43.7 (30.8–62.0)	259 (183–368) **** ####	428 (302–607) **** ####	445 (314–631) **** ####
ALP (U/L)	60.1 ± 4.88	59.6 ± 13.1	45.9 ± 5.18	43.3 ± 1.70	47.0 ± 7.05

$n = 7$. Mean with SD. Compared to control **** $p < 0.0001$*** $p < 0.001$ ** $p < 0.01$. Compared to vHS #### $p < 0.0001$ ### $p < 0.001$
[a] Data analysis performed on log10 transformed data, presented as geometric means with 95 % confidence interval

with results from mice and rats fed sucrose at levels of 32–35 % of total caloric intake [28, 29], circulating levels of TC and TG upon sucrose feeding have also been reported [30–33] It is possible that sucrose induced dyslipidemia differs mechanistically from the high fat induced [31], and moreover very high levels (≥60 %) of sucrose are applied to induce dyslipidemia [31–33]. Thus, it is possible that higher dietary sucrose concentrations may have been necessary to promote dyslipidemia in the current study; indeed calories originating from sucrose and fat were not equal potentially confounding the effect of sucrose. However, the translational relevance of models utilizing extremely high levels of sucrose have been questioned [34]. Consequently, the levels of dietary sucrose in this study may have more relevance to human consumption, albeit still being high.

Decreased levels of hepatic LDL-receptors and concomitant increased levels of circulating TC and LDL-C has been reported in guinea pigs subjected to a high-fat diet [35]. Accordingly, we found that LDL-C and VLDL-C increased upon high-fat feeding, regardless of dietary content of sucrose. This contradicts previous findings of a sucrose imposed elevation of TC and LDL-C when added to a high-fat diet in male guinea pigs [13, 36]. The observed dissimilarity may be due to differences in dietary composition as the latter high-fat diets did not contain excess cholesterol [13, 36] and/or a gender associated effect, as female rats—in contrast to males—proved resistant to sucrose-induced hypertriglyceridemia [37]. In agreement with our findings, circulating levels of lipids did not increase in humans placed on eucaloric diets, consuming 20 % of calories as sucrose for 10 weeks [38].

BW increased in all groups over time, but high-fat fed animals do not become obese and compared to the control and vHS groups, this is in accordance with previous results by us and others [11, 13, 16, 35]. Lack of comparable weight gain despite similar caloric intake might partly be due to hepatic lipid accumulation, rendering lipids unavailable for other tissues. After 16 weeks, HFHS and HFvHS increased energy-intake compared to control and consequently weight differences were eliminated between HFHS, HFvHS and control. Compared to control, HF also tended to increase energy-intake, but not enough to completely eliminate the weight difference. The apparent increase in BW observed for vHS after week 16 was due to the randomization procedure: animals were randomized, but not block-randomized based on weight at euthanasia and by chance, most of the animals with the highest BW were randomly chosen to continue on the diet. Regardless, vHS did not develop dyslipidemia or NAFLD. Thus, weight differences are not thought to influence the results of this study, and animals exposed to either vHS or high-fat diet were not obese compared to controls.

FFA release increases with increasing fat mass in humans [39] and the lack of increased FFA concentrations is likely to reflect the absence of obesity as reported in a non-obese rabbit-model of NASH [40]. Furthermore, plasma TG was not increased in response to the high-fat diet. This could be due to increased TG clearance from the blood and/or decreased hepatic TG production. Accordingly, guinea pigs on high-fat diet (25.1 % fat) displayed reduced plasma TG compared to their low-fat fed counterparts [13]. Lipoprotein lipase activity was increased by high-fat feeding, most likely contributing to the decreased circulating levels of TG [13]. Similarly, plasma TG was also lower in rats fed a high-fat, high-cholesterol diet compared to chow or a high-fat diet without cholesterol [41]. In these rats, hepatic microsomal triglyceride transfer protein mRNA expression was suppressed, potentially limiting hepatic VLDL-TG production [41]. Indeed, hepatic TG production may be compromised as NAFLD progresses from simple steatosis towards steatohepatitis. In humans, NASH is associated with impaired VLDL synthesis and secretion and reduced apoB100 synthesis [42, 43]. Hence, hepatic retention of TG, limiting TG availability for storage in adipose tissue, may constitute a causal mechanism in the progression of NASH in the

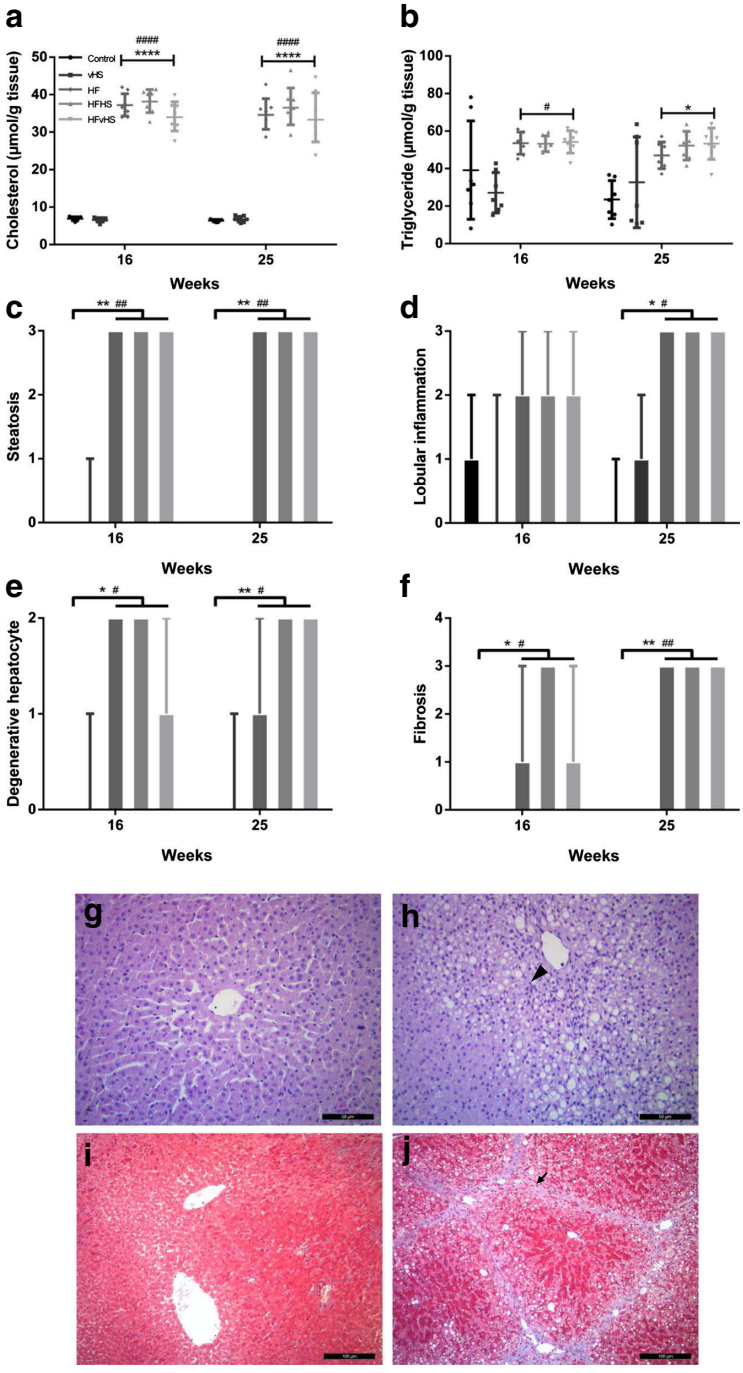

Fig. 3 (See legend on next page.)

(See figure on previous page.)
Fig. 3 Biochemical and histological characteristics of livers. Hepatic lipid content (**a** and **b**), histological scoring of liver sections in accordance to [24] (**c**-**f**) and representative liver sections for control/vHS (**g** and **i**) and HFD (**h** and **j**). The arrow head and arrow indicates inflammatory cells and fibrosis, respectively. Hepatic cholesterol was increased by high-fat feeding at 16 and 25 weeks (**a**), while hepatic triglycerides were significantly increased in HFD groups after 25 weeks compared to control (**b**). Compared to control and vHS, HFD groups had grade 3 hepatic steatosis after 16 weeks, which persisted until the end of the study at 25 weeks (**c**, **g** and **h**). Similarly, high-fat diet induced progressive inflammation (**d**), hepatocyte ballooning (**e**) and fibrosis (**f**, **i** and **j**). Geometric means with 95 % confidence interval (**a**), means with SD (**b**) and medians with range (**c**-**f**). Scale bar 50 μm (**f** and **g**) and 100 μm (**h** and **i**), $n = 6$–7 (HFvHS $n = 6$ for histology at week 16 due to technical difficulties). HFD vs. Control: **** $p < 0.0001$ ** $p < 0.01$ * $p < 0.05$. HFD vs. vHS: #### $p < 0.0001$ ## $p < 0.01$ # $p < 0.05$

non-obese phenotype of the dyslipidemic guinea pig model.

Guinea pig SAA - a systemic marker of inflammation [20] - was not induced by high-fat feeding at any of the two time points, similar to results from high fat fed (15 % fat, 1.35 % cholesterol) mice [44]. Our findings may suggest that systemic inflammation is not prominent in this model, at least when assessed by systemic SAA level. Alternatively, it could be speculated that low SSA levels was due to reduced liver function, supported by histopathology and increased ALT and AST levels, rendering the liver unable to produce and/or secrete SAA.

Our study revealed hepatocyte ballooning after 16 weeks of high-fat feeding, signifying the presence of NASH and distinguishing it from simple steatosis [45, 46]. This is further supported by the recorded inflammatory foci and progression of fibrosis in the HFD groups. While hepatic fibrosis is not necessary for the diagnosis of NASH, it represents a critical step in the progression of the disease, setting the stage for further liver damage such as cirrhosis and hepatocellular carcinoma [41]. However, high-fat diet models often induce mild hepatic fibrosis while rarely leading to severe progressive fibrosis [47, 48], except in guinea pigs [11]. After 16 weeks, guinea pigs in the HF and HFvHS group exhibited mild fibrosis, while the HFHS group already displayed bridging fibrosis (grade 3). After 25 weeks, all three HFD groups had formed bridging fibrosis. Consistent with our findings of circulating levels of lipids, sucrose feeding alone did not induce NAFLD, nor did it affect hepatic outcomes when added to a high-fat diet. Contrary to our results, 60–70 % sucrose promoted development of hepatic steatosis in both rats [49–51] and mice [52, 53]. However, while levels of hepatic inflammatory cytokines were increased [49], hepatic triglycerides were not significantly elevated [49, 51]. Thus, sucrose is seemingly not able to induce NASH.

Diseases characterized by chronic tissue regeneration, such as cirrhosis ensuing from progressive NASH, ultimately results in telomere shortening [54]. This promotes genomic instability paralleled by DNA strand damage which may constitute an underlying disease aspect playing an important role in NAFLD, especially with regards to fibrosis progression [54]. However, DNA strand breaks and telomere length was not different between groups. Consequently, these do not seem to be underlying mechanisms of NAFLD and NASH in this particular animal model. The null results on hepatic DNA damage is in keeping with earlier results in rats showing no altered levels of DNA strand breaks after feeding with saturated fats [55] and sucrose [56–59]. Alternatively, livers may have to become cirrhotic before notable telomere shortening can be detected, which may also explain the absence of telomere shortenings.

Conclusion

Dietary sucrose alone or in combination with a high-fat diet did not affect the development of dyslipidemia or NASH. Thus, disease development appears to be driven mainly by dietary fat and cholesterol, but the current study is not able to distinguish between effects of dietary fat and cholesterol. Furthermore, the present diets contained high levels of saturated fatty acids and extrapolation of the results to dietary regimes differing in fatty acid composition and content should be done with caution. However, encompassing a similar histopathology indicates that the model may closely resemble the human condition. Based on the systemic and hepatic changes observed, our findings may reiterate the idea of fat and cholesterol as critical dietary factors with regards to disease progression.

Additional files

Additional file 1: Exact dietary composition. The complete dietary composition including fatty acid composition. *Vitamin & trace element content (addition per kg feed): 25.0 IU Vitamin A (E672), 1.50 IU, Vitamin D3 (E671), 0.125 g Vitamin E (all-rac-alpha-tocopherylacetate) (3a700), 0.08 g Vitamin K3 (MNB), 0.08 g Vitamin B1 (Thiamine mononitrate), 0.03 g Vitamin B2 (Riboflavin), 0.05 g Ca Pantothenate, 0.025 g Vitamin B6 (pyridoxol hydrochloride) (3a831), 0.00015 g Vitamin B12 (Cyanocobalamine), 0.09 g Niacin, 0.009 g Folic acid, 0.0005 g Biotin, 0.100 g Inositol, 0.100 g Iron (II)-sulfate monohydrate (E1), 0.005 Copper (II)-sulfate pentahydrate (E4), 0.03 g Manganese (II)-sulfate monohydrate (E5), 0.002 g Cobalt (II)-carbonate monohydrate (E3), 0.05 g Zinc sulfate monohydrate (E6), 0.002 g Calcium iodate anhydrate (E2), 0.0001 g Sodium selenite (E8). ** 1.00 g NaCl added to HFvHS as soybean isolate contains approximately 1.5 % NaCl). (DOCX 17 kb)

Additional file 2: Oral glucose tolerance tests. High-fat diets did not induce glucose intolerance as shown by oral glucose tolerance tests conducted after 15 (A) and 24 weeks (B). Means with SD, $n = 7$. HFD vs.

Control: **** $p < 0.0001$ ** $p < 0.01$ * $p < 0.05$. HFD vs. vHS: #### $p < 0.0001$ ### $p < 0.001$ # $p < 0.05$. Control vs. vHS: θθθθ $p < 0.0001$ θθ $p < 0.001$ θ $p < 0.05$. (TIF 497 kb)

Additional file 3: Absolute liver weight. The absolute liver weights were increased in HFD compared to Control and vHS after 16 and 25 weeks. Means with SD, $n = 7$. HFD vs. Control: **** $p < 0.0001$. HFD vs. vHS: ### $p < 0.001$. HFD vs. vHS: ## $p < 0.01$. (TIF 211 kb)

Additional file 4: Hepatic telomere length and DNA strand breaks. T/S expresses the ratio of the mean telomere repeat copies (T) to a reference single copy gene (S) and did not differ between groups (A). Additionally, the extent of DNA damage, measured as strand breaks, did not differ between groups (B). Medians with range, $n = 5–7$. (TIF 442 kb)

Additional file 5: Frequencies of hepatic steatosis, lobular inflammation, ballooning hepatocytes and fibrosis. Histopathological scoring of hepatic steatosis, lobular inflammation, ballooning (degenerative) hepatocytes and fibrosis done according to Kleiner et al. (20). Scores are listed as 16 weeks | 25 weeks (HFvHS $n = 6$ for fibrosis scoring at week 16 due to technical difficulties). (DOCX 15 kb)

Abbreviations

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BW, body weight; FFA, free fatty acids; H&E, Mayer's haematoxylin and eosin; HDL-C, high density lipoprotein cholesterol; HF, high-fat; HFD, high-fat diet; HFHS, high-fat high-sucrose; HFvHS, high-fat very high-sucrose; LDL-C, low density lipoprotein cholesterol; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OGTT, oral glucose tolerance test; SD, standard deviation; T/S ratio, ratio of telomere repeat copy number (T) to single copy gene (S) copy number; TC, total cholesterol; TG, triglycerides; vHS, very high-sucrose; VLDL-C, very low density lipoprotein cholesterol

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Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

The study was designed by DHI, PTN, BR, GR and JL and conducted by DHI, PTN, MB, LWM, LF, PMHH and PM. DHI, PTN, BR, GR, MB, LWM, LF, PMHH, PM and JL analyzed and interpreted the data. DHI wrote the draft manuscript, which was subsequently edited by all authors; JL had responsibility for the final content. All authors have read and approved the final manuscript.

Competing interests

DHI and JL are supported by the LifePharm Centre for In Vivo Pharmacology. BR and GR are employed at Novo Nordisk. The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All experiments were approved by the Danish Animal Experiments Inspectorate under the Ministry of Food, Agriculture and Fisheries and in accordance with European Union directive 2010/63/EU.

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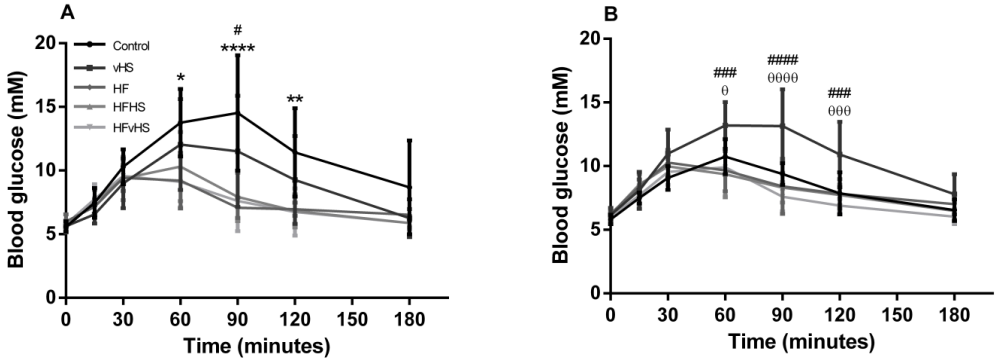
Additional File 1 Exact dietary composition. The complete dietary composition including fatty acid composition.

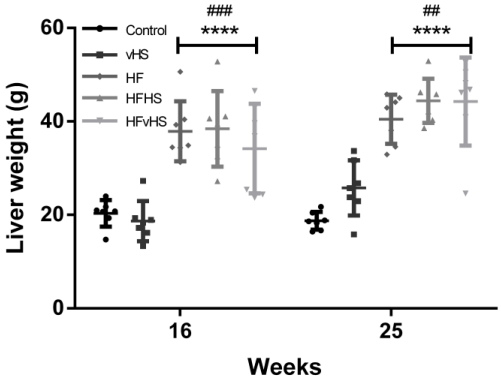
Nutrients (g/kg diet)	Control	vHS	HF	HFHS	HFvHS
Alfalfa	220	220	220	220	220
Wheat	283	185	290	103	27.0
Barley	180	-	-	-	-
Sucrose	-	250	-	150	250
Cellulose (lignocellulose)	46.0	44.0	40.0	46.0	56.0
Sunflower meal	30.0	30.0	30.0	30.0	30.0
Soybean meal	120	40.0	70.0	60.0	30.0
Soybeans (full fat)	26.0	26.0	26.0	26.0	46.0
Soybean concentrate	20.0	120	80.0	120	-
Soybean isolate (90% protein)	-	-	-	-	10.8
Amino acids	5.00	5.50	5.90	5.00	5.50
Vitamins & trace element*	10.0	10.0	10.0	10.0	10.0
Vitamin C (Stay-C)	29.0	29.0	29.0	29.0	29.0
NaCl**	4.0	4.0	4.0	4.0	1.0
Calcium phosphate (monobasic)	12.9	15.4	15.4	16.4	17.4
Calcium propionate	5.5	5.5	5.5	5.5	5.5
Calcium carbonate	2.5	1.5	1.5	1.5	1.5
Choline Cl	3.0	3.0	3.0	3.0	3.0
Sugar beet pulp	10.0	10.0	10.0	10.0	10.0
Cholesterol	-	-	3.50	3.50	3.50
Coconut oil, hydrogenated	-	-	180	180	180
Soybean oil	21.0	27.0	2.0	5.0	5.0

Crude protein	168	168	168	167	168
Crude fat	42	43	200	200	199
Crude fiber	126	113	113	114	113
Crude ash	65	65	65	66	58
Starch	279	127	189	77	29
Sugar	38	276	32	176	267
Carbohydrates	471	535	363	379	411
Fatty acids					
C 6:0	-	-	0.6	0.6	0.6
C 8:0	-	-	9.9	9.9	9.9
C 10:0	-	-	9.3	9.3	9.3
C 12:0	-	-	81.9	81.9	81.9
C 14:0	0.2	0.1	35.4	35.4	35.4
C 16:0	6.3	5.9	22.9	22.7	22.6
C 18:0	1.6	1.5	21.9	22.0	22.0
C 20:0	0.2	0.1	0.3	0.3	0.3
C 16:1	0.3	0.1	0.1	0.1	0.1
C 18:1	8.7	9.4	5.3	5.6	5.5
C 18:2	21.9	22.0	10.0	9.8	9.2
C 18:3	3.7	3.5	2.3	2.2	2.1

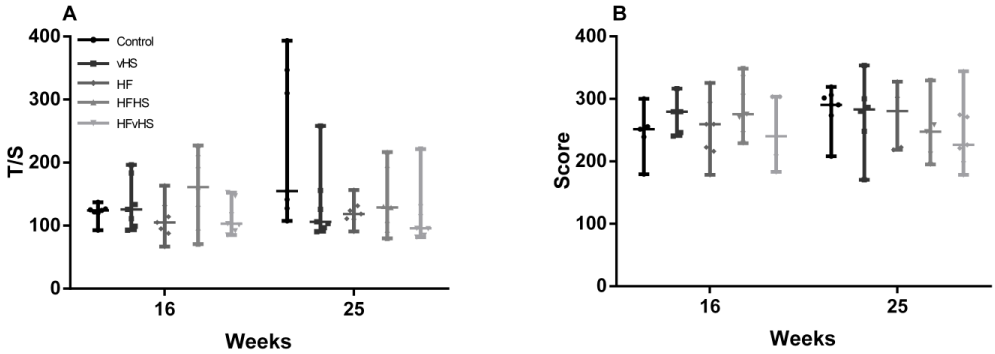
Additional File 2 Oral glucose tolerance tests.



Additional File 3 Absolute liver weight.



Additional File 4 Hepatic telomere length and DNA strand breaks.



Additional file 5 Frequencies of hepatic steatosis, lobular inflammation, ballooning hepatocytes and fibrosis.

	Control	vHS	HF	HFHS	HFvHS
Degree of steatosis					
0	7/7 | 7/7	6/7 | 7/7	0/7 | 0/7	0/7 | 0/7	0/7 | 0/7
1	0/7 | 0/7	1/7 | 0/7	0/7 | 0/7	0/7 | 0/7	0/7 | 0/7
2	0/7 | 0/7	0/7 | 0/7	0/7 | 0/7	1/7 | 0/7	2/7 | 1/7
3	0/7 | 0/7	0/7 | 0/7	7/7 | 7/7	6/7 | 7/7	5/7 | 6/7
Lobular inflammation					
0	2/7 | 4/7	5/7 | 2/7	0/7 | 0/7	1/7 | 0/7	1/7 | 0/7
1	3/7 | 3/7	1/7 | 4/7	2/7 | 0/7	0/7 | 0/7	1/7 | 1/7
2	2/7 | 0/7	1/7 | 1/7	4/7 | 0/7	3/7 | 0/7	4/7 | 0/7
3	0/7 | 0/7	0/7 | 0/7	1/7 | 7/7	3/7 | 7/7	1/7 | 6/7
Ballooning hepatocytes					
0	7/7 | 7/7	6/7 | 6/7	0/7 | 0/7	0/7 | 0/7	0/7 | 0/7
1	0/7 | 0/7	1/7 | 1/7	3/7 | 5/7	3/7 | 2/7	5/7 | 3/7
2	0/7 | 0/7	0/7 | 0/7	4/7 | 2/7	4/7 | 5/7	2/7 | 4/7
Fibrosis					
0	7/7 | 7/7	7/7 | 7/7	0/7 | 0/7	1/7 | 0/7	1/6 | 0/7
1	0/7 | 0/7	0/7 | 0/7	0/7 | 0/7	0/7 | 0/7	0/6 | 0/7
1A	0/7 | 0/7	0/7 | 0/7	6/7 | 1/7	1/7 | 1/7	4/6 | 2/7
1B	0/7 | 0/7	0/7 | 0/7	0/7 | 0/7	0/7 | 0/7	0/6 | 0/7
1C	0/7 | 0/7	0/7 | 0/7	0/7 | 0/7	0/7 | 0/7	0/6 | 0/7
2	0/7 | 0/7	0/7 | 0/7	0/7 | 0/7	0/7 | 0/7	0/6 | 0/7
3	0/7 | 0/7	0/7 | 0/7	1/7 | 6/7	5/7 | 6/7	1/6 | 5/7
4	0/7 | 0/7	0/7 | 0/7	0/7 | 0/7	0/7 | 0/7	0/6 | 0/7

Paper III

Liraglutide decreases hepatic inflammation and injury in advanced nonalcoholic steatohepatitis. Ipsen DH, Rolin B, Rakipovski G, Skovsted GF, Madsen A, Kolstrup S, Schou-Pedersen AM, Lykkesfeldt J and Tveden-Nyborg P
(*Ready for submission to EBioMedicine*)

Liraglutide decreases hepatic inflammation and injury in advanced nonalcoholic steatohepatitis

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Abbreviations

AA: ascorbic acid. ADMA: asymmetric dimethylarginine. ALP: alkaline phosphatase. ALT: alanine aminotransferase. AST: aspartate aminotransferase. BH_2: di-hydrobiopterine. BH_4: tetra-hydrobiopterine. BID: bis in die (twice a day). DHA: dehydroascorbic acid. FFA: free fatty acids. GLP-1: glucagon-like peptide 1. GSH: glutathione. GSSG: oxidized glutathione. HDL: high density lipoprotein. JNK: c-Jun N-terminal kinase-1. L-NAME: N^{ω}-Nitro-L-arginine methyl ester hydrochloride. LDL: low density lipoprotein. MDA: malondialdehyde. MTTP: microsomal triglyceride transfer protein. NAFLD: non-alcoholic fatty liver disease. NASH: non-alcoholic steatohepatitis. RA: receptor agonist. SD: standard deviation. SOD: superoxide dismutase. TC: total cholesterol. TG: triglycerides. VLDL: very low density lipoprotein.

Abstract

Background & Aims

Non-alcoholic fatty liver disease (NAFLD) affects one fourth of the world's population and may progress to non-alcoholic steatohepatitis (NASH) with irreversible liver damage and increased mortality. Currently, no approved treatment for NASH exists. Exploring possible interventions, this study investigated the effect of liraglutide (GLP-1 receptor analogue) and dietary change in the high fat fed guinea pig NASH model.

Methods

Following 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 four 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).

Results

The HF group displayed advanced NASH with severe fibrosis. Liraglutide treatment (HFL) significantly decreased inflammation and hepatocyte ballooning ($p<0.05$), while increasing α-tocopherol levels in the liver ($p<0.05$). Dietary intervention *per se* (HFC) decreased plasma total cholesterol, low density lipoprotein-cholesterol and very low density lipoprotein-cholesterol ($p<0.05$), decreased hepatic cholesterol ($p<0.001$), increased hepatic vitamin C ($p<0.01$) and non-significantly improved NASH. HFCL animals experienced a rapid weight-loss, necessitating periodical a dose-adjustment/discontinuation of liraglutide, potentially limiting the strength of the findings in this group.

Conclusions

Treatment with liraglutide for four weeks significantly reduced key histological features of NASH even in advanced disease with severe fibrosis**.** Dietary intervention reduced dyslipidemia and resulted in a non-significant improvement of hepatic health.

Key words

NAFLD, NASH, dietary intervention, liraglutide, glucagon-like peptide 1, guinea pig.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is estimated to affect about 25% of the world's population and represents an important risk factor for metabolic dysfunction and associated co-morbidities, such as cardiovascular disease (1-3). No targeted pharmacological treatment option is presently available and the effect of dietary intervention on disease progression remains unclear (4). The pathogenesis of NAFLD is not fully elucidated, however, patients are characterized by dyslipidemia—not necessarily obesity—a result of an unhealthy dietary pattern, including high intakes of saturated fat, cholesterol and non-complex carbohydrates (4-6). Fueled by persistent dyslipidemia, the metabolic capacity of the liver is exceeded, prompting the accumulation of intracellular lipids and propagation of redox imbalance and oxidative stress (1, 7). NAFLD may progress to non-alcoholic steatohepatitis (NASH), promoting irreversible liver fibrosis and predisposing to cirrhosis and heptocellular carcinoma (8). Animal models of NASH display increased levels of reactive oxygen species and impairment of the antioxidant system (9, 10), linking hepatic oxidative stress to lipid accumulation, inflammation, fibrosis and hepatocyte damage (1, 10).

Exploring pharmacological treatment options, glucagon-like peptide 1 (GLP-1) receptor agonists (RA) have been found to improve both hepatic steatosis and oxidative stress in mice and rats (11-18). GLP-1 RAs promote glucose-dependent insulin secretion, decrease inflammation and suppress appetite, facilitating a reduction in food intake with subsequent weight loss (19). In humans, a recent investigator initiated randomized controlled clinical trial reported histological resolution of NASH in patients treated for 48 weeks with the GLP-1 RA liraglutide (20). This suggests that liraglutide intervention may constitute a treatment option of both NAFLD and NASH though underlying mechanisms and response relative to disease-stage (i.e. NAFLD to advanced NASH) remains poorly understood.

The present paper investigates the effect of liraglutide and dietary intervention on the progression of NASH, fibrosis and oxidative stress in our validated guinea pig NAFLD/NASH model (21, 22). Unlike rats and

mice, the lipoprotein profile of the guinea pig and human is low density lipoprotein (LDL) dominant and NAFLD and NASH develops with relatively low levels of dietary cholesterol (around 0.35%). This increases the translational value potentially making the guinea pig a more relevant model (21-24). In accordance with a relationship between NAFLD and increased occurrence of cardiovascular disease in humans, 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 (25, 26).

Methods

Animals and experimental design

All animal experimentation was approved by the Animal Experimentation Council under the Danish Ministry of Environment and Food, and in accordance with the European Legislation of Animal Experimentation 2010/63/EU.

Forty female Dunkin Hartley guinea pigs (Envigo, NM Horst, The Netherlands) weighing 500-650 grams were group-housed in floor pens with wood shavings, hay, straw and environmental enrichment and maintained on a 12h light-dark cycle with temperatures between 20-24^{o}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 blocked-randomized based on weight into four groups (n=10): A high fat diet (HF) group, which continued on the startup 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 four weeks until study termination, at which animals were euthanized as previously described (21, 22). All diets (Table 1 and Supplementary table 1) (Ssniff Spezialdiäten GmbH, Soest, Germany) were stored at -20^{o}C until use. 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 slowly titrated over a period of nine days to 30 nmol/kg body weight

(BW) twice a day (BID) administered by subcutaneous injections. During dose escalation, guinea pigs received 5.4 nmol/kg BW BID for two days, then 10.8 nmol/kg BW BID for two days, followed by 16.2 nmol/kg BW BID for three days and lastly 21 nmol/kg BW BID for two 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 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. Consequently, none of the guinea pigs in the HFCL group received uninterrupted treatment with liraglutide during the four week intervention period.

Plasma samples

Blood samples for the analysis of total cholesterol (TC), triglycerides (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lipoprotein fractions (very low density lipoprotein (VLDL), LDL and high density lipoprotein (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 (27). At these time-points, guinea pigs from each of the four groups were block-randomized based on weight and plasma analyzed for either lipids (n=5) or ALT and AST (n=5). At euthanasia (week 4), blood samples were collected intra-cardially using a K_3-EDTA flushed syringe except for samples analyzed for free fatty acids (FFA) and alkaline phosphatase (ALP), which were collected in NaF and heparin microvettes (Sarstedt, Nümbrecht, Germany), respectively. Plasma was obtained by centrifuging blood samples at 2000 g for 4 minutes at 4^{o}C. AST, ALT, ALP and FFA were analyzed on a Cobas 6000 (Roche

Diagnostic Systems, Berne, Switzerland) according to the manufacturer's specifications. TC, TG and lipoprotein fractions were analyzed at the *Lipoprotein Analysis Laboratory* (Wake Forest School of Medicine, Winston-Salem, North Carolina, USA) as previously described (28). L-arginine and asymmetric dimethylarginine (ADMA) levels were measured by HPLC (29) and plasma samples for quantification of ascorbic acid (AA) and dehydroascorbic acid (DHA) were stabilized metaphosphoric acid before analysis by HPLC as previously described (30-32). 8-Isoprostanes were analyzed by ELISA according to the manufacturer's specifications (Cayman Chemicals, Ann Arbor, Michigan, USA). For the determination of di-hydrobiopterine (BH_2) and tetra-hydrobiopterine (BH_4), blood was stabilized in 0.1% dithioerythritol and centrifuged (2000 g, 4 min, 4^{o}C), yielding a plasma fraction which was analyzed by HPLC as described elsewhere (33).

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, Massachusetts, USA) and weighed. TG, TC and glycogen content were analyzed on homogenates from the left lateral lobe (*lobus hepatis sinister lateralis*) on a Cobas 6000 according to the manufactures specifications, as described previously (21, 22). 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^{o}C water bath for two minutes, before cooling on ice, and supplemented with 0.5 ml extraction buffer. 500 µl homogenate was then centrifuged at 5500 RPM for 10 min at 4°C and the supernatant analyzed for TG, TC and free glucose. Total glucose was measured in 400 µl homogenate after over-night incubation at room temperature with 20 µl amyloglucosidase (Sigma, St. Louis, Missouri, USA). Subsequently, glycogen concentrations were calculated by subtracting free glucose from total glucose.

Markers of oxidative stress

Vitamin C, L-arginine and ADMA was quantified in liver homogenates as stated above. Hepatic levels of reduced (GSH) and oxidized (GSSG) glutathione were measured according to Hissin and Hilf (34) and malondialdehyde (MDA) was measured as described (35). Superoxide dismutase (SOD) was measured using colorimetry (Randox Superoxide Dismutase (Ransod assay), Randox Laboratories Ltd, Crumlin, United Kingdom) according to the manufacturer's specifications. α-Tocopherol and γ-tocopherol were analyzed by stabilizing liver homogenates with butylated hydroxytoluene and otherwise proceeding as described by Burton et al. (36).

Histology

Sections of the left lateral lobe (*lobus hepatis sinister lateralis*) were fixed in 4% paraformaldehyde in phosphate buffered saline for 72 hours at 4^{o}C, after which the tissue was stored in 1% paraformaldehyde at 4^{o}C prior to embedding in paraffin (21, 22). Tissue samples were cut into 2-4 µm sections and stained with haematoxylin & eosin and Masson's trichrome and scored in a blinded fashion in accordance with the semi-quantitative scoring scheme suggested by Kleiner et al. (37). 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). 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).

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 >2 mN were included in the study (corresponding to exclusion of segments with a tension <1mN as segments were 2 mm long). Vasodilator responses were determined by adding increasing concentration of the muscarinic acetylcholine receptor agonist carbachol (from 10^{-9} to $3{\cdot}10^{-5}$ M) (Sigma-Aldrich, St. Louis, Missouri, USA) to 60 mM potassium pre-contracted segments. In order to elucidate the carbachol-vasodilator responses, the carbachol concentration-response curves were obtained in absence (controls) or in presence of either the cyclooxygenase inhibitor indomethacin (10^{-4} M) (Sigma-Aldrich, St. Louis, Missouri, USA) or the endothelial nitric oxide synthase inhibitor N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) (10^{-5} M) (Sigma-Aldrich, St. Louis, Missouri, USA). 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 Enterprice Guide 7.1 (SAS Institute Inc, Cary, North Carolina, USA) and graphs were made using GraphPad Prism 7 (GraphPad Software, La Jolla, California, USA). Weight, energy intake and endothelial function was analyzed by repeated measures 2-way ANOVA, while plasma TG, VLDL-TG, TC, VLDL-C, LDL-C, HDL-C, ALT and AST were analyzed with an ANCOVA with baseline (week 0) as covariate. All other continuous data were analyzed using ANOVA. Tukey's post-hoc test was applied in all cases and, unless otherwise stated, data are presented as means with standard deviations (SD). Ordinal data (liver histology) are presented as medians and were analyzed with Kruskal-Wallis test followed by Dunn's post-hoc test (38). Data with inhomogeneous variances were log transformed before analysis and then back-transformed and presented as geometric means with 95% confidence intervals.

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 HFLC compared to HF ($p<0.01$) and HFC ($p<0.05$). 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 (Figure 1A). The average daily energy intake was decreased for HFL compared to HF at week 1, 2 and 4 post intervention ($p<0.01$) and in HFC and HFCL compared to HF at all post intervention time points ($p<0.01$). 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 week 1 and 2 post intervention ($p<0.001$) (Figure 1B).

Liver histology and status

High fat feeding induced NASH and severe (grade 3) fibrosis with close resemblance to human histopathology, i.e. bridging fibrosis and a pericellular/perisinoisodal (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 HF ($p<0.05$) and inflammation in HFL and HFCL compared to HF ($p<0.05$) (Figure 2A and B). Though not reaching statistical significance, hepatocyte ballooning was reduced in HFC and HFCL. Hepatic steatosis was lowered following dietary intervention in HFC and HFCL compared to HFL ($p<0.05$) and also tended to be decreased compared to HF. Bridging fibrosis was observed in most HF animals (80%) and was not significantly affected by treatments. Portal inflammation was not significantly different among groups, although a nominal reduction was observed by treatment with liraglutide in HFL compared to HF.

In all three intervention groups (HFL, HFC and HFCL) the absolute liver weight was decreased compared to HF animals (data not shown). Normalized to body-weight only HFC and HFCL animals displayed significantly reduced liver weights compared to HF ($p<0.05$). Likewise, hepatic cholesterol levels were decreased in HFC and HFCL compared to HF and HFL ($p<0.05$), but not in HFL compared to HF. Hepatic triglyceride levels did not differ between groups. Liver glycogen was not different between HF, HFL and HFC, but reduced in HFCL compared to HF ($p<0.05$) (Table 2). ALT and AST was elevated in HFCL compared to HF, HFL and HFC two weeks after intervention ($p<0.001$ and $p<0.05$, respectively), but did not differ between groups at any other time points (Figure 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, HFL and HFCL ($p<0.01$) 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 ($p<0.05$), HFC and HFCL ($p<0.01$) and γ-tocopherol compared to HFC ($p<0.05$). MDA, a marker of lipidperoxidation, was increased in HFCL compared to HF, HFL and HFC ($p<0.05$). There were no differences in hepatic anti-oxidants GSH, GSSG, %GSSG, SOD, or markers of oxidative stress L-Arginine, ADMA and the L-Arginine/ADMA ratio between groups (Table 2).

Dyslipidemia

Plasma TC, TG and fractionated (VLDL-TG, VLDL-C, LDL-C and HDL-C) lipoprotein levels confirmed an equal degree of dyslipidemia in all groups before intervention (week 0). Measurements were obtained at two additional time-points; week 2 and 4 (termination) after intervention (Table 3). HFL animals displayed an increase in VLDL-TG ($p<0.001$) at week 2, but was otherwise not significantly different from HF animals. In the HFC group, TC was decreased compared to both HF and HFL animals on both time points ($p<0.05$). A similar pattern was observed in HFCL, although only reaching statistical significance compared to HFL animals at week 2 ($p<0.001$). VLDL-C and LDL-C decreased in HFC animals on both time-points compared to HF ($p<0.05$). In the HFCL group, VLDL-C was increased compared to HF at week 2 ($p<0.05$) and compared to HFL at week 2 and 4 ($p<0.001$ and $p<0.05$, respectively), whereas LDL-C was decreased at week 2 compared to HFL ($p<0.001$). VLDL-TG was also found to be decreased in HFCL animals compared to HF animals at week 2 ($p<0.05$). At week 4, FFA was significantly increased in HFL compared to HF and HFC ($p<0.05$) and in HFCL compared to HF ($p<0.05$) (Table 4). On all other time-points and measures of dyslipidemia, 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 (data not shown) were equal between groups. Circulating levels of L-arginine, ADMA (data not shown), the L-Arginine/ADMA ratio and 8-isoprostanes were not different between groups. The BH_2/BH_4 ratio was increased in HFLC compared to HF and HFC (Table 4).

Endothelial dysfunction

The acetylcholine receptor agonist carbachol induced vasodilation that was completely abolished by the nitric oxide synthase inhibitor L-NAME ($p<0.001$) in both HF and HFL. The cyclooxygenase inhibitor,

indometacin, also reduced carbachol-induced vasodilation, although this was only significant for the HF group at carbachol concentrations of 10^{-7}, $3{\cdot}10^{-7}$ and 10^{-6} M ($p<0.05$) (Figure 4).

Discussion

The GLP-1 analogue liraglutide reduced NASH progression, by ameliorating hepatic inflammation and hepatocyte damage (ballooning) compared to high fat fed counterparts. Dietary intervention reduced dyslipidemia and tended to reduce hepatic steatosis and ballooning compared to HF. This directs attention towards the different targeted outcomes by the two applied interventions and suggests a putative additive or even synergistic effect of treatment combination in NASH, albeit the limitations of the present study weakens conclusions regarding such effects.

GLP-1 modulates inflammatory processes in multiple tissues, but it is currently not clear if these anti-inflammatory effects results from direct actions on immune cells or are secondary to the metabolic and weight-loss effects (19). The absence of hepatocyte GLP-1 receptors suggests that histological improvements are mediated by indirect effects of liraglutide (39, 40). Liraglutide has been found to inactivate the key pro-inflammatory c-Jun N-terminal kinase-1 (JNK) pathway in the liver (15) and to decrease circulating levels of CD163, a marker of macrophage activation which is increased in patients with NAFLD (41, 42), potentially accounting for some of the anti-inflammatory effects of liraglutide. The histological improvement by liraglutide was associated enhanced hepatic α-tocopherol (vitamin E) content and randomized clinical trials have found that vitamin E improves NASH histology in both adults and children (43, 44). Thus, it could be speculated that the beneficial effects of liraglutide may be linked to vitamin E associated effects. Liraglutide did not affect other included markers of hepatic redox-status, suggesting the effect of liraglutide is not exclusively linked to a reduction in hepatic oxidative (45). As previously demonstrated, long term high fat feeding in guinea pigs induces NASH with concomitant fibrosis (22). Similar hepatic changes were confirmed in the HF group of the current study, clearly indicating that

the observed histological improvement in HFL animals was due to liraglutide. However, since biopsies were not obtained pre-intervention, it cannot be ruled out that liraglutide delays the development of, rather than reversing NASH. The ability of liraglutide to decrease hepatic inflammation and hepatocellular ballooning is in agreement with previous studies in humans. Treatment with liraglutide for 48 weeks in patients with biopsy confirmed NASH facilitated histological resolution of NASH, including improvements in steatosis and hepatocyte ballooning, compared to placebo (20). Liraglutide also prevented progression, but did not induce regression, of fibrosis (20). In the current study, interventions may have been initiated after the development of fibrosis, obscuring a potential effect on fibrosis progression.

Similar to studies in humans in which liraglutide therapy for 12 weeks did not affect liver fat assessed by imaging techniques (46, 47), liraglutide did not decrease steatosis score or liver triglycerides. In humans and rats, NASH may decrease the expression of microsomal triglyceride transfer protein (MTTP) and compromise lipid export from the liver (48-50). It has been proposed that liraglutide reduces hepatic lipid content by increasing MTTP, which may increase VLDL-TG export (13). However, there was no effect on hepatic triglyceride levels in the current study. This could be due to the already advanced NASH at the time of intervention as well as the relatively short intervention-period, preventing clearly manifested effects. In murine models, decreased steatosis following liraglutide treatment has been reported (13-18, 51, 52). These models were all obese and glucose intolerant/insulin resistance, thus the effect of liraglutide may partly be mediated by a reduction in hyperglycemia and subsequent prevention of glucose-induced *de novo* lipogenesis in the liver (21, 22, 24, 53). The absence of glucose intolerance and obesity, in the high fat fed NAFLD/NASH guinea pig (21, 22), may then account for the unaffected hepatic steatosis following liraglutide treatment. In addition, progressive NASH may not have been present in the murine models as NASH was only histologically confirmed by a single study (17) and fibrosis described as mild in another (52), rendering the reported effects targeting NAFLD and possibly early stage NASH. Whether intervention at an earlier disease stage and/or longer duration of treatment will exert more profound effects on steatosis remains to be investigated.

Dyslipidemia was improved by dietary intervention reducing circulating levels of TC, VLDL-C and LDL-C in HFC and HFCL groups. 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 (54). Rats subjected to a high fat diet (10% lard, 20% sucrose, 2% cholesterol, 0.3% cholate) for eight weeks and then fed a low fat diet (4% fat) for an additional eight weeks had reduced dyslipidemia and improved hepatic steatosis and inflammation (55). This effect was more pronounced compared with the current study, in which dietary intervention tended to improve hepatocellular ballooning and steatosis, a tendency that may have been statistically significant had the duration been prolonged. Also, two animals of the HF group presented a very low steatosis grade. This was highly unexpected in comparison with our previous studies (21, 22), where a high degree of steatosis is consistently found in HF groups. However, as HFC and HFC 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.

In addition to utilizing a shorter induction and longer intervention period, hepatic fibrosis was not apparent in the rat model (55). The presence of advanced NASH in the guinea pigs could render them less responsive to treatment. In support of this, switching spontaneous hypertensive stroke prone rats from a high fat diet (35% fat, 5% cholesterol, 2% cholic acid) to a control diet (4.8% fat) reversed hepatic steatosis and down-regulated inflammatory genes and liver enzymes, but only if the intervention took place before the development of fibrosis (56). Following dietary intervention, oxidative stress was decreased in the liver, evident by increased vitamin C levels. We have previously reported that high fat diets decrease hepatic vitamin C levels suggesting that inadequate vitamin C status may contribute to the development of NASH (21, 57). Importantly, absolute food intake was not increased in HFC compared to HF (data not shown), suggesting that increased levels of hepatic vitamin C reflects an improvement in the anti-oxidant system and not increased dietary intake. The decreased circulating levels of vitamin C in HFL and HFCL may, however, be explained by lower absolute food intake in these groups compared to HF.

Endothelial function was investigated in coronary arteries from HF and HFL. Vasodilation was completely inhibited by L-NAME, indicating that carbachol-induced vasodilation was primarily mediated through a nitric oxide-dependent mechanism. Prostaglandin-induced vasodilation contributed to a smaller degree, since the cyclooxygenase inhibitor indometacin did reduce carbachol-induced vasodilation, although less than L-NAME. Liraglutide did not improve vasodilation in coronary arteries of guinea pigs fed a high fat diet, in agreement with unchanged circulating levels of 8-isoprostanes and BH_2/BH_4 and L-Arginine/ADMA ratios. We have previously reported the absence of atherosclerotic lesions in the aorta of guinea pigs fed a high fat diet for 16 weeks, thus it is possible the current experimental setup may not induce vascular dysfunction (21). Alternatively, potential effects 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 in liraglutide dose during the intervention period in the HFCL group, necessary to comply with the humane endpoint of 20% weight loss. Also, the four-week duration of intervention may have limited our ability to detect changes in steatosis and fibrosis in the current model.

In conclusion, liraglutide improved key histological features of NASH, even in the setting of advanced disease with severe fibrosis and a persistent intake of high fat/high cholesterol. Dietary intervention reduced dyslipidemia; however, longer treatment duration may be required to affect NASH. The combination of dietary intervention with liraglutide seems a promising approach targeting both NASH and dyslipidemia, but requires further investigation.

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Conflicts of interest

DH Ipsen, GF Skovsted, AM Schou-Pedersen and J Lykkesfeldt are supported by the LifePharm Centre for In Vivo Pharmacology. B Rolin and G Rakipovski are employed by Novo Nordisk A/S that produces liraglutide. The remaining authors declare no conflicts of interest that could influence the present work.

Author Contribution

The study was designed by DH Ipsen, P Tveden-Nyborg, B Rolin, G Rakipovski and J Lykkesfeldt and conducted by DH Ipsen, P Tveden-Nyborg, GF Skovsted, A Madsen, S Klostrup and AM Schou-Pedersen. All authors analyzed and interpreted the data. DH Ipsen wrote the draft manuscript, which was subsequently edited by all authors; PTN had responsibility for the final content. All authors have read and approved the final manuscript.

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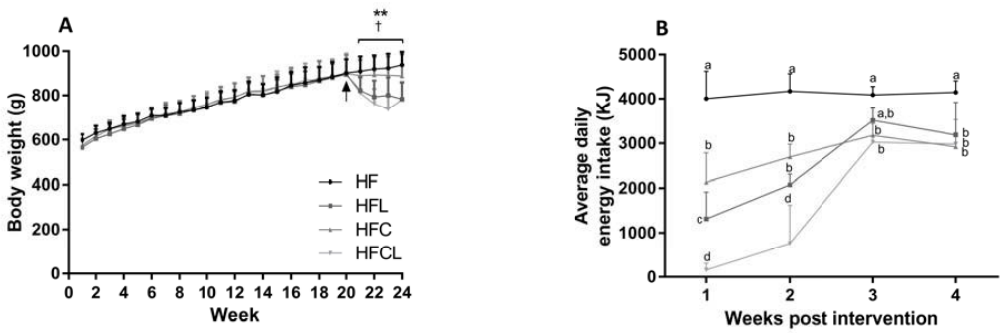
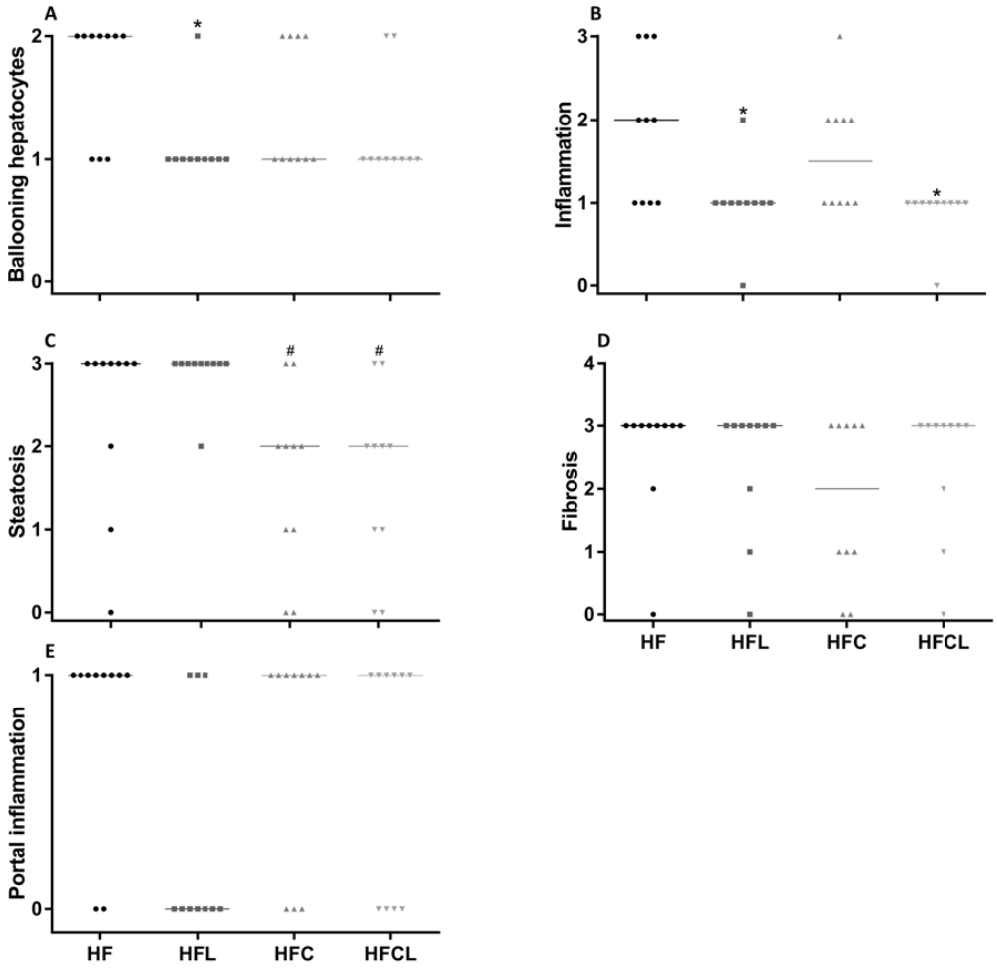
Figure 1 Body weight and energy intake



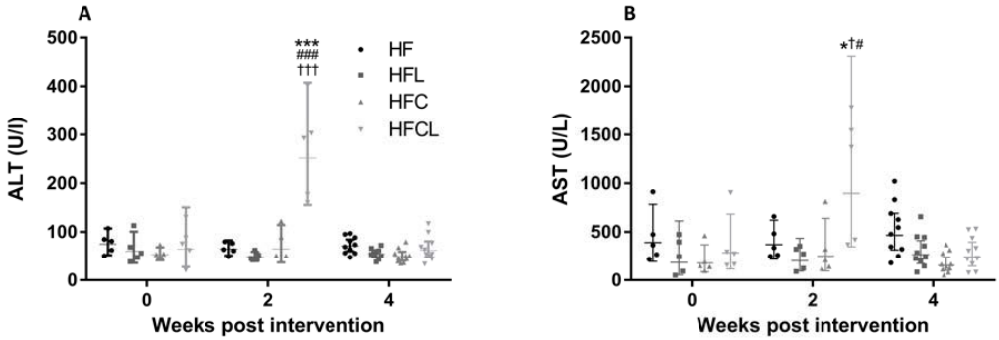
Body weight increased similarly in all groups prior to intervention (indicated by the arrow) after which the body weight stagnated in HFC and decreased significantly in HFL and HFCL compared to HF and HFC (A). The average daily energy intake followed the changes in body weight and decreased in HFL, HFC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

Figure 2 Liver histology



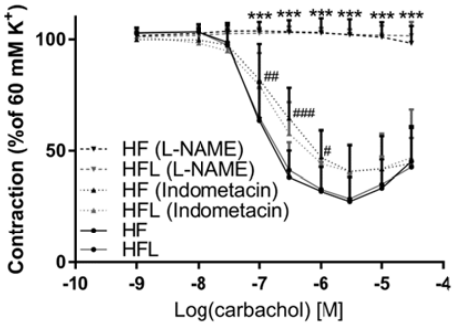
HFL displayed significantly fewer ballooning (degenerative) hepatocytes compared to HF animals (A), while HFL and HFCL reduced the degree of inflammation (B). Dietary intervention reduced hepatic steatosis compared to HFL and tended to reduce steatosis grade compared to HF (C). Bridging fibrosis was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

Figure 3 Liver enzymes



ALT (A) and AST (B) was 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. ALT: alanine aminotransferase. AST: aspartate aminotransferase.

Figure 4 Endothelial function



Carbachol induced vasodilation in HF and HFL treated animals, which could be inhibited by L-NAME. Indometacin tended to reduce carbachol-induced vasodilation, but this was only significant for HF at carbachol concentrations of 10^{-7}, $3{\cdot}10^{-7}$ and 10^{-6} M. *** p<0.001 HF(L-NAME) and HFL(L-NAME) vs. HF/HFL or HF/HFL(Indometacin). # p<0.05 ## p<0.01 ### p<0.001 HF(Indometacin) vs. HF. Means with SD. n = 6-8 (due to exclusion of segments with a tension <1mN). L-NAME: N^{ω}-Nitro-L-arginine methyl ester hydrochloride.

Table 1 Overview of 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	-

Table 2 Liver status at euthanasia (week four)

	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‡ (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	25.5±10.0	41.8±17.6*	21.9±8.31##	20.7±6.90##
γ-Tocopherol	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[a]	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‡	621 (351-1101)	909 (513-1609)	364 (206-646)	433 (245-767)

* p<0.05 ** p<0.01 *** p<0.001 vs. HF. # p<0.05 ## p<0.01 ### p<0.001 vs. HFL. † p<0.05 †† p<0.01 vs. HFC. Means with SD. ‡ Geometric means with 95% confidence intervals. [a] Calculated as GSSG/(GSH+GSSG). n = 10. ADMA: asymmetric dimethylarginine. ALP: alkaline phosphatase. DHA: dehydroascorbic acid. GSH: gluthathion. GSSG: oxidized glutathione. MDA: malondialdehyde. SOD: superoxide dismutase.

Table 3 Plasma lipids prior to and two and four weeks post intervention

Group	Week	TG	VLDL-TG	TC	VLDL-C	LDL-C	HDL-C
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)

* p<0.05 *** p<0.001 vs. HF. # p<0.05 ## p<0.01 ### p<0.001 vs. HFL. Geometric means with 95% confidence intervals. n = 5-10. HDL-C: high density lipoprotein cholesterol. LDL-C: low density lipoprotein cholesterol. TC: total cholesterol. TG: triglycerides. VLDL-C: very low density lipoprotein cholesterol. VLDL-TG: very low density lipoprotein triglycerides.

Table 4 Plasma lipids and markers of oxidative stress at euthanasia (week 4)

	HF	HFL	HFC	HFLC
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‡	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*†

* p<0.05 ** p<0.01 vs. HF. $^{\#}$ p<0.05 vs. HFL. † p<0.05 vs. HFC. Means with SD. ‡ Geometric means with 95% confidence intervals. n = 10. ADMA: asymmetric dimethylarginine. BH_2: di-hydrobiopterine. BH_4: tetra-hydrobiopterine. DHA: dehydroascorbic acid. FFA: free fatty acids.

Supplementary Table 1 Exact dietary composition

Nutrients (g/kg diet)	High-fat high sucrose	Chow
Alfalfa	220	220
Wheat	100	283
Barley	-	180
Sucrose	150	-
Cellulose (lignocellulose)	46.0	46.0
Sunflower meal	30.0	30.0
Soybean meal	60.0	120
Soybeans (full fat)	26.0	26.0
Soybean concentrate	120	20.0
Amino acids	5.00	5.00
Vitamins & trace element*	10.0	10.0
Vitamin C (Stay-C)	4.40	4.40
Salt (NaCl)	4.0	4.0
Calcium phosphate (monobasic)	16.4	12.9
Calcium propionate	5.5	5.5
Calcium carbonate	1.5	2.5
Choline Cl	3.0	3.0
Sugar beet pulp	10.0	10.0
Cholesterol	3.50	-
Coconut oil, hydrogenated	180	-
Soybean oil	5.0	21.0
Crude protein	167	168

Crude fat	200	42
Crude fiber	114	126
Crude ash	66	65
Starch	77	279
Sugar	176	38
Carbohydrates	379	471
Fatty acids		
C 6:0	0.6	-
C 8:0	9.9	-
C 10:0	9.3	-
C 12:0	81.9	-
C 14:0	35.4	0.2
C 16:0	22.7	6.3
C 18:0	22.0	1.6
C 20:0	0.3	0.2
C 16:1	0.1	0.3
C 18:1	5.6	8.7
C 18:2	9.8	21.9
C 18:3	2.2	3.7

Diets were manufactured by Ssniff Spezialdiäten GmbH, Soest, Germany.

*Vitamin & trace element content (addition per kg feed): 25.0 IU Vitamin A (E672), 1.50 IU, Vitamin D3 (E671), 0.125 g Vitamin E (all-rac-alpha-tocopherylacetate) (3a700), 0.08 g Vitamin K3 (MNB), 0.08 g Vitamin B1 (Thiamine mononitrate), 0.03 g Vitamin B2 (Riboflavin), 0.05 g Ca Pantothenate, 0.025 g Vitamin B6 (pyridoxol hydrochloride) (3a831), 0.00015 g Vitamin B12 (Cyanocobalamine), 0.09 g Niacin, 0.009 g

Folic acid, 0.0005 g Biotin, 0.100 g Inositol, 0.100 g Iron(II)-sulfate monohydrate (E1), 0.005 Copper(II)-sulfate pentahydrate (E4), 0.03 g Manganese (II)-sulfate monohydrate (E5), 0.002 g Cobalt(II)-carbonate monohydrate (E3), 0.05 g Zinc sulfate monohydrate (E6), 0.002 g Calcium iodate anhydrate (E2), 0.0001 g Sodium selenite (E8).

Paper IV

Normal weight dyslipidemia: Is it all about the liver? Ipsen DH, Tveden-Nyborg P, Lykkesfeldt J. Obesity (Silver Spring). 2016 Mar;24(3):556-67.

Review
OBESITY BIOLOGY AND INTEGRATED PHYSIOLOGY

Obesity

Normal Weight Dyslipidemia: Is It All About the Liver?

David Højland Ipsen, Pernille Tveden-Nyborg, and Jens Lykkesfeldt

Objective: The liver coordinates lipid metabolism and may play a vital role in the development of dyslipidemia, even in the absence of obesity. Normal weight dyslipidemia (NWD) and patients with nonalcoholic fatty liver disease (NAFLD) who do not have obesity constitute a unique subset of individuals characterized by dyslipidemia and metabolic deterioration. This review examined the available literature on the role of the liver in dyslipidemia and the metabolic characteristics of patients with NAFLD who do not have obesity.
Methods: PubMed was searched using the following keywords: nonobese, dyslipidemia, NAFLD, NWD, liver, and metabolically obese/unhealthy normal weight. Additionally, article bibliographies were screened, and relevant citations were retrieved. Studies were excluded if they had not measured relevant biomarkers of dyslipidemia.
Results: NWD and NAFLD without obesity share a similar abnormal metabolic profile. When compared with patients with NAFLD who have obesity, the metabolic abnormalities of NAFLD without obesity are similar or less severe. Furthermore, hepatic lesions develop independent of obesity, and the extent of dyslipidemia seems comparable.
Conclusions: NAFLD may impair hepatic lipid handling, causing faulty lipid homeostasis, and serves as a likely starting point for initiation and propagation of dyslipidemia along with associated comorbidities in patients without obesity.

Obesity (2016) **24**, 556–567. doi:10.1002/oby.21443

Introduction

Individuals with dyslipidemia, a distorted metabolic profile, and nonalcoholic fatty liver disease (NAFLD) often have obesity (1). However, individuals without obesity but with dyslipidemia and metabolic abnormalities constitute another important patient group here termed as normal weight dyslipidemia (NWD). The exact incidence of NWD in the general population is unclear; however, the current estimates suggest prevalence ranging from 10% to 37% (2). This subpopulation is also at increased risk of developing morbidities such as NAFLD, cardiovascular disease (CVD), and diabetes (3,4). NAFLD is the most common liver disease in the Western world, and prevalence rates are rising disturbingly in the Asia-Pacific regions (5,6). The disease comprises a range of hepatic conditions, extending from simple, reversible steatosis to nonalcoholic steatohepatitis (NASH) imposing irreversible liver damage, with current estimates suggesting that up to 20% of NASH cases will progress to cirrhosis and resulting liver failure (5). Furthermore, NAFLD is closely linked with dyslipidemia, hypertension, diabetes, CVD, and chronic kidney disease and constitutes an important health risk factor, although the exact pathogenesis remains incompletely understood (1). Even though obesity is recognized as an important risk factor, its causality to NAFLD is not clear, made evident by the fact that the disease is also common in individuals without obesity. The prevalence of NAFLD without obesity has been reported to range from 3.3% to 25.6% (7-11). This relatively large discrepancy regarding the prevalence of NAFLD without obesity may be explained by differences in lean body mass index (BMI) cutoffs; thus, lean BMI was defined as <24 kg/m^2 (10), <25 kg/m^2 (7-9), and <26 kg/m^2 (11). Additionally, two of the studies were conducted in populations of mixed ethnicity (7) or Turkish descent (9), and the remaining studies were conducted using Indians (8), Chinese (10), or Japanese (11) subjects, offering another potential explanation for the variation in prevalence. Although the exact reasons for ethnic differences in NAFLD are not clear, genetic modifiers (see later) have been identified as important contributing elements (12). Hence, NAFLD may develop independently of obesity and should not merely be considered a comorbidity of this condition. Both conditions share a similarly impaired metabolic homeostasis characterized by dyslipidemia, low-grade systemic inflammation, and insulin resistance (IR), hallmarking the metabolic syndrome (MS) (6, 13). As high levels of circulating lipids are thought to be essential in the development of many of the comorbidities commonly seen in metabolically unhealthy individuals (14), dyslipidemia may be a driving force behind disease development. Indeed, orchestrating lipid metabolism and regulating plasma lipid levels,

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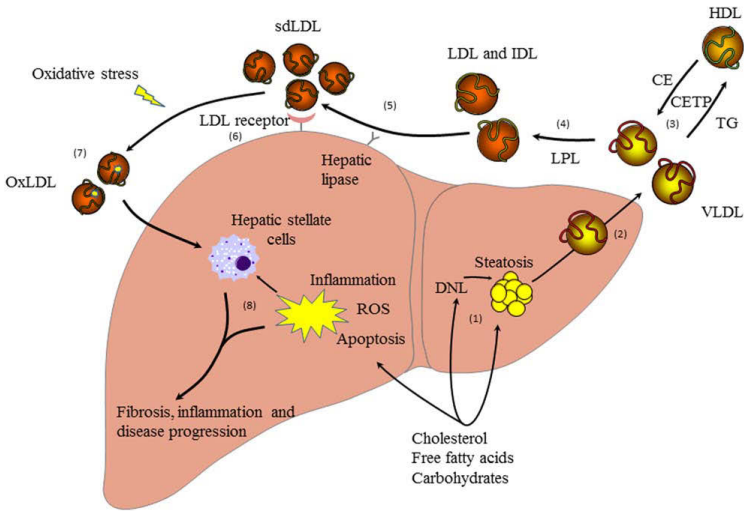

Figure 1 Pathophysiological relationship between NAFLD and dyslipidemia. **(1)** Dietary carbohydrates, free fatty acids, and cholesterol may all contribute to the development of NAFLD by inducing steatosis. Despite the hepatic fat accumulation, carbohydrates and cholesterol activate ChREBP and SREBP1c, respectively, enhancing *de novo* lipogenesis. **(2)** The liver not only stores excess lipids as triglycerides but also increases export of cholesterol and triglycerides through elevated synthesis and secretion of VLDL particles. **(3** However, enhanced CETP activity in the face of NAFLD increases the exchange of cholesteryl esters and triglycerides between HDL and VLDL. Thus, VLDL-cholesterol concentrations are raised, whereas HDL concentrations are decreased, promoting dyslipidemia and an atherogenic lipoprotein profile. **(4)** In the periphery, lipoprotein lipase converts circulating VLDL into LDL. **(5)** LDL may then be converted to small, dense LDL particles by hepatic lipase, which exhibits enhanced expression in NAFLD. **(6)** Normally, LDL is cleared from the circulation through the LDL receptor. However, NAFLD is also characterized by decreased expression of the LDL receptor, ultimately increasing circulating levels of LDL. **(7)** Oxidative stress facilitates generation of oxLDL that may be taken up by the liver through CD36 and subsequently activate hepatic stellate cells. **(8)** Furthermore, cholesterol and free fatty acids induce macrophage and hepatocyte apoptosis alongside inflammation and ROS, all leading to activation of hepatic stellate cells. Subsequently, the activated hepatic stellate cells deposit collagen and α-smooth muscle actin, resulting in hepatic fibrosis and disease progression. CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; CD36, cluster of differentiation 36; ChREBP, carbohydrate regulatory element binding protein; DNL, *de novo* lipogenesis; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; LPL, lipoprotein lipase; NAFLD, nonalcoholic fatty liver disease; OxLDL, oxidized low-density lipoprotein; ROS, reactive oxygen species; sdLDL, small, dense low-density lipoprotein; SREBP1c, sterol regulatory element binding protein 1c; TG, triglyceride; VLDL, very low-density lipoprotein. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the liver plays a central role in advancing an unhealthy lifestyle and diet into an unbalanced metabolic profile and further into increased morbidity and mortality. This narrative review discusses the role of the liver in dyslipidemia in individuals with normal weight and in relation to NAFLD. The literature was searched using the following keywords: nonobese, dyslipidemia, NAFLD, NWD, liver, and metabolically obese/unhealthy normal weight through the PubMed database, and screening included articles' bibliography, subsequently including relevant citations. The measurement of dyslipidemia was regarded as inclusion criterion.

Dyslipidemia and NAFLD

Dyslipidemia is defined by abnormal levels of lipids, for example, cholesterol, triglycerides, and/or specific lipoproteins, in the circulation and is an important component of NAFLD and CVD (14,15). In the vast majority of cases, dyslipidemia is due to lifestyle or disease (16). As such, the so-called Western diet comprising high amounts of sugar, saturated fatty acids, cholesterol, and trans-fatty acids alters lipid metabolism and promotes dyslipidemia (17). Increased lipids in the circulation cause ectopic deposition of lipids and promote development of atherosclerosis and NAFLD (1).

However, the progression from a stage of dyslipidemia to NAFLD and toward NASH seems to involve multiple hits as the development of steatosis, inflammation, and fibrosis takes place in parallel (Figure 1) (18). Hepatic lipids may originate from the diet, free fatty acids released from peripheral adipose tissue, and increased *de novo* lipogenesis (19). In cases with IR, hepatic lipid deposition is further propagated as the resulting hyperinsulinemia and hyperglycemia activate sterol regulatory element binding protein (SREBP) and carbohydrate regulatory element binding protein (ChREBP), respectively. These transcription factors upregulate lipogenetic genes, increasing *de novo* lipogenesis and subsequently hepatic lipid accumulation (20,21). Furthermore, cholesterol metabolites such as oxysterols serve as endogenous ligands for the liver X receptor α, which also mediates SREBP1c activation, ultimately enhancing fatty acid synthesis and steatosis (22). Additionally, both free fatty acids and

cholesterol may promote macrophage, hepatocyte apoptosis, and formation of reactive oxygen species (18,23,24). Oxidative stress is independently associated with NAFLD (25) and promotes IR, release of inflammatory cytokines, DNA damage, and impairment of membrane structures through lipid peroxidation (20,26). Disconcerted production of inflammatory cytokines further drives the development of NASH by promoting hepatocyte apoptosis and by activating hepatic stellate cells (27). The latter activation promotes the deposition of collagen and α-smooth muscle actin leading to hepatic fibrosis (28). Moreover, oxidized low-density lipoprotein (LDL) is also able to activate hepatic stellate cells, reiterating the link between dyslipidemia and NAFLD (29). NAFLD itself also contributes to dyslipidemia, as NAFLD has been shown to predict atherogenic dyslipidemia [low high-density lipoprotein (HDL) and high triglyceride levels] even after correcting for metabolic risk factors, adiposity, and IR (30). Accordingly, Siddiqui et al. (31) reported that NAFLD induced atherogenic dyslipidemia and that these changes were driven by increased hepatic lipid synthesis. Indeed, the lipid metabolism is altered in the face of NAFLD causing dyslipidemia: Very low-density lipoprotein (VLDL) secretion is increased in an attempt to compensate for hepatic triglyceride accumulation (32). The activity of cholesteryl ester transfer protein is enhanced, whereas LDL receptor expression is decreased, leading to increased circulating levels of VLDL-cholesterol and LDL (13). Additionally, enhanced expression of hepatic lipase increases the amount of small, dense LDL particles, which are more susceptible to oxidation and thought to play a pivotal role in the development of both NAFLD and CVD, one of the most common causes of death in NAFLD (1,13,29).

Several new studies suggest an important role of intrahepatic fat in the development of dyslipidemia and hepatic and peripheral IR in humans, which are pivotal components of type 2 diabetes (33-37). Accordingly, in a prospective study involving 109 patients with NAFLD and 249 healthy controls (a subset of the population-based Busselton Health study), patients with NAFLD at baseline were more likely to develop diabetes when compared with patients without NAFLD at baseline (38). Additionally, reduction of intrahepatic fat through dietary energy restrictions for 8 weeks decreased hepatic IR, but not peripheral IR, in 11 patients with type 2 diabetes (35). Fabbrini et al. (33) also emphasized the importance of intrahepatic fat contrary to visceral fat. By matching 31 subjects by visceral adipose tissue volume and intrahepatic fat content, they were able to show that intrahepatic fat, but not visceral adipose tissue, was independently associated with IR (33). The important role of the liver in the development of systemic IR and diabetes is further supported by two studies conducted in 42 subjects with nondiabetic obesity, but with varying amounts of hepatic fat (34), and 69 clinically diagnosed patients with NAFLD, respectively (36). Intrahepatic fat was associated with insulin action and IR in liver, skeletal muscles, and adipose tissue (34,36). This is important as dyslipidemia may be exacerbated by IR, causing even further metabolic imbalance (29). In addition, results from a study of 42 patients with obesity divided into groups based on BMI suggest that increased BMI and body fat may not necessarily cause dyslipidemia and metabolic abnormalities. Instead, increased intrahepatic fat seems more important, as increased visceral adipose tissue without increased intrahepatic fat did not alter secretion and clearance of VLDL in men and women with obesity *per se* (37). Clearly, intrahepatic fat accumulation and an unhealthy metabolic profile are important factors influencing long-term health and mortality.

Furthermore, genetic constitution has been shown to be involved in hepatic lipid droplet remodeling and VLDL secretion and is a contributing factor in the development of hepatic steatosis and progression toward NASH (12). In humans, the single-nucleotide polymorphism encoding isoleucine to methionine at position 148 in the patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) gene has been associated with increased liver fat, and it currently constitutes the largest known genetic impact on the development of NAFLD (12,39) and may also contribute directly to hepatic fibrogenesis and carcinogenesis (12). Additionally, a loss-of-function mutation in the transmembrane 6 superfamily member 2 gene substituting lysine for glutamic acid at position 167 has been associated with increased circulating lipoproteins, hepatic triglycerides, and NASH-related liver damage (12).

Normal Weight Dyslipidemia Phenotype

Excess body fat does not always appear to be associated with metabolic abnormalities (40), and although mechanisms underlying late-complications associated to NWD is currently not completely disclosed, increased ectopic fat accumulation particularly in the liver could be a risk factor directly associated with disease progression. This is supported by decreased amounts of intrahepatic fat in metabolically healthy individuals with obesity when compared with a general population with obesity (40). Additionally, in a cohort of 8,090 Japanese individuals without diabetes, an increased odds ratio of ultrasonographic fatty liver was found for NWD when compared with healthy controls with normal weight (41). When compared with obesity, NWD individuals display a similar metabolic profile, both with regards to lipid abnormalities, inflammatory cytokines, and IR (2,42,43), further supporting the notion that increased adiposity alone cannot fully account for an unhealthy metabolic profile and increased mortality.

Table 1 outlines the anthropomorphic and biochemical characteristics of NWD, although varying inclusion criterions and differences in the exact definitions of NWD between studies must be noted. However, generally applied features of NWD include dyslipidemia and increased blood glucose, waist circumference, and BMI when compared with controls (42-51). Although overall gender-specific differences were not marked in older (≥60 years) men and women from the NHANES III cohort (49), women with NWD ($n = 168$) had higher short-term CVD mortality, whereas men with NWD ($n = 200$) had higher long-term CVD mortality when compared with controls ($n = 597$ women and $n = 563$ men) (49). Additionally, in a Caucasian subset of participants from the Firenze-Bagno A Ripoli study comprising 490 individuals with obesity, 500 NWD, and 2,040 controls, women with NWD had higher levels of circulating lipids when compared with healthy individuals without obesity than did male NWD (43). Like in individuals with obesity and dyslipidemia, most NWD studies identify a dyslipidemia characterized by increased levels of total cholesterol, triglycerides, and LDL-C, whereas HDL-C is decreased (44-46,48,51). Hyun et al. (46) studied a group of 25 female NWD and 25 female controls pair-matched for age, height, weight, and menopausal status. Although LDL-C concentrations were not increased, LDL particle size was decreased signifying the development of an atherogenic lipoprotein profile (46). In contrast, other studies have found unaltered total cholesterol levels when compared with controls; however, subjects commonly

TABLE 1 Dyslipidemic and metabolic characteristics of normal weight dyslipidemia

Study design	Definition of NWD	Anthropomorphic and biochemical characteristics	Primary findings
Cohort NWD ($n = 187$) vs. controls ($n = 263$) or obesity ($n = 317$) (42)	BMI ≤ 23 kg/m^2 Presence of MS[a]	WC TG ↑ TC LDL-C ↔ HDL-C ↓ TC TG LDL-C HDL-C ↔ WC ↓	NWD is independently associated with coronary artery disease
Cohort NWD ($n = 500$) vs. controls ($n = 2,040$) (43)	BMI < 30 kg/m^2 Increased WC	TC TG LDL-C HDL-C Glu ↔	NWD with hypercholesterolemia may have increased risk of abnormal glucose metabolism
Cohort NWD ($n = 428$) vs. controls ($n = 2,745$) (44)	HOMA-IR within highest quartile	BMI WC TC TG LDL-C Glu MS ↑ HDL-C ↓	Lipid profiles identify high CVD risk groups such as NWD
Cohort NWD ($n = 28$) vs. controls ($n = 23$) (45)	VFA ≥ 100 cm^2 BMI ≤ 25 kg/m^2	BMI TC TG Insulin 8-epi-prostaglandin-F2α ↑ Glu ↔ Adiponectin HDL-C ↓	Increased oxidative stress may decrease adiponectin levels in NWD
Cohort NWD ($n = 25$) vs. controls ($n = 25$) (46)	VFA ≥ 100 cm^2 BMI ≤ 25 kg/m^2	TG FFA TNFα IL6 leptin oxLDL ↑ BF% sc. AT ↔ Adiponectin LDL-C ↔ TC LDL-size ↓	NWD has an atherogenic LDL profile
Prospective NWD ($n = 121$) vs. controls ($n = 542$) (47)	BMI ≤ 23 kg/m^2 Presence of MS[b]	BMI WC TG Glu ↑ TC ↔ HDL-C ↓	NWD has greater all-cause mortality
Prospective NWD ($n = 186$) vs. controls ($n = 1,267$) (48)	BMI < 25 kg/m^2 Presence of MS[c]	BMI WC TG Glu insulin IR ↑ HDL-C ↓	Obesity-related risk factors for CVD are already present in NWD
NHANES III NWD ($n = 505$ or 368) vs. controls ($n = 503$ or 1,160) (49)	BMI < 25 kg/m^2 BF% in upper tertile or BF% > 25% (men) and 35% (women)	WC TC TG leptin ↑ HDL-C Glu ↔	NWD is associated with increased risk for cardiovascular mortality
Cohort NWD ($n = 13$) vs. controls ($n = 58$) (50)	BMI < 26 kg/m^2 Impaired insulin sensitivity	BF% TC insulin ↑ WC TG LDL-C HDL-C Glu ↔	NWD may predispose to diabetes and CVD
Cohort NWD ($n = 111$) vs. controls ($n = 1,111$) (51)	BMI < 25 kg/m^2 Skin-fold measurement in 90th percentile or BF% > 23% (men) and 30% (women)	BMI WC TG LDL-C Glu IR ↑	NWD is associated with MS and IR

[a]MS defined as ≥ 3 metabolic abnormalities (systolic and diastolic blood pressure ≥ 130 and 85 mm Hg, respectively, or treatment for hypertension; TG ≥ 150 mg/dl; fasting glucose ≥ 100 mg/dl or treatment of type 2 diabetes; HDL-C <40 or <50 mg/dl for men and women, respectively; and WC ≥ 90 or 85 cm for men and women, respectively).
[b]MS defined as ≥ 1 metabolic abnormalities (systolic and diastolic blood pressure ≥ 130 and 85 mm Hg, respectively, or treatment for hypertension; TG ≥ 150 mg/dl; fasting glucose ≥ 100 mg/dl or treatment of type 2 diabetes; HDL-C <40 or <50 mg/dl for men and women, respectively).
[c]MS defined as ≥ 2 metabolic abnormalities (systolic and diastolic blood pressure ≥ 130 and 85 mm Hg, respectively, or treatment for hypertension; TG ≥ 150 mg/dl; fasting glucose ≥ 100 mg/dl or treatment of type 2 diabetes; HDL-C <40 or <50 mg/dl for men and women, respectively; and HOMA-IR > 5.13).
BF%, body fat percentage; BMI, body mass index; CVD, cardiovascular disease; FFA, free fatty acids; Glu, glucose; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; IL, interleukin; IR, insulin resistance; LDL-C, low-density lipoprotein cholesterol; MS, metabolic syndrome; NHANES, National Health and Nutrition Examination Survey; NWD, normal weight dyslipidemia; oxLDL, oxidized low-density lipoprotein; Sc. AT, subcutaneous adipose tissue; TC, total cholesterol; TG, triglycerides; TNFα, tumor necrosis factor α; VFA, visceral fat area; WC, waist circumference; ↑, increase; ↓, decrease; ↔, no change.

displayed significantly elevated plasma triglycerides, supporting the connection between dyslipidemia and NWD (42,46,47). Additionally, high levels of total cholesterol and triglycerides were independently associated with NWD in a cohort of 428 NWD and 2,745 non-NWD Korean subjects (44). High levels of circulating lipids and atherogenic alterations of lipoproteins suggest that the lipid metabolism of NWD may be affected and that the liver of NWD may not function optimally. Accordingly, results from a study equally dividing 30 men without obesity into two groups based on hepatic fat content found that subjects with increased hepatic fat also had increased levels of plasma triglycerides and insulin alongside lower HDL-C levels when compared with men without obesity and normal hepatic fat content (52). Furthermore, in a small cohort study, circulating levels of triglycerides were higher in male patients characterized by overweight and NASH ($n = 45$) when compared with male patients characterized by overweight without NASH ($n = 45$) matched for age, BMI, waist circumference, and visceral adipose tissue (53).

Induction of inflammatory cytokines and oxidative stress has been linked to the propagation of a dysfunctional metabolism in individuals with NWD. As such, a study of 28 NWD and 23 age-matched male controls with normal glucose tolerance found increased concentrations of 8-epi-prostaglandin-F2α and decreased levels of the anti-inflammatory adipokine adiponectin in NWD when compared with controls (45). In contrast, unchanged adiponectin levels have been reported in female NWD when compared with controls (46). However, in the subjects with NWD, oxidized LDL, tumor necrosis factor α, and interleukin 6 were all increased (46). Furthermore, oxidative stress correlated inversely with adiponectin levels, independent of visceral adipose tissue suggesting that oxidative stress may contribute to metabolic impairment in NWD (45). Interestingly, the liver has been implicated in systemic inflammation as NASH was reported to predict increased inflammatory markers in male patients with overweight, independently of visceral fat (53). Collectively, these metabolic abnormalities appear to maintain and facilitate CVD progression. Indeed, in a cohort of 187 NWD and 263 controls, previously admitted for evaluation of angina pectoris and/or positive stress findings, individuals with NWD had a higher prevalence and severity of angiographic coronary artery disease when compared with controls; however, this was not observed for metabolically healthy or unhealthy patients with obesity (42). The prospective South-West Seoul study investigated mortality of 121 NWD, 429 metabolically healthy patients with obesity, and 542 control subjects aged more than 60 years (47). Interestingly, individuals with NWD had higher all-cause mortality when compared with healthy controls or metabolically healthy individuals with obesity, even after adjusting for age, sex, smoking, alcohol drinking, diabetes, hypertension, and CVD (47). The authors speculated that low-grade systemic inflammation, oxidative stress, atherogenic LDL particles, and dyslipidemia may be underlying causes (47).

Poor diet and lack of exercise may increase the amount of circulating lipids and ectopic fat deposition thereby contributing to the development of NAFLD, diabetes, coronary artery disease, hypertension, and thus the NWD phenotype (3,18). Dietary energy intake has been reported to be similar between NWD and controls (46,50), although increased consumption of saturated fatty acids (46) and carbohydrates (54) has been reported in the former. Thus, high insulin levels following a meal rich in carbohydrates may predispose to fat storage and potential metabolic imbalance (54). Additionally, physical activity energy expenditure and level of exercise were lower in NWD (50,54), which could enhance the development of peripheral IR, decrease glucose tolerance, and induce unfavorable changes to the lipid profile (3). Therefore, the relative inactivity of NWD may be of importance in the pathogenesis. Ultimately, poor dietary composition may also facilitate the development of NAFLD in individuals with NWD (27).

NAFLD and its Comorbidities

Like NWD, NAFLD without obesity is characterized by impaired metabolic homeostasis, frequently with systemic inflammation and dyslipidemia with raised total cholesterol, triglycerides, LDL-C, and low HDL-C. When compared with healthy controls, and in accordance with compromised hepatic function, liver enzymes are elevated. Moreover, these patients display increased IR and higher blood glucose levels when compared with healthy controls (Table 2). Importantly, the liver is a key source of metabolic impairment, being responsible for overproduction of triglycerides and glucose, two main components of MS (63). It is therefore interesting that NAFLD—but not obesity—has been reported to be closely related to CVD risk factors (55,56). As such, NAFLD without obesity correlated independently with arterial stiffness in a cohort of subjects without obesity, diabetes, and hypertension, but with ($n = 246$) or without ($n = 1{,}050$) NAFLD (55). In addition, an Asian study of 2,108 patients with NAFLD who did not have obesity and 7,035 controls found that cardiovascular risk factors and the odds ratio for coronary events were increased in NAFLD without obesity when compared with controls without obesity (56). Vendhan et al. (64) examined South Indian patients with NAFLD, but with ($n = 125$) or without ($n = 48$) obesity, as well as 368 controls. The odds ratio for hypercholesterolemia was higher for patients with NAFLD but not obesity when compared with patients with obesity and NAFLD, despite displaying a similar metabolic profile (64). This increased risk of severe dyslipidemia in patients with NAFLD but without obesity could predispose them to CVD. In support of this, a retrospective study of 435 patients with NAFLD (mainly Caucasians) found coronary artery disease among the leading causes of death in patients with NAFLD (65). Furthermore, NAFLD without obesity has been reported to be associated with coronary artery disease even after adjusting for confounders, although this was not the case for NAFLD with obesity (64). These studies suggest an integral role of the liver and NAFLD in the development of both dyslipidemia and CVD, independent of obesity. Indeed, dyslipidemia was found in almost 90% of 205 consecutive patients with NAFLD but without obesity, constituting the most important metabolic risk factor in this subgroup (57), and thus, dyslipidemia and NAFLD seem tightly related. Applying the Ossabaw miniature pig as an experimental model, it has been shown that MS induced by a high-fructose diet without concomitant dyslipidemia does not cause liver injury hereby linking dyslipidemia to NAFLD development (66). Dyslipidemia also plays a pivotal role in endothelial dysfunction and atherosclerosis: elevated LDL-C levels are harmful as they build up in arterial walls, initiating the formation of atherosclerotic plaques (67). In addition, circulating levels of small, dense LDL particles which are increased in NAFLD (1,13,29) are closely related to CVD (14). Thus, increased uptake of oxidized LDL through upregulation of oxidized LDL receptors in blood vessels stimulates the expression of adhesion molecules and reduces nitric oxide bioavailability (68).

TABLE 2 Dyslipidemic and metabolic profiles in NAFLD without obesity when compared with healthy controls

Study design	Diagnosis	Liver function	Dyslipidemia	Metabolic profile	Primary findings
NHANES III mixed ethnicity cohort Nonobese ($n = 431$) vs. control ($n = 4,026$) (7)	Ultrasound Nonobese if BMI < 25 kg/m^2	ALT AST ↑	Hypercholesterolemia ↑	IR MS ↑	A distorted metabolic profile may be an underlying cause of NAFLD without obesity
Chinese cohort Nonobese ($n = 134$) vs. control ($n = 597$) (10)	Ultrasound Nonobese if BMI < 24 kg/m^2	ALT AST ALP γGT ↑	TC TG LDL-C ↑ HDL-C ↓	Insulin IR UA WBC ↑	NAFLD without obesity adds significant risk for diabetes and MS, but not hyperlipidemia
Japanese cohort Nonobese ($n = 64$) vs. control ($n = 1,718$) (11)	Ultrasound Nonobese if BMI < 26 kg/m^2		TC TG LDL-C ↑ HDL-C ↓		NAFLD without obesity is independently correlated with coronary risk factors
Chinese cohort Nonobese ($n = 246$) vs. control ($n = 1,050$) (55)	Ultrasound Nonobese if BMI < 28 kg/m^2	ALT AST ↑	TC LDL-C ↑ HDL-C ↓	IR MS WBC ↑	NAFLD without obesity is independently associated with arterial stiffness
Asian cohort Nonobese ($n = 2,108$) vs. control ($n = 7,035$) (56)	Ultrasound No exact definition of nonobese, but mean BMI 23.1 ± 3.0 kg/m^2		TC TG LDL-C ↑ HDL-C ↓	Glu WBC ↑	NAFLD without obesity increases the risk of CVD
Indian cohort Nonobese ($n = 27$) vs. control ($n = 131$) (57)	Ultrasound and biopsy Nonobese if BMI < 23 kg/m^2	ALT AST ALP ↑	TC TG LDL-C ↑ HDL-C ↓	MS ↑	Dyslipidemia is found in the vast majority of patients with NAFLD but without obesity
Asian cohort Nonobese ($n = 120$) vs. control ($n = 240$) (58)	Ultrasound Nonobese if BMI < 25 kg/m^2	ALT γGT ↑	TC TG LDL-C ↔ HDL-C ↓	Glu insulin IR UA ↑	IR and systemic inflammation may be key risk factors in inducing NAFLD without obesity
Korean cohort Nonobese ($n = 74$) vs. control ($n = 386$) (59)	Ultrasound Nonobese if BMI < 25 kg/m^2	ALT AST ↑	TC TG ↑ LDL-C ↔ HDL-C ↓	Glu insulin IR ↑	NAFLD is an early predictor of metabolic disorders in patients without obesity
Korean cohort Nonobese ($n = 1,611$) vs. control ($n = 4,267$) (60)	Ultrasound Nonobese if BMI < 23 kg/m^2	ALT γGT ↑	TC TG LDL-C ↑ HDL-C ↓	Glu insulin IR UA MS↑	NAFLD independently predicts risk of IR
Korean, retrospective Nonobese ($n = 107$) vs. control ($n = 1,054$) (61)	Ultrasound Nonobese if BMI < 25 kg/m^2		TC TG LDL-C ↑ HDL-C ↓	IR UA↑	Sustained NAFLD increases the risk of developing type 2 diabetes
Italian cohort Nonobese ($n = 20$) vs. control ($n = 30$) (62)	Ultrasound Nonobese if BMI < 30 (men) and < 28 (women) kg/m^2	ALT AST ALP γGT ↑	TG ↑ TC LDL-C ↔ HDL-C ↔	UA insulin ↑ Glu TNFα ↔ Adiponectin ↓	Patients with NAFLD but not obesity have increased cholesterol consumption

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CVD, cardiovascular disease; Glu, glucose; HDL-C, high-density lipoprotein cholesterol; IR, insulin resistance; LDL-C, low-density lipoprotein cholesterol; MS, metabolic syndrome; NHANES, National Health and Nutrition Examination Survey; TC, total cholesterol; TG, triglycerides; TNFα, tumor necrosis factor α; UA, uric acid; WBC, white blood cell count; γGT, γ-glutamyl transferase; ↑, increase; ↓, decrease; ↔, no change.

This offers some explanation to the increased risk of CVD in the dyslipidemic patient with NAFLD but no obesity. Dyslipidemia may be further augmented by peripheral IR as the activity of lipoprotein lipase in the endothelium is diminished, giving rise to hypertriglyceridemia (69). In addition, IR may enhance hepatic inflammation (18) and steatosis as hyperinsulinemia, regardless of IR, can promote hepatic lipid accumulation by activating anabolic pathways resulting in lipid synthesis and storage (69).

Several studies have reported increased levels of insulin and glucose alongside IR in patients with NAFLD and no obesity when compared with healthy controls without obesity (7,10,55,56,58-61). These studies imply that the sustained presence of NAFLD promotes the development of IR and diabetes. Accordingly, a retrospective study of Korean controls without obesity ($n = 1{,}054$) and patients with NAFLD ($n = 107$) reported that patients with NAFLD but not obesity had a higher odds ratio of having type 2 diabetes, IR, high triglycerides, and impaired fasting glucose when compared with controls without obesity (61). In addition to this, the odds ratio for IR was also higher for Korean patients with NAFLD but without obesity when compared with patients with NAFLD who were overweight (59). Pancreatic β-cell dysfunction preceded glucose intolerance in patients with NASH but without obesity ($n = 20$) compared to both insulin-sensitive ($n = 30$) and -insensitive ($n = 15$) controls (matched for age, sex, and BMI) and could contribute to the development of type 2 diabetes in these patients (62). From a metabolic point of view, several studies reported that NALFD without obesity may be comparable with NAFLD with obesity (Table 3) (8,9,59, 70). In contrast, other authors have found the metabolic profile of NAFLD without obesity to be less severe when compared with NAFLD with obesity (10,57,64,71). NAFLD without obesity and with almost no or only minor IR has also been reported (57), obscuring the connection between NAFLD without obesity and IR. However, even though metabolic abnormalities were less severe in Chinese subjects with NAFLD but without obesity ($n = 134$) when compared with NAFLD with obesity ($n = 764$), the patients without obesity were still dyslipidemic and more likely to develop diabetes, hypertension, and MS, prompting the authors to suggest that NAFLD without obesity may in fact be a more deleterious condition than NAFLD with obesity (10). Interestingly, not all cases of fatty liver are accompanied by a metabolic decline, suggesting the existence of a metabolically benign fatty liver (39) proposed to be more effective in detoxifying hepatic lipids. A degree of genetic predisposition is also suggested which could explain some, if not all, of the variations in metabolic profiles associated with NAFLD and degree of disease progression (73).

Inflammation is another key feature of the metabolic collapse linked to dyslipidemia and CVD, and a significant association has been reported between systemic inflammation and NAFLD without obesity (10,58). Consequently, in NAFLD without obesity, markers of systemic inflammation, that is, C-reactive protein, total leukocyte, and white blood cell count were all increased, whereas the anti-inflammatory adipokine adiponectin was decreased when compared with controls (10,56,58,62). As adiponectin acts as an insulin sensitizer, it may offer some explanation to the high levels of insulin and IR reported for NAFLD without obesity (18). When compared with patients with obesity and NAFLD, the degree of inflammation was less severe or similar (8,10,57,72). The liver has been implicated as an active participator in the development and maintenance of systemic inflammation, as hepatic inflammation caused by NAFLD has been suggested to contribute to the systemic inflammation in individuals with MS (74). In addition to this, inflammatory cytokines correlate with increased levels of circulating lipids and may even contribute to the development of dyslipidemia by reducing the activity of lipoprotein lipase in adipocytes (14).

Over time, NAFLD may progress to NASH—a more severe and irreversible stage of the disease characterized by inflammation, oxidative stress, hepatocellular ballooning, and fibrosis. Liver enzymes are most often increased regardless of obesity, suggesting a similarly impaired or in some cases even worse liver function in NAFLD without obesity (8-10,57,59,70,71). Accordingly, studies including liver biopsies from 229 (8) and 56 (70) patients with NAFLD reported the prevalence of NASH to be similar between subjects with or without obesity. Additionally, disease severity was not different, supported by equal NAFLD activity scores and uniform degrees of steatosis, fibrosis, and inflammation in subjects both with and without obesity; thus, once progressed, hepatic disease advancement occurs irrespective of obesity status (8,70). Conversely, liver biopsies from consecutive patients with NAFLD who were overweight/obese ($n = 446$) or lean ($n = 37$) displayed similar degrees of steatosis, inflammation, and hepatocellular ballooning, *albeit* less fibrosis in patients without obesity when compared with patients with obesity (9). However, the patients without obesity also had lower prevalence of MS when compared with patients with obesity (9), and as the presence of MS may increase the severity of hepatic lesions (70), this offers a potential explanation to the different degrees of fibrosis. In addition to this, decreased adiponectin levels correlated with steatosis, fibrosis, and inflammation grade regardless of obesity, thereby, constituting another potential factor in the development of NAFLD without obesity (62). Unfortunately, the studies (8,9,57,70,71) included both patients with steatosis and NASH and did not evaluate the contribution of advanced liver disease when compared with steatosis in patients with and without obesity, making the differentiation between disease stage and degree of obesity difficult. Nevertheless, the studies do suggest that the development and severity of NASH is independent of obesity and consequently other factors must drive in the development of NAFLD.

Accordingly, the diet influences the development of the NWD phenotype and may also affect the development of NAFLD in patients without obesity. Although dietary intake of protein, carbohydrates, and fat was found to be similar in healthy controls and patients with NAFLD who were lean or obese, an increased intake of dietary cholesterol and saturated fatty acids together with decreased intake of polyunsaturated fatty acids (PUFAs) has been noted for patients with NAFLD but not obesity (62,72). However, similar dietary cholesterol consumption between healthy controls and patients with NAFLD but without obesity has also been reported (7). In a controlled randomized trial, Caucasian patients with biopsy-confirmed NASH were treated with 3,000 mg omega-3 PUFA or placebo per day (75). After 1 year, NAFLD activity score and blood lipid composition were not improved; however, treatment did reduce liver fat (75). Accordingly, a meta-analysis including nine studies found a beneficial effect of omega-3 PUFA treatment on liver fat reduction (76). Interestingly, daily treatment with either 2,000 or 4,000 mg of omega-3 PUFA for 24 (77) or 8 weeks (78), respectively, also improved dyslipidemia (77,78). In contrast, a randomized controlled clinical trial found no effect of daily treatment with 945 mg omega-3 PUFA for 6 months. However, off-protocol intake of PUFA was likely increased in the

TABLE 3 Comparison of the dyslipidemic and metabolic profiles in NAFLD without obesity when compared with NAFLD with obesity

Study design	Diagnosis	Liver function	Dyslipidemia	Metabolic profile	Primary findings
NHANES III mixed ethnicity cohort Nonobese ($n = 431$) vs. obese ($n = 2,061$) (7)	Ultrasound Nonobese if BMI < 25 kg/m^2 Obese if BMI > 25 kg/m^2	Patients with NAFLD ALT AST ↓	Hypercholesterolemia ↓	IR MS ↓	The metabolic profile of patients with NAFLD differs based on obesity status
Indian cohort Nonobese ($n = 119$) vs. obese ($n = 346$) (8)	Ultrasound and biopsy Nonobese if BMI < 25 kg/m^2 Obese if BMI > 25 kg/m^2	Patients with NAFLD and NASH[a] ALT AST γGT ↔ Steatosis ↔ Inflammation ↔ Ballooning ↔ Fibrosis ↔	TC TG LDL-C HDL-C ↔	Glu IR↔	The severity of NAFLD is independent of obesity
Turkish cohort Nonobese ($n = 37$) vs. obese ($n = 446$) (9)	Biopsy Nonobese if BMI < 25 kg/m^2 Obese if BMI > 25 kg/m^2	Patients with NAFLD and NASH[a] Steatosis ↔ Inflammation ↔ Ballooning ↔ Fibrosis ↓	TC TG LDL-C HDL-C ↔	IR ↔ MS ↓	Excluding fibrosis, the severity of NAFLD is independent of obesity
Japanese cohort Nonobese ($n = 134$) vs. obese ($n = 764$) (10)	Ultrasound Nonobese if BMI < 26 kg/m^2 Obese if BMI > 26 kg/m^2	Patients with NAFLD ALT ↑ AST ALP ↔	HDL-C ↑ TC TG LDL-C ↔	UA insulin WBC ↓	NAFLD without obesity may be a more dangerous condition than NAFLD with obesity
Indian cohort Nonobese ($n = 48$) vs. obese ($n = 125$) (64)	Ultrasound Nonobese if BMI < 23 kg/m^2 Overweight/obese if BMI > 23 kg/m^2	Patients with NAFLD ALT AST γGT ↔	HDL-C ↑ TC TG LDL-C ↔	Glu IR ↓	NAFLD without obesity, but not NAFLD with obesity, was associated with coronary heart disease
Indian cohort Nonobese ($n = 27$) vs. obese ($n = 141$) (57)	Ultrasound and biopsy Nonobese if BMI < 23 kg/m^2 Obese if BMI > 25 kg/m^2	Patients with steatosis and NASH[a] ALT AST ALP ↔ Fibrosis ↓	TC TG HDL-C LDL-C ↔	Insulin IR ↓	The metabolic profile and hepatic fibrosis of NAFLD without obesity is less severe than NAFLD with obesity
Korean cohort Nonobese ($n = 74$) vs. obese ($n = 106$) (59)	Ultrasound Nonobese if BMI < 25 kg/m^2 Obese if BMI > 25 and < 30 kg/m^2	Patients with NAFLD ALT AST ↔	TC TG LDL-C HDL-C ↔	Glu IR ↔ Insulin ↓	NAFLD is closely associated with metabolic disorders regardless of obesity

TABLE 3. (continued).

Study design	Diagnosis	Liver function	Dyslipidemia	Metabolic profile	Primary findings
Greek cohort Nonobese ($n = 19$) vs. obese ($n = 143$) (70)	Ultrasound and biopsy Nonobese if BMI < 25 kg/m^2 Obese if BMI > 25 kg/m^2	Patients with NAFLD and NASH[a] ALT AST ↑ Steatosis ↔ Inflammation ↔ Fibrosis ↔	TC TG LDL-C HDL-C ↔	Glu UA MS ↔	The severity of NAFLD is independent of obesity
Indian cohort Nonobese ($n = 30$) vs. obese ($n = 120$) (71)	Ultrasound and biopsy Nonobese if BMI < 23 kg/m^2 Obese if BMI > 23 kg/m^2	Patients with NAFLD and NASH[a] ALT γGT ↔	TC TG LDL-C HDL-C ↔	IR ↓	The metabolic profile of NAFLD without obesity is similar to that of NAFLD but not obesity
Japanese cohort Nonobese ($n = 12$) vs. obese ($n = 44$) (72)	Ultrasound, computed tomography, or biopsy Nonobese if BMI < 25 kg/m^2 Obese if BMI > 25 kg/m^2	Patients with NAFLD	TC TG LDL-C HDL-C ↔	Insulin ↑ Glu IR ↔ Adiponectin ↔ TNFα leptin ↔	Patients with NAFLD but not obesity have increased cholesterol and decreased polyunsaturated acid consumption

[a]Results are based on pooled data from patients with NAFLD and patients with NASH.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Glu, glucose; HDL-C, high-density lipoprotein cholesterol; IR, insulin resistance; LDL-C, low-density lipoprotein cholesterol; MS, metabolic syndrome; NHANES, National Health and Nutrition Examination Survey; TC, total cholesterol; TG, triglycerides; TNFα, tumor necrosis factor α; UA, uric acid; WBC, white blood cell count; γGT, γ-glutamyl transferase; ↑, increase; ↓, decrease; ↔, no change.

TABLE 4 Dyslipidemic and metabolic characteristics in animal models of NAFLD without obesity relating to dyslipidemia

Species/diet %w/w)/duration	Lipid profile	Metabolic profile	Hepatic histology	Comments
Japanese white rabbits/1% cholesterol/12 weeks (82)	TC HDL-C LDL-C ↑ TG ↓	TGFβ ↑ Insulin ↔	Thin, fibrosis (occasionally bridging), steatosis, ballooning hepatocytes, Mallory bodies, inflammation	Steatosis was microvesicular Inflammation not prominent
Japanese white rabbits/ HFD + 0.75% cholesterol/ 9 months (85)	TC TG VLDL-C LDL-C ↑ FFA HDL-C ↔	Glu Insulin IR ↓	Steatosis, inflammation, bridging fibrosis (grade 3[a]), ballooning hepatocytes	Histopathology resembles humans Hepatic TG not increased
C57BL/6J mice/HFD +1.25% cholesterol + 0.5% cholate/24 weeks (83)	TC FFA ↑ TG ↓	IR ↑	Steatosis, inflammation, fibrosis, ballooning hepatocytes	Weight loss on diet
Hartley guinea pigs/ HFD + 0.25% cholesterol/ 12 weeks (86)	LDL-C ↑ FFA HDL-C ↔ VLDL-C TG ↓		Steatosis (grade 3[b]), mild inflammation (grade 1[c]), no Mallory bodies, no fibrosis	Both diets contain cholesterol but varying amounts of fat
Hartley guinea pigs/ HFD + 0.33% cholesterol/ 6 months (87)	TC ↑		Steatosis, fibrosis	

[a]Grade 1: zone 3 perisinusoidal/pericellular fibrosis; focally or extensively present. Grade 2: zone 3 perisinusoidal/pericellular fibrosis with focal or extensive periportal fibrosis. Grade 3: zone 3 perisinusoidal/pericellular fibrosis and portal fibrosis with focal or extensive bridging fibrosis. Grade 4: cirrhosis.
[b]Based on the amount of hepatic fat infiltration: grade 1: <5%; grade 2: <33%; grade 3: 34-66%; and grade 4: >66%.
[c]Rare inflammatory foci.
FFA, free fatty acids; Glu, glucose; HDL-C, high-density lipoprotein cholesterol; HFD, high-fat diet; IR, insulin resistance; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides; TGFβ, transforming growth factor β; VLDL-C, very low-density lipoprotein cholesterol; ↑, increase; ↓, decrease; ↔, no change.

placebo group, possibly confounding the results (79). Thus, PUFA supplementation may reduce the adverse effects of developing dyslipidemia and NAFLD, whereas a decreased dietary intake of PUFA could accelerate the development of dyslipidemia and hepatic fat accumulation. Cholesterol also plays a key role in the induction of NAFLD: cytotoxic free hepatic cholesterol increases with disease severity in humans (80,81), and dietary cholesterol induces NAFLD in animal models, even in the absence of obesity (82,83). As enhanced cholesterol levels may mediate increased *de novo* lipogenesis through the liver X receptor α-pathway (22), it is of interest that hepatic gene expression of liver X receptor α was higher in patients with NAFLD without obesity ($n = 10$) when compared with patients with NAFLD with obesity ($n = 23$) and that the expression was higher for both groups when compared with healthy controls (22). Additionally, pharmacological inhibition of cholesterol absorption has a beneficial effect on liver histology in patients with NAFLD (84). Thus, increased dietary cholesterol may play a central role in hepatic fat accumulation, especially in NAFLD without obesity. Furthermore, both cholesterol and saturated fatty acids mediate an increase in circulating levels of total cholesterol and LDL-C, thereby contributing to the development of dyslipidemia as well as NAFLD (17). Moreover, saturated fatty acid intake as a percentage of caloric intake correlated negatively with insulin sensitivity (62). Collectively, abundant cholesterol and saturated fatty acid consumption appears to act in concert with decreased intake of PUFA to induce and maintain hepatic steatosis and to fuel the progression toward NASH in subjects without obesity.

Animal Models of NAFLD Without Obesity Related to Dyslipidemia

Animal models using diet-induced dyslipidemia to model NAFLD without obesity are sparse (Table 4). Feeding rabbits with 0.75-1% cholesterol with or without a concomitant high-fat diet induced dyslipidemia and hepatic NAFLD-like changes (82,85). Although evidence of hepatic DNA damage and oxidative stress was present on cholesterol feeding (82), the histopathological changes were most severe when combined with high-fat feeding, in which case the hepatic changes closely resembled with those of humans (85). In contrast to humans, however, plasma cholesterol was mainly found in VLDL particles and hepatic triglycerides were not increased (85). Notably, neither alanine aminotransferase nor aspartate aminotransferase was elevated (82,85), supporting an intact liver function and in agreement with the apparent lack of hepatocellular triglyceride accumulation. This is in contrast to humans, in which triglyceride deposition with subsequent liver damage and reduced liver function are hallmarks of developing NAFLD (7,10,55,57-60,62). Mice placed on a high-fat diet supplemented with 1.25% cholesterol and 0.5% cholate for 24 weeks displayed progressive liver steatosis, inflammation, and fibrosis in a time-dependent manner. These changes were accompanied by ballooning hepatocytes, Mallory bodies, hepatic IR, and oxidative stress (83). Still, very high cholesterol concentrations were used to produce this effect, and the mice lost weight during the study period, most likely due to a protein content of the diet below the maintenance requirement for mice (88). Similarly, diets deficient in methionine and choline induce hepatic

steatosis, inflammation, and the characteristic chicken-wire fibrosis in rats and mice (89). However, deficiency of methionine or choline is rarely the cause of NAFLD in humans. Moreover, plasma triglycerides and cholesterol are decreased and accompanied by severe weight loss in these models (89). Consequently, they do not recapitulate the central dyslipidemic aspect of NAFLD. In humans, LDL is the predominant lipoprotein. However, most rodent species transport circulating cholesterol in HDL particles and lack several important enzymes involved in the lipid metabolism of humans. This constitutes a distinct and noteworthy difference that may influence the validity of the models. In contrast, the lipoprotein profile of the guinea pig mirrors that of humans, and both species have cholesteryl ester transfer protein activity. Therefore, the guinea pig may be a more appropriate model in these regards (90). Indeed, guinea pigs fed a high-fat diet supplemented with cholesterol developed dyslipidemia with enhanced LDL-C concentrations, and NAFLD was confirmed by hepatic steatosis and inflammation (86,87). With increased dietary cholesterol, hepatic fibrosis was also induced, hereby demonstrating the model's ability to mimic human dyslipidemia and evolving hepatic damage including progression from NAFLD to NASH (87). Currently, no single experimental model recapitulates the dyslipidemic and metabolic profile of human NAFLD, highlighting the necessity of investigating novel approaches to increase animal model validity and translational value in the quest for elucidating the etiology and treatment of this complex disease.

Concluding Remarks

Compromised liver metabolism in the face of hepatic lipid accumulation may lead to increased synthesis and secretion of lipids, thereby causing dyslipidemia and creating a vicious cycle in which circulating lipids exacerbate NAFLD even further. Indeed, patients with NAFLD exhibit dyslipidemia to a similar extent regardless of obesity status. Although the exact pathophysiology of NAFLD without obesity remains to be established, increased intake of dietary cholesterol and saturated fatty acids alongside reduced intake of PUFA may be the underlying causes of dysregulated hepatic lipid metabolism, subsequently promoting disease development. Thus, hepatic steatosis and NASH hamper the ability of the liver to adequately control systemic lipid homeostasis, thereby singling out the liver as the likely starting point for systemic dyslipidemia and its associated comorbidities.

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Paper V

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METABOLISM (J PROIETTO, SECTION EDITOR)

Dyslipidemia: Obese or Not Obese—That Is Not the Question

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Abstract
Purpose of Review Purpose of review: It is becoming increasingly clear that some obese individuals do not develop dyslipidemia and instead remain healthy, while some normal weight individuals become dyslipidemic and unhealthy.
Recent Findings The present review examines the similarities and differences between healthy and unhealthy individuals with and without obesity and discusses putative underlying mechanisms of dyslipidemia.
Summary The presence of dyslipidemia and compromised metabolic health in both lean and obese individuals suggests that the obese phenotype per se does not represent a main independent risk factor for the development of dyslipidemia and that dyslipidemia, rather than obesity, may be the driver of metabolic diseases. Notably, adipose tissue dysfunction and ectopic lipid deposition, in particular in the liver, seems a common trait of unhealthy individuals.

Keywords Dyslipidemia · Obesity · Metabolically healthy obese · Metabolically unhealthy obese · Metabolically unhealthy normal weight

This article is part of the Topical Collection on *Metabolism*

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Abbreviations

BMI	Body mass index
FFA	Free fatty acids
FGF21	Fibroblast growth factor 21
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
HN	Healthy normal weight
HO	Healthy obese
MS	Metabolic syndrome
PPAR	Peroxisome proliferator-activated receptor
UN	Unhealthy normal weight
UO	Unhealthy obese
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
SAT	Subcutaneous adipose tissue
VAT	Visceral adipose tissue
VLDL	Very low-density lipoprotein

Introduction

Obesity currently affects one third of the World's population [1] and is associated with increased mortality as well as being considered an important risk factor for cardiovascular diseases, type-2 diabetes, and non-alcoholic fatty liver disease (NAFLD) also known as lifestyle-associated diseases [2]. Though an association between obesity and health problems is well established, the link may—in reality—not be as simple. In fact, findings from experimental animal models and human studies collectively suggest that the increased morbidity in obesity is mainly due to increased levels of circulating lipids resulting in ectopic lipid deposition and lipotoxicity, which in turn negatively impacts metabolism and tissue function, suggesting dyslipidemia as the primary inducer of disease development [3, 4].

Recently, two subgroups have attracted particular attention: the healthy obese (HO) and the unhealthy normal weight (UN) individuals. Despite being overweight or obese, HO individuals display normal levels of circulating lipids and decreased risk for cardiovascular and other related diseases [5, 6]. Contrary to this, UN individuals display increased levels of circulating lipids and increased risk of disease and mortality despite being categorized as normal weight [6]. This implies that it is not obesity per se which promotes dyslipidemia, metabolic imbalance, and increased mortality. Instead, this may be facilitated by an excessive intake of dietary fat which increases hepatic and circulating lipids resulting in a dyslipidemic plasma profile, characterized by elevated levels of triglycerides, cholesterol, free fatty acids (FFA), and/or decreased levels of high-density lipoprotein (HDL) [7]. To accommodate persistent dyslipidemia, adipocytes increase the storage of triglycerides through hyperplasia and/or hypertrophy. Whereas hyperplasia allows for increased triglyceride storage in the adipose tissues without fuelling the detrimental dyslipidemic cascade, hypertrophy results in pathological alterations in adipocyte metabolism and macrophage infiltration of the adipose tissue [8, 9]. This leads to the release of inflammatory cytokines and FFA to the circulation, further exacerbating the dyslipidemic state [3, 4].

This review investigates the current literature concerning the underlying mechanisms of dyslipidemia by searching PubMed using the following keywords: dyslipidemia/hyperlipidemia, metabolically healthy/normal obese, metabolically unhealthy/abnormal obese, metabolically unhealthy/abnormal normal weight, and metabolically healthy/normal normal weight. Subsequently, article bibliographies were screened and relevant citations included. The current review proposes that ectopic lipid deposition, adverse lipid metabolism, and dysfunctional adipose tissue, rather than obesity, are the primary driving forces when regarding dyslipidemia.

The Influence of the Adipose Tissue on Development of Dyslipidemia

Energy is mainly stored in the form of triglycerides in white adipose tissue found predominantly in subcutaneous (SAT) and visceral (VAT) depots. The adipose tissue is able to expand and remodel hereby adapting to alternating energy resource levels and requirements. However, in case of chronic over-nutrition and excess of dietary fat and cholesterol, adipocyte dysfunction and ectopic deposition of excess lipids induces lipotoxicity [8, 10]. Eventually, this leads to improper lipid handling, mitochondrial and endoplasmatic reticulum dysfunction, oxidative stress, and inflammation [10]. Adipocyte hypertrophy seems particularly important in the propagation of adipose tissue impairment; isolates from human adipocytes show that the release of pro-inflammatory cytokines and chemo attractants increases with the size of adipocytes, suggesting that hypertrophied adipocytes promote adipose tissue inflammation and attract macrophages [11]. Concurrently, macrophages impair adipogensis, prompting adipocyte hypertrophy, which further enhances the recruitment of inflammatory cells [12]. Thus, a vicious cycle is established, continuously exacerbating the dysfunctional state of the adipose tissue. Macrophages are present, mainly as M2 anti-inflammatory macrophages, in the adipose tissue prior to inflammation. However, adipose tissue dysfunction is characterized by macrophage infiltration and, importantly, a phenotypic switch from M2 towards M1 inflammatory macrophages [13]. M1 macrophages contribute to development and maintenance of adipose tissue dysfunction by secreting inflammatory cytokines, thereby impairing insulin signaling and causing systemic inflammation [14]. Inflammation also decreases the release of adiponectin, a key anti-inflammatory and insulin-sensitizing adipokine [15, 16]. The ensuing insulin resistance enhances lipolysis resulting in increased levels of circulating FFA [8, 10]. Notably, hypertrophied adipocytes have increased rates of lipolysis and are able to cause insulin resistance independent of inflammation [17, 18]. In turn, increased lipolysis may promote macrophage infiltration [19] and thereby maintain or even promote adipocyte dysfunction and the inflammatory state of the adipose tissue. Conversely, inhibition of lipolysis without changing fat mass or adipose tissue inflammation results in improved insulin sensitivity and glucose homeostasis in mice [20]. Dysfunctional adipocytes are resistant to the anti-lipolytic effects of insulin which further increases the release of FFA, inflammatory cytokines, and adipokines [9, 21]. Furthermore, FFA may then themselves contribute to development and maintenance of adipocyte insulin resistance. In addition to this, FFA exerts lipotoxic effects on other tissues and organs causing systemic metabolic impairment which adversely affects the lipid homeostasis even further [22, 23] (Fig. 1a).

Whole-Body and Adipose Tissue Metabolism is Altered in Unhealthy Individuals

Whole-body and tissue-specific metabolism differs between healthy and unhealthy individuals (Table 1), and dysfunctional metabolism may mediate the progression towards an unhealthy phenotype. In a cross-sectional study of 172 overweight and obese subjects (body mass index (BMI) >24.9 kg/m^2), fat utilization, assessed by the Respiratory Quotient, was decreased in unhealthy obese individuals (UO) with the metabolic syndrome (MS) compared to HO without MS and correlated with insulin resistance [49]. Though not included as a primary endpoint, the finding suggests that the ability of healthy individuals to properly utilize fat may be protective from the adverse effects of ectopic fat deposition [49]. Additionally, the metabolism of amino acids,

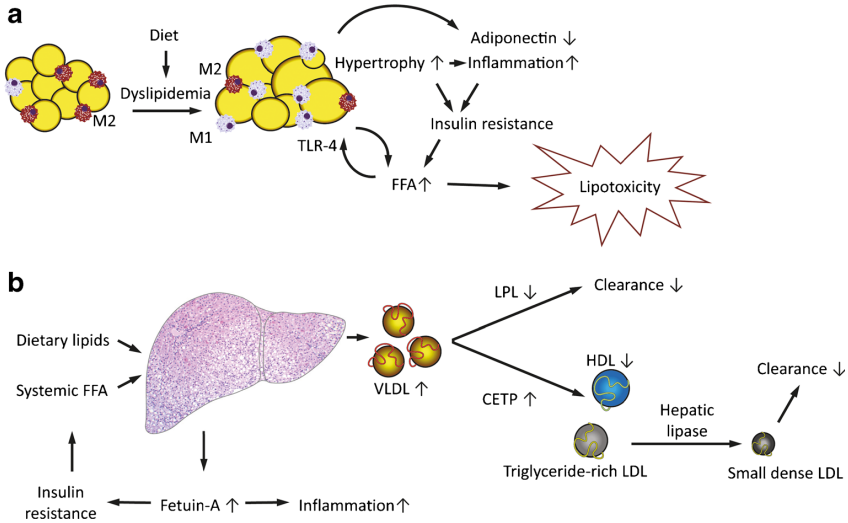

Fig. 1 **a** *The role of adipose tissue dysfunction in dyslipidemia*. A diet rich in fat and cholesterol and/or chronic over-nutrition causes dyslipidemia, resulting in adipocyte hypertrophy. Additionally, this induces a phenotypic switch from anti-inflammatory M2 macrophages to pro-inflammatory M1 macrophages resulting in adipose tissue dysfunction [8, 13]. Levels of inflammatory cytokines are increased and adiponectin levels decreased which, alongside adipocyte hypertrophy, promotes insulin resistance. In the course of insulin resistance and adipocyte hypertrophy, lipolysis is increased causing the adipose tissue to release more FFA. FFA may then cause lipotoxicity in other tissues, leading to systemic metabolic impairment. However, FFA also directly affects the adipose tissue by binding to TLR-4, thereby activating the nuclear factor kappa B pathway and promoting the production of inflammatory cytokines [24]. Ultimately, this results in propagation of inflammation and insulin resistance [22, 24], establishing a vicious cycle which continuously promotes dyslipidemia and metabolic deterioration. **b** *The role of non-alcoholic fatty liver disease in dyslipidemia*. Excess intake of dietary lipids alongside increased levels of systemic FFA may accumulate in the liver as triglycerides. Consequently, hepatic triglyceride export is enhanced, resulting in hypertriglyceridemia. Decreased LPL activity due to inflammation and insulin resistance reduces the clearance of VLDL particles, thus augmenting hypertriglyceridemia [25–28]. Concomitantly, increased activity of CETP and hepatic lipase reduces the levels of HDL particles while increasing the levels of small dense LDL particles, thereby decreasing their affinity for the LDL-receptor and promoting a state of atherogenic dyslipidemia [29]. Hepatic fat accumulation also alters hepatokine secretion by increasing secretion of Fetuin-A, which facilitates insulin resistance and ultimately increases circulatory levels of FFA. Importantly, Fetuin-A also acts as an adaptor protein, facilitating the binding of FFA to TLR-4, providing a link to the crosstalk between the liver and adipose tissue which ultimately results in dyslipidemia [30–32]. *CETP* cholesteryl ester transfer protein, *FFA* free fatty acids, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *LPL* lipoprotein lipase, *TLR-4* Toll-like receptor 4, *VLDL* very low-density lipoprotein

fatty acids, and cell membrane components is altered in primary adipocytes isolated from the subcutaneous adipose tissue of insulin-resistant UO ($n = 10$, BMI >40 kg/m^2) compared to insulin-sensitive HO individuals ($n = 10$, BMI

Table 1 Characteristics of non-obese and obese individuals with dyslipidemia

	Non-obese		Obese		Reference
	Healthy	Unhealthy	Healthy	Unhealthy	
Visceral fat area	–	+	–	+	[33–35, 36••, 37•]
Subcutaneous fat area	–	–/+	–	–/+	[34, 35, 36••, 37•]
Adipose tissue dysfunction and inflammation	–	+	–	+	[38–41, 42•]
Systemic inflammation	–	+	–	+	[33, 38, 40, 41, 42•, 43, 44, 45•, 46–48]
Insulin resistance	–	+	–	+	[35, 36••, 37•, 39–41, 42•, 43, 44, 47–49]
Adiponectin	–	÷	–	÷	[35, 36••, 43, 45•, 46, 50–52]
Non-alcoholic fatty liver disease	–	+	–	+	[42•, 44]
Hepatic dysfunction[a]	–	+	–	+	[33, 43, 46, 52]

Compared to healthy counterpart: ÷ decreased, − normal, + increased

[a] Altered lipid metabolism and/or altered secretion of hepatokines

>40 kg/m^2) and may underlie metabolic disease by affecting membrane fluidity and increasing adipose tissue inflammation and insulin resistance [38]. Likewise, gene expression analysis implicated that branch-chained amino acid catabolism and the tricarboxylic acid cycle in subcutaneous adipose tissue from obese men (BMI ≥28 kg/m^2) and women (BMI ≥24 kg/m^2) are impaired, regardless of metabolic health status [53]. However, the extent of this adipocyte energy metabolism impairment was greater in individuals characterized as UO with MS compared to HO without MS [53]. Moreover, Arner and coworkers demonstrated that lipid turnover differed between healthy individuals and obese and non-obese individuals with dyslipidemia [54]. Consequently, non-obese individuals (BMI <30 kg/m^2) with familial combined hyperlipidemia ($n = 13$) displayed reduced triglyceride storage and removal (lipolysis followed by oxidation), while storage was increased and removal decreased in obese individuals ($n = 30$, BMI >30 kg/m^2) [54]. This may suggest differences in the pathogenesis of dyslipidemia in UO and UN individuals. Furthermore, gene expression analysis of hormone-sensitive lipase, uncoupling protein 2, and peroxisome proliferator-activated receptor (PPAR) δ revealed decreased levels in dyslipidemic UO ($n = 10$, BMI ≥30 kg/m^2) with MS compared to HO ($n = 9$, BMI ≥30 kg/m^2) without MS, suggesting that alterations in lipid homeostasis and metabolism may accelerate dyslipidemia in the unhealthy phenotype [43].

Adipocyte Hypertrophy Is Associated with the Unhealthy Phenotype

In agreement with the proposed role of adipose tissue dysfunction and hypertrophied adipocytes in development of dyslipidemia, several studies have found hypertrophied adipocytes in both UO [33, 39, 55] and UN individuals [39]. Similar adipocyte size in HO and UO has also been reported [44]. However, the peak adipocyte diameter was larger in insulin resistant subjects ($n = 74$) despite having fewer hypertrophied adipocytes in the subcutaneous adipose tissue compared with insulin-sensitive subjects matched for BMI ($n = 55$) [56]. This supports the notion of a critical adipocyte size preceding metabolic dysfunction through impairment of adipogenesis. However, overfeeding men ($n = 29$, study start BMI 22–32 kg/m^2) 40 % of their daily energy requirement for 8 weeks promoted insulin resistance and unexpectedly increased the numbers of subcutaneous small adipocytes while the number of large adipocytes did not increase [57]. As the authors sampled subcutaneous adipose tissue, the role of adipocyte hypertrophy in visceral adipose tissue was not addressed. Nevertheless, this suggests that small adipocytes are not necessarily protective and/or that other tissues are important in promoting metabolic damage. A study of 150 lean (BMI 18.5–24.99 kg/m^2) and overweight/obese women (BMI 25–39.99 kg/m^2) reported that lean and obese women with omental adipocyte hypertrophy displayed dyslipidemia compared with lean and obese women with omental adipocyte hyperplasia [40]. Lipid profiles were similar regardless of the size of the subcutaneous adipocytes [40]. Though not an absolute marker of disease, results thus point towards adipocyte phenotype, primarily hypertrophy in VAT, as associated with dyslipidemia and concurrent metabolic alterations.

Inflammation, Insulin Resistance, and Decreased Adiponectin Promotes the Unhealthy Phenotype

Increased inflammatory cell infiltration and production of interleukin 1β was found in the visceral adipose tissue of UO ($n = 23$, BMI >30 kg/m^2) with MS compared to HO ($n = 21$, BMI >30 kg/m^2) without MS [58]. Moreover, the activity of the NLPR3 inflammasome—which has been suggested to play a role in insulin resistance—was also increased in macrophages isolated from the visceral adipose tissue of UO [58]. Like in their obese counterparts, adipose tissue inflammation is also present in UN: Macrophage-associated genes were upregulated in the subcutaneous, but not visceral, adipose tissue of UN ($n = 21$, BMI ≤25 kg/m^2) with MS when compared to HN individuals ($n = 71$, BMI ≤25 kg/m^2) without MS suggesting macrophage infiltration and that the subcutaneous compartment may also contribute to the unhealthy phenotype [41]. However, macrophages are not the only inflammatory cells, which are present in dysfunctional adipose tissue. Instead, a unique immune cell signature is found in the adipose tissue of UO compared to HO. Thus, interleukin 17 and 22 producing CD4+ T cells were increased in subcutaneous adipose tissue of insulin resistant UO ($n = 13$) compared to insulin sensitive HO ($n = 12$) and lean ($n = 9$) individuals [42•]. Additionally, circulating levels of interleukin 22 were increased in UO and both interleukin 17 and 22 decreased insulin sensitivity in vitro, possibly contributing to insulin resistance in the unhealthy phenotype [42•]. Likewise, the visceral adipose tissue of UO is characterized by a decreased percentage of anti-inflammatory regulatory T cells compared to HO and lean subjects [58]. Obese mouse models have shown that regulatory T cells confer insulin sensitivity and induce the M2 macrophage phenotype [59, 60]. Such a specific immune cell signature may help explain development of dyslipidemia, insulin resistance, and metabolic decline in individuals with otherwise similar degrees of obesity/leanness.

Consistent with an inflamed adipose tissue, both UO and UN individuals display low-grade systemic inflammation [34, 45•]. Insulin-resistant, post-menopausal UO women ($n = 22$, BMI >27 kg/m^2) displayed increased systemic inflammation and dyslipidemia compared to insulin sensitive HO women ($n = 22$, BMI >27 kg/m^2) [34]. Additionally, a cross-sectional study of 2047 men and women utilizing five different definitions of metabolic health reported low-grade systemic inflammation in metabolically unhealthy individuals regardless of

obesity status [45•]. Inflammation in unhealthy individuals may propagate insulin resistance, thereby contributing to the vicious cycle and enhancing dyslipidemia and metabolic dysfunction [45•]. Furthermore, individuals characterized as unhealthy had lower circulating levels of adiponectin [45•]. In a cohort of UN women ($n = 25$, BMI <25 kg/m^2) characterized by a visceral fat area ≥100 cm^2, circulating levels of interleukin 6 and tumor necrosis factor α were both increased while adiponectin was decreased compared to controls with a visceral fat area <100 cm^2 ($n = 25$, BMI <25 kg/m^2) pair-matched for weight, age, height, and menopausal status [35]. Additionally, individuals characterized as UN were more insulin resistant and had dyslipidemia with smaller, more atherogenic, low-density lipoprotein (LDL) particles [35]. Active high molecular weight adiponectin levels were also decreased in UN men ($n = 24$, BMI <25 kg/m^2) with a visceral fat area ≥100 cm^2 compared to age-matched controls with a visceral fat area <100 cm^2 ($n = 28$, BMI <25 kg/m^2) [50]. Thus, reductions in adiponectin seem to occur irrespective of gender and BMI among unhealthy individuals. Conversely, total adiponectin levels were not different between insulin-resistant UN ($n = 12$) and insulin-sensitive HN ($n = 84$) women; however, the study did not differentiate between total and active high molecular weight adiponectin [51]. Decreased levels of adiponectin could be an important factor in promoting the unhealthy phenotype by causing insulin resistance and inflammation, compromising lipid metabolism and facilitating dyslipidemia. This is supported by animal experiments, in which increased adiponectin status is associated with metabolic flexibility and promotes the healthy phenotype. Accordingly, transgenic adiponectin *ob/ob* mice are more obese than ordinary *ob/ob* mice, but are protected from hypertriglyceridemia and insulin resistance and display decreased systemic inflammation and macrophage infiltration into the adipose tissue [61]. *Ob/ob* mice with adipocyte-specific overexpression of mitoNEET (a protein central to mitochondrial function) develop excessive obesity with weight gain exceeding that of *ob/ob* littermates [62]. However, the *ob/ob* mitoNEET mice do not become dyslipidemic, remain insulin sensitive and euglycemic, and have lower hepatic levels of ceramide and diacylglycerol as well as higher levels of plasma adiponectin. The protective effects associated with mitoNEET overexpression were abolished in mitoNEET adiponectin-knockout mice, demonstrating a pivotal role of adiponectin in mediating the HO phenotype [62].

The Role of Hepatic Function and Lipid Metabolism in the Development of Dyslipidemia

The increased amount of visceral fat characteristic of the unhealthy phenotype [33–35, 36••, 37•] leads to adipocyte FFA release that is transported to the liver through the portal vein [63]. The proportion of FFA delivered to the liver from VAT increases with increasing VAT area, suggesting that the livers of unhealthy, visceral obese individuals are exposed to high concentrations of FFA [64]. In the liver, FFA are stored as triglycerides leading to intracellular lipid accumulation and resulting hepatic steatosis, clinically termed NAFLD. Though initially reversible, NAFLD and the progression to irreversible steatohepatitis (NASH) currently constitute the most prevalent chronic liver disease in humans and may progress further to cirrhosis, hepatocellular carcinoma, and/or liver failure [65–67]. Exposed to chronic dyslipidemia and concurrent steatosis, the liver will attempt to decrease the intrahepatic lipid content by enhancing the export of triglycerides packed in very low-density lipoproteins (VLDL). In accordance, hepatic VLDL-triglyceride secretion was almost doubled in subjects with high intra-hepatic fat content compared to normal counterparts [68, 69]. Concurrently, FFA [25], insulin resistance [26], and inflammatory cytokines [27, 28] decrease the activity and expression of lipoprotein lipase in adipose tissue and skeletal muscles. Consequently, the clearance of circulating VLDL-triglycerides is reduced. Decreased clearance with concurrent increased hepatic triglyceride export then causes hypertriglyceridemia. Hypertriglyceridemia and elevated levels of hepatic lipids enhances cholesteryl ester transfer protein activity, which in turn lowers the level of circulating HDL particles and creates triglyceride-rich LDL particles [7, 70]. Hepatic lipase subsequently converts the triglyceride-rich LDL-particles into small, dense LDL-particles that are highly atherogenic and display decreased affinity for the LDL receptor, increasing their circulatory half-life and exacerbating hyperlipidemia [29]. Furthermore, the liver also influences lipid and glucose metabolism by releasing a range of hepatokines. Of these, recent interest has focused on Fetuin-A and fibroblast growth factor 21 (FGF21). Hepatic lipid retention alters the hepatokine secretion pattern, and both Fetuin-A and FGF21 are increased in patients with NAFLD [30]. Fetuin-A causes hepatic and skeletal muscle insulin resistance through inhibition of insulin tyrosine kinases alongside inflammation by acting as an adaptor protein and facilitating the binding of FFA to toll-like receptor 4 [30, 31]. This links Fetuin-A to lipid-induced inflammation and the ensuing insulin resistance [32]. In mice, FGF21 regulates metabolism by decreasing the level of circulating lipids and enhancing both hepatic and peripheral insulin sensitivity. The role in humans is less clear as FGF21 concentrations are unexpectedly high in patients suffering from obesity, NAFLD and/or insulin resistance, suggesting that FGF21 resistance may disrupt potential beneficial effects [30] (Fig. 1b). A recent paper by Berti et al. questioned the protective role of FGF21 as the authors found FGF21 concentrations twofold higher in insulin-resistant UO ($n = 10$, BMI >40 kg/m^2) compared to insulin-sensitive HO ($n = 10$, BMI >40 kg/m^2) [71]. Furthermore, FGF21 decreased adiponectin secretion while

enhancing interleukin 6 secretion in differentiating pre-adipocytes isolated from the subcutaneous adipose tissue. The authors suggested that NAFLD—a hallmark in metabolically unhealthy individuals—may increase hepatic FGF21 secretion which, in turn, suppresses adiponectin release from the adipocytes [71]. Thus, hepatic fat accumulation with resulting alterations in lipid metabolism and hepatokine secretion may contribute directly to the development of dyslipidemia.

NAFLD and Impaired Hepatic Metabolism Are Key Facilitators of the Unhealthy Phenotype

Ectopic fat accumulation, particularly in the liver, is often increased in UO and UN individuals compared to their healthy counterparts [72, 73] and hepatic fat accumulation appears to be pivotal for dyslipidemia. Obese-discordant monozygotic twins pairs ($n = 16$) apparently separated into a metabolically unhealthy and a metabolically healthy subgroup based on their amount of liver fat [36••]: When liver fat was increased, the obese twin displayed dyslipidemia, insulin resistance, mitochondrial dysfunction in the subcutaneous adipose tissue alongside decreased expression of genes associated with amino acid and fat oxidation. However, when the amount of liver fat did not differ, the obese twin was not dyslipidemic and did not display any metabolic abnormalities. Accordingly, obese individuals with normal levels of liver fat are seemingly protected from metabolic decline [74] and NAFLD may induce dyslipidemia even in lean individuals [75]. Dyslipidemia is enhanced in patients with NAFLD due to compromised hepatic metabolism [29, 76]. As such, consumption of carbohydrate-rich meals promoted an increase in hepatic de novo lipogenesis and triglyceride synthesis in insulin-resistant UN ($n = 12$) compared to insulin-sensitive controls matched for age, weight, BMI, and activity ($n = 12$) [46]. This induced atherogenic dyslipidemia with increased plasma triglycerides and decreased levels of HDL particles [46]. Regardless of obesity status, post-prandial triglyceride metabolism was impaired in unhealthy individuals ($n = 808$), resulting in hypertriglyceridemia and increased large triglyceride-rich lipoproteins compared to healthy individuals ($n = 184$) [47]. The importance of hepatic fat with regards to dyslipidemia is further emphasized by a study of HO ($n = 12$) individuals with normal levels of liver fat and UO individuals ($n = 8$) with increased levels of liver fat subjected to moderate (~6 %) weight gain [37•]. Weight gain was found to only cause dyslipidemia and insulin resistance in the unhealthy subjects [37•]. In accordance with compromised hepatic function, the secretion of hepatokines was also affected in unhealthy individuals; Fetuin-A levels were higher in insulin-resistant UO compared to insulin-sensitive HO individuals and is likely to predispose to inflammation and insulin resistance [33]. Similarly, circulating levels of both Fetuin-A and FGF21 were higher in UO (classified by BMI percentiles) adolescents with type 2 diabetes ($n = 74$) compared to HO adolescents without type 2 diabetes ($n = 74$) [52].

Conclusion

Dyslipidemia is a key factor in the promotion of metabolic disease and is present in both lean and obese individuals. Consequently, obesity per se does not fully account for the development of dyslipidemia and associated lifestyle diseases. Instead, ectopic lipid deposition, particularly in the liver, alongside adipose tissue dysfunction results in adverse lipid metabolism. This fuels and maintains the development of dyslipidemia and associated metabolic stress, ultimately, propagating disease, regardless of obesity status.

Compliance with Ethical Standards

Conflict of Interest David H. Ipsen, Pernille Tveden-Nyborg, and Jens Lykkesfeldt declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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•• Of major importance

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Paper VI

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Review

Does Vitamin C Deficiency Promote Fatty Liver Disease Development?

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Abstract: Obesity and the subsequent reprogramming of the white adipose tissue are linked to human disease-complexes including metabolic syndrome and concurrent non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH). The dietary imposed dyslipidemia promotes redox imbalance by the generation of excess levels of reactive oxygen species and induces adipocyte dysfunction and reprogramming, leading to a low grade systemic inflammation and ectopic lipid deposition, e.g., in the liver, hereby promoting a vicious circle in which dietary factors initiate a metabolic change that further exacerbates the negative consequences of an adverse life-style. Large epidemiological studies and findings from controlled *in vivo* animal studies have provided evidence supporting an association between poor vitamin C (VitC) status and propagation of life-style associated diseases. In addition, overweight per se has been shown to result in reduced plasma VitC, and the distribution of body fat in obesity has been shown to have an inverse relationship with VitC plasma levels. Recently, a number of epidemiological studies have indicated a VitC intake below the recommended daily allowance (RDA) in NAFLD-patients, suggesting an association between dietary habits, disease and VitC deficiency. In the general population, VitC deficiency (defined as a plasma concentration below 23 μM) affects around 10% of adults, however, this prevalence is increased by an adverse life-style, deficiency potentially playing a broader role in disease progression in specific subgroups. This review discusses the currently available data from human surveys and experimental models in search of a putative role of VitC deficiency in the development of NAFLD and NASH.

Keywords: antioxidants; obesity; oxidative stress; non-alcoholic fatty liver disease; non-alcoholic steatohepatitis; reactive oxygen species; vitamin C; vitamin C deficiency

1. Introduction

Obesity has become an epidemic with detrimental effects on health and wellbeing. In 2013, more than one-third of the world's adult population could be characterized as being overweight or obese (body mass index (BMI) ≥25 kg/m^2) [1]. Obesity has been found to result in dysfunctional adipose tissue, low-grade systemic inflammation and redox imbalance with increased oxidative stress [2–4]. As part of the so-called metabolic syndrome, known associations with obesity include a wide range of diseases, e.g., atherosclerosis, insulin resistance (IR), type 2 diabetes and non-alcoholic fatty liver disease (NAFLD) [5]. The development of NAFLD is closely linked to increased levels of circulating lipids, IR, inflammation and oxidative stress [6,7]. In agreement with a putative role of oxidative stress in the etiology of NAFLD, important antioxidant enzymes and vitamins are decreased in obese and NAFLD patients [4,8,9].

Vitamin C (VitC) is a water-soluble, chain-breaking antioxidant capable of scavenging essentially all physiologically relevant free radicals [10]. Additionally, it serves as donor of reducing equivalents in multiple enzymatic reactions, of which its role in proline and lysine hydroxylation during collagen synthesis is probably most widely known [11]. Humans and a few other species are unable to synthesize VitC, and must instead acquire it through dietary means. Inadequate dietary intake results in VitC deficiency defined as plasma levels below 23 μM [12]. Cross-sectional population studies have shown that about 10%–20% of the western population can be diagnosed with VitC deficiency and that poor VitC status is associated with increased all-cause mortality [13–17] Moreover, the prevalence of VitC deficiency in specific subgroups may be considerably higher, potentially enhancing susceptibility to oxidative stress and disease [18]. Accordingly, VitC status correlates inversely with e.g., BMI and is significantly decreased in obese compared with lean individuals [9,19–22]. Thus, the apparent link between obesity, NAFLD and oxidative stress suggests that NAFLD progression may be accelerated by poor VitC status.

The aim of this review is to discuss the current data from human and animal studies in relation to a putative role of VitC in the development of NAFLD.

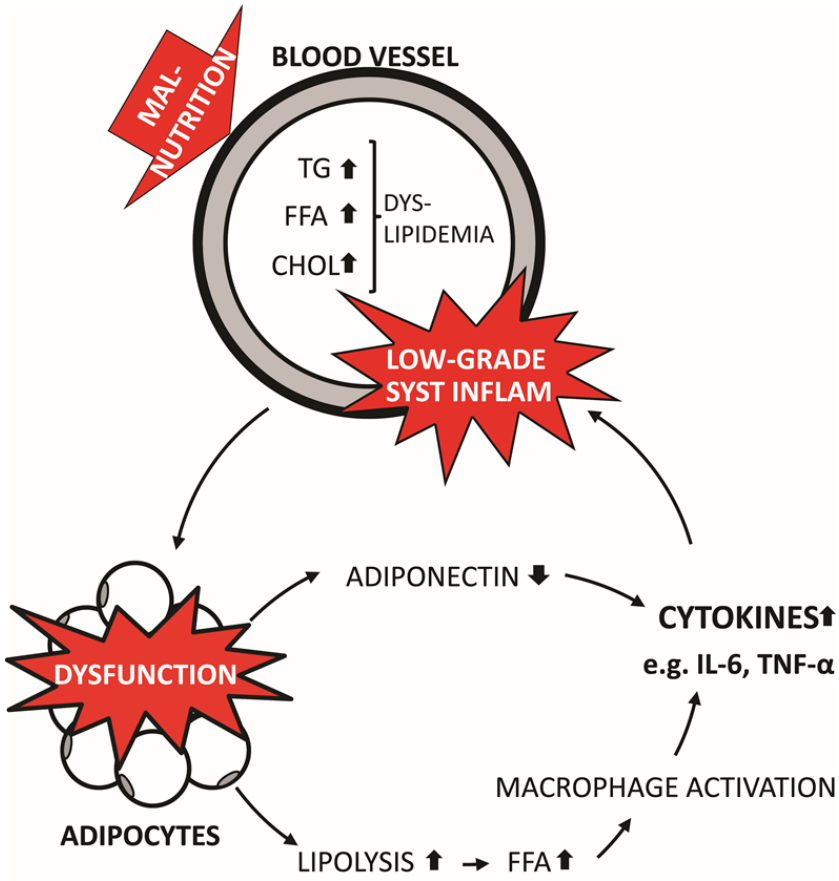
2. Obesity, Systemic Inflammation and NAFLD

2.1. Obesity and Systemic Inflammation

Obesity is primarily a consequence of a sedentary lifestyle and the intake of an excess amount of calories. As a result, unused resources are accumulated as fat in adipose tissue, facilitating adipocyte hypertrophy and hyperplasia, alongside increased production of chemokines, cytokines, reactive oxygen species (ROS), hypoxia and cell death, ultimately leading to macrophage infiltration and adipocyte dysfunction [23–25]. Macrophages initiate and maintain inflammation by secreting a variety of cytokines, such as tumor necrosis factor α (TNFα) and interleukin (IL) 6 [2,23] At the same time, dysfunctional

adipocytes increase their secretion of pro-inflammatory cytokines, while decreasing secretion of anti-inflammatory cytokines [24,26]. Decreased secretion of adiponectin by adipocytes and concurrent interference with the insulin signaling pathway by inflammatory cytokines promotes the development of IR [2,27,28]. Furthermore, inflammation of the adipose tissue may participate in the induction of hepatic IR as well [29]. Inflammation, IR and adipocyte hypertrophy per se can increase the rate of lipolysis and subsequent release of free fatty acids (FFA) [2,30,31]. FFAs have been shown to directly activate macrophages through Toll-like receptors 2 and 4 and enhance the production of inflammatory cytokines [32]. Thus, a vicious cycle is established: Adipocyte dysfunction induces a low-grade inflammation, which increases the release of FFA. In turn, the FFAs stimulate macrophage activation and additional production of inflammatory cytokines [5] (Figure 1). Ultimately, this may result in a state of chronic, low-grade systemic inflammation and oxidative stress [33]. Low levels of antioxidants may enhance oxidative stress, aggravating an already vulnerable state of redox imbalance.

Figure 1. Propagation of low-grade systemic inflammation from adipose tissues.



Dietary induced dyslipidemia leads to increased fat deposition and promotes hypertrophy and hyperplasia of adipocytes as well as cellular reprogramming leading to altered secretory functions of adipocytes. The tissue expansion and associated adipocyte dysfunction increases the release of inflammatory cytokines (such as IL6 and TNFα) and activates macrophages. Furthermore, release of the anti-inflammatory adipokines, e.g. adiponectin, is decreased. This induces a state of low-grade systemic inflammation, which in turn compromises insulin sensitivity and increases lipolysis in adipose tissues, increasing the release of free fatty acids (FFA) and exacerbating the dietary induced dyslipidemia. Moreover, FFAs also promote macrophage activation by binding to Toll-like receptors 2 and −4, further propagating the production of inflammatory cytokines. Thus a viscous cycle is established in which dyslipidemia and systemic inflammation is propagated through dysfunctions in adipocyte metabolism.

Multiple cross-sectional studies have found decreased levels of VitC and several other antioxidants in obese men and women, and observed an inverse correlation between VitC levels and BMI, waist circumference and body fat percentage [15,19–22]. The third Glasgow MONICA population survey investigated the plasma VitC status of men and women aged 25–74 years and found 44% of the participants (n = 1267) to be VitC deficient, with a VitC plasma concentration below 23 μM [15]. Additionally, using data from 8808 U.S. adult men and women participating in the Third National Health and Nutrition Examination Survey (NHANES III), Ford *et al.* reported that the metabolic syndrome was associated with decreased levels of VitC and other antioxidants such as vitamin E (VitE) [34]. Dietary habits may be an important factor contributing to the poor VitC status in obesity. Obese individuals have been shown to consume less dietary VitC [22]. Similarly, obese women were found to consume less fruits and vegetables compared to lean controls [35].

A putative role of VitC in inflammation has also been suggested, since VitC levels have been found to be inversely associated with inflammatory markers such as C-reactive protein (CRP) and myeloperoxidase [19,22]. Furthermore, VitC may be involved in the regulation of the key adipokine, adiponectin, supported by a recent study reporting dietary VitC intake to correlate with adiponectin levels in adolescent girls [36]. *In vitro* studies have suggested that VitC increases the secretion of high molecular weight (HMW) adiponectin in fully differentiated human adipocytes without changing total adiponectin secretion [37]. Contrary to this, however, supplementation with VitC (500 mg/day) in obese patients did not change adiponectin levels [21]. Quantification of total adiponectin, instead of the bioactive HMW isomer alone, may account for the conflicting results. Alternatively, the dose of VitC might have been too low, as 1000 mg/day was shown to improve several other metabolic parameters in patients with type 2 diabetes, *i.e.*, HbA1c, fasting blood sugar, serum insulin, triglycerides (TG) and low-density lipoprotein (LDL) [38]. Further cell studies have shown that increased oxidative stress decrease the expression of adiponectin mRNA in adipocytes and that adiponectin levels are inversely correlated with 4-hydroxynoneal, a maker of lipid peroxidation [39]. Adiponectin levels were also inversely associated with the severity of hepatic steatosis and necroinflammation [40]. Thus, a role of VitC in adiponectin regulation could have important effects on progression of inflammation, IR and NAFLD.

2.2. NAFLD

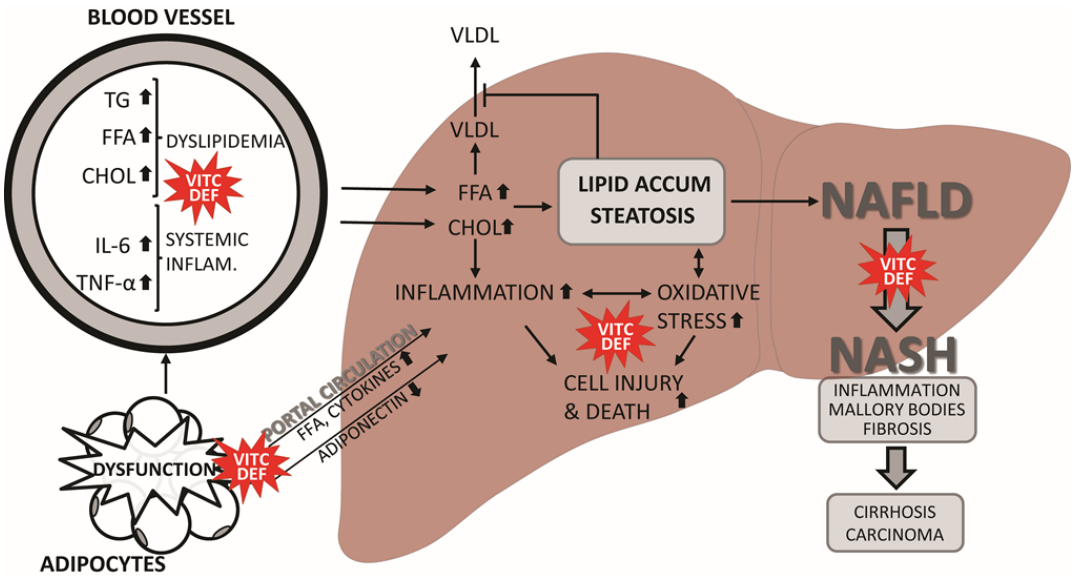
NAFLD is estimated to affect 6% to 33% (median 20%) of the world's population and constitutes the most common liver disease in the western world [41,42]. NAFLD comprises a cluster of hepatic conditions ranging from simple, initially benign and reversible steatosis to hepatocyte ballooning, Mallory bodies, fibrosis and inflammation in non-alcoholic steatohepatitis (NASH), [6,43–45]. However, in some patients, hepatic steatosis progresses to NASH, increasing the risk of hepatic cirrhosis and hepatocellular carcinoma [43,46]. NAFLD is strongly associated with obesity, dyslipidemia, IR, oxidative stress, inflammation and the metabolic syndrome [47,48]. Indeed, the prevalence of NAFLD in obese patients has been reported to be 74%, the amount of visceral adipose tissue in particular being highly correlated with increased risk of NAFLD [6,49]. The pathogenesis of NAFLD is not yet completely understood. However, it is currently believed that initial hepatic fat accumulation is followed by multiple parallel hits that promote inflammation and disease progression. The most important events believed to be involved in this process include; IR, increased FFA, dietary factors, cytokines derived from the adipose tissue and ROS formation [7].

IR is a key mechanism in hepatic dyslipidemia. Expectedly, diabetic patients have an increased prevalence of NAFLD compared to non-diabetic individuals, supporting the link between IR and NAFLD [50]. While IR increases circulating levels of FFAs, hyperinsulinemia during IR, paradoxically, apparently inhibits hepatic lipid oxidation and increases *de novo* lipogenesis [51]. Concurrently, saturation of very-low-density lipoprotein (VLDL) secretion may facilitate lipid accumulation as well [52]. In patients with NAFLD, the majority of hepatic lipids originate from circulating FFA [53]. FFA from visceral adipose tissue can drain directly into the portal vein and be transported straight to the liver. As hepatocytic FFA uptake is mediated by passive diffusion, increased FFA levels result in increased FFA uptake, which is subsequently stored as TG [6,54]. Interestingly, a meta-analysis of clinical trials investigating the effect of VitC intervention on LDL, high-density lipoprotein (HDL) and TGs in patients with hypercholesterolemia suggested that VitC supplementation can reduce the concentration of lipids in circulation (TG and LDL-C) [55]. Additionally, adipose tissue-derived cytokines may contribute to the development of NAFLD, and inflammation of the adipose tissue may indeed precede hepatic inflammation [56–58]. Adiponectin has been shown to reduce ectopic hepatic lipid accumulation, systemic IR and inflammation [59]. In addition, TNFα and IL6 have received much attention as putative key cytokines involved in hepatic lipid accumulation, inflammation and IR [60].

Dietary factors also play an important role in NAFLD. High dietary fructose, trans-fatty acids, and cholesterol have all been found to contribute to the development of dyslipidemia, hepatic fat accumulation and subsequent disease progression [7,61–65]. FFA and cholesterol not only induce hepatic lipid deposition, but may also have injurious effects in the liver, in part by inducing hepatocyte apoptosis, lipotoxicity, mitochondrial dysfunction and endoplasmic reticulum stress with subsequent ROS formation and inflammation [24,66–71]. Furthermore, activation of hepatic stellate cells and Kupffer cells, e.g., by hepatocyte apoptosis and ROS, also appear to promote disease progression through the secretion of TNFα, IL6, transforming growth factor β and collagen, which induces inflammation and fibrosis [72] (Figure 2). Indeed, increased generation of ROS and lipid peroxidation are believed to be key pathogenic components in NAFLD, perpetuating inflammation, fibrogenesis, and development of cirrhosis and hepatocellular carcinoma [73,74]. Oxidative stress alone has been found to be associated with NAFLD

both in patients with, but also without type 2 diabetes [75]. VitC has been shown to decrease mitochondrial ROS formation and stimulate the activity of manganese superoxide dismutase (SOD) and glutathione peroxidase (GPx) in isolated rat liver mitochondria [76]. However, dietary intake of VitC and VitE has been reported to be lower in NAFLD patients, potentially predisposing them to oxidative stress [77,78].

Figure 2. Putative effects of vitamin C deficiency on the progression of NAFLD.



As co-factor for the 7α-hydroxylase, catalyzing the conversion of cholesterol to 7α-hydroxycholesterol, VitC deficiency may increase circulating levels of cholesterol (CHOL) by reducing its excretion through the bile. Systemic inflammation and dyslipidemia is propagated through adipocyte dysfunction, e.g., releasing fatty acids (FFA) and inflammatory cytokines to the blood stream. Moreover, by affecting adiponectin regulation, lack of VitC may further increase dyslipidemia, systemic inflammation and oxidative stress. As VitC is also a powerful antioxidant, deficiency per se promotes cellular oxidative stress, e.g., in the liver. The excess lipids induce mitochondrial dysfunction and stress of the endoplasmic reticulum (ER) further propagating hepatic oxidative stress and inflammation. FFA and CHOL is taken up by hepatocytes and processed to be exported from the liver by VLDL. When overloaded, hepatic VLDL export is saturated and lipids are accumulated in hepatocytes. Ultimately, NAFLD progression is driven by increased oxidative stress and inflammation, promoting activation of hepatic stellate and Kupffer cells, which alongside an increased hepatic production of TNFα, IL6, transforming growth factor β and collagen, amplifies the induction of cellular damage and fibrosis. Combined with key pathogenic changes taking place during the advancement from simple steatosis (NAFLD) to steatohepatitis (NASH), e.g., hepatocyte ballooning, formation of Mallory bodies and fibrosis, NASH may progress even further to hepatic cirrhosis and hepatocellular carcinoma.

Consistent with this notion, a number of investigators have found that markers of oxidative damage such as malondialdehyde (MDA) and protein carbonyls are elevated, while antioxidants like catalase and SOD are decreased in the plasma and livers of animals and humans with NAFLD [8,65,79,80].

This has led to the hypothesis that VitC therapy could prove beneficial in the treatment of NAFLD (Figure 2).

3. Does Vitamin C Deficiency Promote NAFLD?

3.1. Animal Studies of Vitamin C Deficiency

Several *in vivo* studies have investigated the role of poor VitC status in relation to dyslipidemia and NAFLD (Table 1). Like humans, guinea pigs are unable to synthesize VitC due to a non-functional gene encoding for L-gulonolactone oxidase (gulo), making them an excellent model for the study of VitC deficiency. Moreover, with regards to, e.g., cholesterol distribution, activity of enzymes involved in lipoprotein metabolism, LDL receptor binding domain characteristics and cholesterol synthesis, guinea pigs are comparable to humans in contrast to other rodents [81]. Contrary to the guinea pig, the gulo gene is functional in mice and rats and both species are able to synthesize VitC. However, the effect of VitC deficiency can be examined in genetically engineered rodent models, although the validity and human relevance of such models may be difficult to assess.

Guinea pigs kept on a diet without VitC for 4 weeks showed elevated levels of hepatic cholesterol and TG compared to control animals on a VitC supplemented diet [82]. Similarly, another study in guinea pigs showed increased hepatic triacylglycerol and cholesteryl esters after 6 weeks of VitC deficiency [83]. Scurvy can be avoided by supplementing the diet with small amounts of VitC, allowing investigators to study the long-term effects of non-scorbutic VitC deficiency. After up to 31 weeks on such a diet, guinea pigs had elevated levels of TG in the liver, thoracic aorta and serum. This was due to decreased conversion of cholesterol to bile acid, revealing a crucial role of VitC in this process [84]. Specifically, VitC is a co-factor for the 7α-hydroxylase catalyzing the conversion of cholesterol to 7α-hydroxycholesterol, constituting the rate-limiting step in bile acid formation, and thus, VitC deficiency results in reduced excretion of cholesterol in animals [85]. The addition of cholesterol to a VitC deficient diet exacerbated the effects of VitC deficiency per se by increasing the amount of hepatic TG, cholesterol, focal fibrosis and proliferation of connective tissue. Moreover, high dose (100 mg/day, p.o.) of VitC significantly diminished these changes [86]. Interestingly, dietary cholesterol also appears to have effects on the VitC status. In a long-term dietary intervention study in guinea pigs, high cholesterol fed animals consistently showed 33% (range 27%–43%) lower plasma and liver VitC concentrations compared to control animals regardless of low or high dietary VitC intake [87]. In the osteogenic disorder shionogi (ODS) rat—a mutant rat strain unable to synthesize VitC—VitC deficiency with or without cholesterol and cholic acid supplementation for 19 days increased hepatic cholesterol and total lipids [88]. Furthermore, regardless of the amount of cholesterol added to the diet, Uchida *et al.* did not find any effect of VitC relating to changes in hepatic lipids in ODS rats [89]. These discrepancies may reflect the significant differences between guinea pigs and rats in the way lipids are handled and stored [90,91]. Collectively, the above results suggest that VitC is involved in the regulation of both circulating and hepatic lipid homeostasis, supporting VitC as an important factor in the development of NAFLD.

Table 1. Animal studies of vitamin C deficiency.

Species	Age/Weight	Design	Outcome	Ref.
Guinea Pigs	200–300 g	Control (VitC 5 mg VitC/day) Deficient (0.5 mg VitC/day) High VitC (100 mg VitC/day) ± 100 mg chol/day Duration: 16 weeks	VitC deficiency, liver: TG ↑ Chol ↑ PL ↔ VitC deficiency + chol, liver: TG ↑ Chol ↑ PL ↔ Histology reveal fat infiltration, local necrosis and proliferation of connective tissue	[86]
Guinea Pigs	400–450 g	Control (1000 mg VitC/kg diet) VitC deficient (0 mg VitC/kg diet) VitC high (25,000 mg VitC/kg) Duration: 4 weeks	VitC deficiency, liver: Chol ↑ TG ↑ VitC deficiency, serum: Chol ↑ TG ↑ HDL ↓ LDL ↑ VLDL ↓	[82]
Guinea Pigs	300–450 g	Control (10 mg VitC/day) Deficient (0.5 mg VitC/day) Duration: 22, 28 or 31 weeks	VitC deficiency, liver/serum/thoracic aorta: Chol ↑	[84]
Guinea Pigs	?	Control (660 mg VitC/kg diet) Deficiency (33 mg VitC/kg diet) High (13,200 mg VitC/kg diet) Duration: 5 weeks	VitC deficiency, liver: TBARS ↑ MDA ↑ protein carbonyls ↑	[92]
Guinea Pigs	250–300 g	Control (25 mg VitC/kg/day) Deficiency (0 mg VitC/kg/day) Duration: 21 days	VitC deficiency, liver: TBARS ↑	[93]
Guinea Pigs	600–700 g	Control (500 mg VitC/kg diet) Deficiency (50 mg VitC/kg diet) Duration: 6 weeks	VitC deficiency, liver: TAG ↑ CE ↑ FC ↔	[83]
Guinea Pigs	12 weeks	High or low fat diet with different VitC: Low (100 mg VitC/kg diet) High (691 mg VitC/kg diet) Duration: 6 months	High fat diet, liver: VitC ↓	[87]

Table 1. *Cont.*

Mice, SMP30$^{-/-}$ and WT	30 days	SMP30$^{-/-}$ or WT ±1.5 mg/L VitC in water Duration: 57 days	VitC deficiency, liver: protein carbonyl ↑ SOD-activity ↑ Cu Zn-SOD protein expression ↑ TBARS ↔, CAT protein expression ↓	[94]
Mice, Gulo$^{-/-}$ or WT	20–21 weeks	16 weeks on western diet, then: Control (0.33 g VitC/L in drinking water) Deficiency (0 g VitC/L in drinking water) Duration: 3 weeks	VitC deficiency: GSH ↔ MDA ↑ F2- an F4-isoprostanes ↔	[95]
Mice, Gulo$^{-/-}$	Newborn	Control Gulo$^{+/+}$ (0 mg VitC) Deficiency Gulo$^{-/-}$ (0 mg VitC) Duration: 18 days	Gulo$^{-/-}$, liver: MDA ↑ protein carbonyl ↑ sulfhydryls ↔ GSH ↑	[96]
Rats, ODS or WT	6 weeks	ODS fed 0, 50, 300, 3000 mg VitC/kg diet ± 0.5% chol and 0.25% cholic acid WT fed 0 mg VitC/kg diet ± 0.5% chol and 0.25% cholic acid Duration: 19 days	VitC deficiency, liver: Chol ↑/↔ total lipids ↑/↔ VitC deficiency, serum: Chol ↔ HDL-C ↔ VitC deficiency + chol/cholic acid, liver: Chol ↑, total lipids ↔ VitC deficiency + chol/cholic acid, serum: Chol ↑, HDL-C ↑	[88]
Rats, ODS	6 weeks	Control 300 mg VitC/kg diet Deficient 0 mg VitC/kg diet Duration: 14 days	VitC deficiency, liver: CINC-1 ↑ Apo-A1 mRNA ↓ ApoE mRNA ↔	[97]
Rats, ODS or WT	9 weeks	Control (30 mg VitC/L in drinking water ± 0.5% chol Deficiency 0 mg/L in drinking water Duration: 3 weeks	VitC deficiency, liver: Chol ↔ TG ↔ PL ↔ VitC deficiency, serum: Chol ↑ Total lipoprotein ↓ HDL ↓IDL ↓ LDL ↑ VLDL ↓ VitC deficiency + chol did not affect serum levels further. Chol feeding, regardless of VitC status, increase hepatic lipids	[89]

CAT: Catalase. Chol: Cholesterol. CE: cholesteryl ester. CINC-1: Cytokine-induced neutrophil chemoattractant-1. FC: Free cholesterol. GSH: Glutathione. Gulo: L-gulonolactone oxidase HDL: High-density lipoprotein. IDL: Intermediate-density lipoprotein. LDL: Low-density lipoprotein. MDA: Malondialdehyde. ODS: Osteogenic Disorder Shionogi. PL: Phospholipids. SMP30: Senescence marker protein. SOD: Superoxide dismutase. TAG: Triacylglycerol. TBARS: Thiobarbituric acid reactive substances. TG: Triglyceride. VitC: Vitamin C. VLDL: Very-low-density lipoprotein. WT: Wild type; Ref.: Reference; ↓ Decrease; ↑ Increase; ↔ No change.

In accordance with a central role in the prevention of oxidative stress, VitC deficiency has been shown to cause a reduction in both expression and activity of various antioxidant enzymes with a concurrent rise in markers of oxidative stress. Thus, 3–5 weeks of VitC deficiency caused increased levels of thiobarbituric acid reactive substance (TBARS), MDA and protein carbonyls in the liver of guinea pigs [92,93]. Oxidative stress was also evident in the liver of gulo$^{-/-}$ mice without access to VitC for 57 days. Protein carbonyls, but not TBARS, were increased in the livers of these animals, alongside decreased catalase protein expression compared to WT or gulo$^{-/-}$ supplemented controls [94]. However, SOD activity was increased, likely as a compensatory mechanism [94]. Indeed, SOD appears to play an important role in NAFLD as increased hepatic fibrosis is found in NASH patients with mutations in the gene coding for SOD2 [98]. Pierce *et al.* found increased hepatic MDA in VitC deficient gulo$^{-/-}$ mice placed on a western diet, but with no change in glutathione or isoprostanes [95]. However, other authors report an increase of glutathione a long with increased MDA and protein carbonyls in livers of gulo$^{-/-}$ mice deprived of VitC for similar periods of time [96]. These differences between studies may be attributed to different diet compositions and age of the animals, *i.e.*, a western type diet and 4–5 weeks old animals *vs.* normal chow and newly born animals, respectively. The considerable number of studies reporting increased protein carbonyls, MDA and TBARS in VitC deficient animals, support that VitC has a protective effect against hepatic protein and lipid oxidation. This may be of significance as lipid oxidation has been shown to occur in NAFLD and even correlate with the severity of liver necroinflammation and fibrosis [99]. Furthermore, keeping ODS rats on a VitC free diet for 14 days resulted in increased cytokine-induced neutrophil chemoattractant-1 (CINC-1), a potent inflammatory chemokine, in serum, liver and spleen. Thus, VitC deficiency may mediate the infiltration and accumulation of neutrophils with subsequent inflammatory damage in some tissues [97].

3.2. Animal Studies of VitC Intervention and NAFLD

Methionine and choline are essential for hepatic β-oxidation and VLDL production. Expectedly, methionine and choline deficient (MCD) diets cause accumulation of intra-hepatic lipids and decreased VLDL synthesis [43]. Rezazadeh and coworkers fed rats a MCD diet for 10 weeks, and randomized animals to receive VitC or no supplementation for an additional eight weeks on the diet [100]. While VitC supplementation significantly decreased hepatocellular ballooning and inflammation, liver steatosis still persisted. At the same time, hepatic markers of oxidative stress were decreased, while SOD and catalase were increased [100]. Similar results were obtained by supplementing rats placed on a MCD diet with VitC for 10 weeks. The authors also found that VitC treatment significantly increased glutathione reductase (GR) and GPx activity [101]. Moreover, VitC treatment resulted in a significant decrease in circulating liver enzymes, TG and LDL, showing a protective effect of VitC supplementation on the MCD diet-induced dyslipidemia [100,101]. Conversely, rats fed a choline deficient diet for 4 weeks with concurrent VitC supplementation did not show any improvements in serum TG or aspartate aminotransferase, even though hepatic steatosis was ameliorated and oxidative stress reduced [102]. However, the studies differ considerably in diet composition, duration and rat strain used, confounding compatibility between findings.

Imbalance of the antioxidant status, similar to that observed in the animal studies, has also been found in patients with steatosis while antioxidant status is even further compromised in NASH [80]. Animal

models utilizing MCD or choline deficient diets to induce NAFLD appear to present a similar pathology, but the pathogenesis is different from the human situation. Additionally, most animals lose weight on the MCD diet, contrary to the human situation, in which many NAFLD patients are obese. Results may therefore not be directly translatable.

Guinea pigs placed on an atherogenic diet, high in fat and cholesterol, for 4 months developed dyslipidemia with elevated serum cholesterol and phospholipids in addition to elevated hepatic levels of cholesterol, phospholipids and TG [103]. VitC deficiency exacerbated the dyslipidemia and hepatic consequences, but high VitC reduced the severity of both dyslipidemia and hepatic lipid accumulation [103]. It is noteworthy, that extremely high doses of VitC (13,200 mg/kg diet) have been shown to decrease hepatic markers of oxidation to the same degree as normal VitC doses in guinea pigs, but also cause a reduction of body weight and GR activity, similarly to what was observed in the VitC deficient animals [92]. It is possible that the very high dose of VitC caused adverse effects in the guinea pigs, e.g., gastrointestinal disturbances and osmotic diarrhea [104,105], which could affect bodyweight and general physiology, however, this was not reported by the authors. Conversely, Roomi *et al.* did not report any adverse effects of a mega-dose of VitC (25,000 mg VitC/kg diet) in guinea pigs [82]. Contrary to guinea pigs, supplementation with high levels of VitC in ODS rats did not reduce hepatic cholesterol to a greater extent that normal VitC levels did [88]. Thus, VitC treatment seemingly reduces hepatic oxidative stress, but have variable effect on hepatic steatosis, likely depending on the employed method of induction, *i.e.*, a high fat diet *versus* a MCD or MD diet (Table 2).

3.3. Epidemiological Studies of VitC Intake and NAFLD

A number of cohort studies have been carried out, investigating the dietary habits of patients with NAFLD. Results are conflicting; some studies indicate suboptimal VitC intake by NAFLD patients while others do not (Table 3). Using a 7-day food record Musso *et al.* evaluated dietary habits in patients with NASH and healthy controls. Among other differences, NASH patients consumed significantly less VitC compared to the healthy controls (84.3 ± 43.1 *vs.* 144.2 ± 63.1 mg) [77]. Males constituted the vast majority of the NASH group (24/25 patients) and though below that of healthy controls, dietary intakes were close to the recommended daily intake of 90 mg VitC/day, although this was not the case for smokers with NASH [77,106]. Contrary to this, Ferolla and colleagues found that intake of VitC in NAFLD patients diagnosed with fatty liver, did not meet recommendations when assessed in men and women using 24-h dietary recall and food frequency questionnaires (FFQ) [78]. Unfortunately, the lack of a control group impedes investigation as to whether low VitC intake was also prevalent in a comparable healthy population. Different methodology, ethnicity and patients' disease stage may account for apparent study dissimilarities. Indeed, food records may themselves lead patients to change their dietary patterns [107].

Table 2 Animal studies of VitC intervention in NAFLD.

Species	Age/Weight	Design	Outcome	Ref.
Guinea Pigs	300 g	Normal or atherogenic diet Control (10 mg/kg/day) Deficiency (1 mg/kg/day) High (25 mg/kg/day) VitC administration: Oral Duration: 4 months	Compared to control (normal diet): VitC deficiency, liver: TC ↑ TG ↑ PL ↓ serum: TC ↔ TG ↔ PL ↔ High VitC, liver: TC ↔ TG ↓ PL ↑ serum: TC ↔ TG ↔ PL ↔ Compared to control (atherogenic diet): VitC deficiency, liver: TC ↑ TG ↑ PL ↓ serum: TC ↑ TG ↑ PL ↔ High VitC, liver: TC ↓TG ↔ PL ↓ serum: TC ↓ TG ↓ PL ↓	[103]
Rats	250–300 g	Control (MCD diet for 10 weeks, then 8 additional weeks of MCD diet + vehicle) Treatment (MCD diet for 10 weeks, then 8 additional weeks of MCD diet + VitC 30 mg/kg/day) VitC administration: Oral Duration: 18 weeks	VitC, liver: ballooning ↓ inflammation ↓ steatosis ↔ SOD ↑ CAT ↑ protein carbonyls ↓ VitC, serum: AST ↓ ALT ↓ ALP ↓ γGT ↓ TC ↓ HDL ↑ LDL ↓	[100]
Rats	250–300 g	Control (MCD diet + vehicle) Treatment (MCD diet + VitC 30 mg/kg/day) VitC administration: Oral Duration: 10 weeks	VitC, liver: ballooning ↓ SOD ↑ CAT ↑ GR ↑ GPx ↑ TBARS ↓ protein carbonyls ↓ VitC, serum: AST ↓ ALT ↓ ALP ↓ γGT ↓ TC ↓ HDL ↑ LDL ↓	[101]
Rats	300–350 g	Control (CD diet + vehicle) Treatment (CD diet +30 mg VitC/kg/day) VitC administration: Oral Duration: 4 weeks	VitC, liver: prevents steatosis and reduces oxidative stress VitC, plasma: AST ↔ TG ↔	[102]

ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CAT: Catalase; CD: Choline deficient; GPx: Glutathione peroxidase; GR: Glutathione reductase; γGT: Gamma-glutamyl transferase; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; MCD: methionine choline deficient; PL: Phospholipids; SOD: Superoxide dismutase; TBARS: Thiobarbituric acid reactive substances; TC: Total cholesterol; TG: Triglyceride; VitC: Vitamin C; Ref.: Reference; ↓ Decrease; ↑ Increase; ↔ No change.

Han and co-workers studied the dietary intake of Korean adults [108] and reported no differences in dietary VitC intake between men and women with NAFLD and control subjects However, when adjusted for age, job, education level, exercise frequency, smoking status, energy intake and *n*–3 fatty acid intake, VitC intake was negatively associated with the risk of NAFLD in male patients (OR: 4.23; *p*-trend = 0.014) [108]. In children with steatosis or NASH, Vos *et al.* found dietary intakes of VitC to be similar [109]. However, while differences in steatosis grade were not associated with differences in VitC intake, significantly increased hepatocyte ballooning was indeed observed in children with decreased VitC consumption [109]. In another study, Mager *et al.* reported that daily recommendations of VitC were met by children with NAFLD [110]. However, impaired antioxidant status was implied as measured by low levels of red blood cell-glutathione compared to previous reports in healthy children although the conclusion is weakened by the lack of paired controls [110]. Da Silva and associates concluded that VitC intake was not different between healthy controls and adults with either steatosis or NASH [111]. In this study of 74 NASH patients and 27 healthy controls, the intake correlated with median VitC plasma concentrations, which did not differ between groups 67.6 and 72.1 μM, respectively [111]. Likewise, Madan *et al.* did not find any differences in VitC plasma levels between 29 NAFLD patients and 23 healthy controls [112]. Also, the VitC plasma concentrations were found not to correlate with hepatic inflammation or fibrosis [112]. However, in another study by Cabakan and co-workers, 105 NASH patients had reduced VitC plasma levels alongside increased plasma MDA and nitric oxide compared with steatosis patients [113]. It is possible that this reflects an ability of VitC to slow down the progression of NAFLD.

The conflicting results do not allow a definitive conclusion regarding dietary VitC intake. However, the NAFLD patients included in the studies mentioned above largely seem to have met recommended daily allowance of VitC. Unfortunately, most of the studies only examine the estimated dietary intake of VitC and have not actually measured plasma VitC concentrations. It has been suggested that assessments of dietary VitC only have a moderate relationship with plasma VitC levels, thus the two measures may not describe the VitC status in the same way [114]. Moreover, dietary questionnaires have certain limitations and underreporting is known to occur, which may also skew results [107]. As noted previously, oxidative stress is increased in NAFLD patients [75]. As such, they may have an increased demand of antioxidants, like VitC, and inadequate levels are not likely to be reflected by reports of dietary intake alone. Daily doses of VitC between 30 and 100 mg do not result in plasma saturation, leading some investigators to propose that the daily recommended dose of VitC be increased from 75 and 90 mg/day (US women and men, respectively) [105], to 200 mg/day in healthy individuals [115]. Furthermore, small differences in dietary intake of VitC between 30 and 100 mg/day can lead to vastly different plasma concentrations, as this interval represents a very steep part of the plasma concentration *vs.* dose curve [115]. Therefore, patients with a daily VitC intake in this area and even corresponding to the current recommendation may still be at risk of having plasma VitC concentrations below optimal levels. While they may not strictly be VitC deficient, plasma VitC levels between 23 and 50 μM may still leave patients vulnerable to disease, as, e.g., the relative risk of heart failure has been shown to increase significantly by every 20 μM drop in VitC plasma concentrations [116].

Table 3. Epidemiological studies of VitC status in NAFLD patients.

Design	Groups	Outcome	Ref.
Cross-Sectional	Adults Healthy controls (n = 25) NASH (n = 25)	NASH patients consumes less dietary VitC (p = 0.0001)	[77]
Cross-Sectional	Adults NAFLD patients (n = 96)	Dietary VitC intake was below recommended levels	[78]
Cross-Sectional	Adults Male healthy controls (n = 63) Male NAFLD (n = 103) Women healthy controls (n = 116) Women NAFLD (n = 66)	Dietary intake of VitC was not different in men and women with NAFLD compared with control (p = 0.666) Intake of VitC correlated negatively with the odds-ratio of NAFLD for male patients (OR: 4.23, p-trend = 0.014)	[108]
Cross-Sectional	Children Steatosis (n = 39) Borderline Z3 (n = 27) Borderline Z1 (n = 36) NASH (n = 47)	Dietary VitC intake was similar in all groups (p = 0.15) and above recommended levels High grade of steatosis was not associated with lower dietary VitC intake (p = 0.97) Amount of hepatocyte ballooning increases with lower dietary VitC levels (p = 0.05)	[109]
Cross-Sectional	Children NAFLD (n = 38)	Dietary VitC intake was in agreement with recommended levels	[110]
Cross-Sectional	Adults Healthy control (n = 27) Steatosis (n = 33) NASH (n = 41)	Plasma concentrations of VitC did not differ between groups (p > 0.05) Dietary intake of VitC did not differ between groups (p > 0.05)	[111]
Prospective	Adults Healthy controls (n = 23) NAFLD (n = 29)	Plasma VitC concentrations were not different between groups (p = 0.65) Plasma VitC concentrations did not correlate with inflammatory grade (p = 0.56) or fibrosis stage (p = 0.53)	[112]
Cross-Sectional	Adults Fatty liver disease (n = 38) NASH (n = 67)	Plasma VitC concentrations were lower in NASH patients (p = 0.001)	[113]

NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; OR: Odds ratio; VitC: Vitamin C; Ref.: Reference.

3.4. Clinical Intervention Studies with VitC and NAFLD

To date, no study has directly examined the effect of VitC treatment alone in NAFLD and compared it to a placebo-treated control group. However, studies which combine VitC with other treatment form(s) have been conducted (Table 4). A 12 month, double-blinded, randomized controlled trial (RCT) examined the differences between placebo and combined treatment with VitC (500 mg/day) and E (600 IU/day) in children [117]. Both groups received lifestyle intervention, involving increased physical activity and a tailored diet aimed at inducing weight loss or maintaining weight in overweight and normal weight children, respectively. Liver brightness (as a measure of lipid accumulation), examined by ultrasound, circulating levels of liver enzymes, cholesterol and TG were reduced in both groups compared to baseline, but were not significantly different between groups [117]. Subsequently, the study was continued as an open-label study for an additional 12 months, but with fewer participants and the inclusion of a post-treatment liver biopsy [118]. Compared to baseline, both groups exhibited significant improvements in steatosis grade, lobular inflammation, hepatocyte ballooning and liver enzymes, but not in fibrosis scores. However, again there were no differences between placebo and treatment group. Thus, VitC and E supplementation are seemingly not superior to lifestyle intervention in children with NAFLD [118]. However, due to the study design, it is not possible to determine if vitamin treatment is better than no treatment at all. The authors rightfully suggested that increased fruit and vegetable consumption, augmented by the new diet, may well have minimized the effect of the vitamin treatment [118]. Indeed, VitC absorption is highly dose depended and excess amounts are effectively excreted and can therefore not facilitate additional health benefits [115]. Harrison *et al.* examined the effect of VitC (1000 mg/day) in combination with VitE (1000 IU/day) *versus* placebo after 6 months treatment of NASH patients [119]. Lifestyle interventions in both groups focused on exercise and a low-calorie diet. Hepatic inflammation, necrosis, fibrosis and liver enzymes were not significantly different between the groups. However, a significant, albeit clinically modest, intra-group improvement was observed in the vitamin treated group, with regards to hepatic fibrosis. On the basis of this result, the authors concluded that VitC and VitE treatment seemingly improves hepatic fibrosis in NASH patients [119]. However, this conclusion has been criticized; emphasizing that valid conclusion in placebo-controlled trials must be made by comparing treatment and placebo-groups rather than with historic data [120]. Interestingly, the fibrosis score was not improved in children with NAFLD, when compared to baseline [118]. Both studies implemented lifestyle changes, but doses of VitC and E, study duration and participants were different between the two studies. Additionally, the children with NAFLD had a low fibrosis score (0–1) at baseline, which may impede detection of potential improvements [118]. Importantly, liver physiology, development and the features of NASH may differ considerably between children and adults, which could affect study outcomes [121,122].

Table 4. Clinical intervention studies with VitC in NAFLD.

Design	Groups and Intervention		Outcome	Ref.
12 months, double-blinded, randomized clinical trial	Children Lifestyle intervention + placebo (n = 43) Lifestyle intervention + VitC (500 mg/day) and VitE (600 IU/day) (n = 45)		No differences between groups ($p > 0.05$)	[117]
24 months 12 month double-blinded followed by 12 month open-label, randomized clinical trial	Children Lifestyle intervention + placebo (n = 28) Lifestyle intervention + VitC (500 mg/day) and VitE (600 IU/day) (n = 25)		No differences between placebo and VitE/VitC groups ($p > 0.05$) Compared to baseline, treatment improved steatosis grade ($p < 0.001$), lobular inflammation ($p < 0.001$), hepatocyte ballooning ($p < 0.001$) and NAFLD activity score ($p > 0.001$), but not portal inflammation ($p = 0.1$) and fibrosis stage ($p = 0.6$).	[118]
6 month, double-blinded, randomized clinical trial	Adults Lifestyle intervention + placebo (n = 22) Lifestyle intervention + VitC (1000 mg/day) and VitE (1000 IU/day) (n = 23)		No differences between placebo and VitC/VitE groups ($p > 0.05$) Compared to baseline, VitC/VitE treatment improves fibrosis ($p = 0.002$)	[119]
12 months, pilot study No control group	Adults VitC (300 mg/day) and VitE (300 mg/day (n = 23)		Treatment decreased serum ALT ($p < 0.0001$) and hs-CRP ($p < 0.005$) and improved steatosis (6/10), necroinflammation (8/10), fibrosis (4/10).	[123]
6 month, open-label, randomized study	Adults Ursodeoxycholic acid (10 mg/kg/day) (n = 29) VitC (500 mg/day) and VitE (600 IU/day) (n = 27)		No differences between ursodeoxycholic acid and VitC/VitE treatment ($p > 0.05$)	[124]
4 years, randomized clinical trial	Adults NAFLD Placebo (n = 36) Treatment (1000 mg VitC/day, 1000 IU VitE/day, 20 mg atorvastatin/day) (n = 44)	Adults Normal liver Placebo (n = 190) Treatment (1000 mg VitC/day, 1000 IU VitE/day, 20 mg atorvastatin/day (n = 185)	Treatment reduced risk of having moderate to severe hepatic steatosis (OR = 0.36, $p < 0.017$)	[125]

ALT: Alanine aminotransferase; Hs-CRP: High sensitivity C-reactive protein; NAFLD: Non-alcoholic fatty liver disease; OR: Odds ratio; VitC: Vitamin C; VitE: Vitamin E; Ref.: Reference.

In a pilot study, NASH patients were treated with VitC (300 mg/day) and VitE (300 mg/day) for 12 months and changes were compared to baseline. BMI remained unchanged, but serum alanine aminotransferase and high-sensitivity-CRP were significantly reduced by the antioxidant treatment [123]. High-sensitivity-CRP has been shown to be associated with the severity of liver pathology in NASH [126]. Ten of the 23 study participants underwent liver biopsy post-treatment and of these, steatosis improved in 6/10, necroinflammation in 8/10 and fibrosis in 4/10 [123]. However, the lack of a placebo group confounds these findings with time-induced changes and complicates the interpretation of the potential effect of the combined VitC and E treatment. The effect of ursodeoxycholic acid (UDCA) (10 mg/kg/day) compared to that of VitC (500 mg/day) and VitE (600 IU/day) was examined in a 6 month, open-label, randomized study [124]. Compared to baseline, both treatments decreased circulating levels of liver enzymes without inducing changes in liver echogenicity. Side effects were reported in the UDCA treated group, whereas no side effects were reported in the vitamin treated group [124]. This is in agreement with other studies reporting that VitC and E are well tolerated [119,123]. Foster and coworkers evaluated the combination of atorvastatin (20 mg/day), VitC (1000 mg/day) and VitE (1000 IU/day) [125]. CT-scans were used to identify NAFLD. Subsequently, the participants were divided into NAFLD patients and normal liver patients. These two groups were further allocated to either the treatment combination or placebo. The four groups were reexamined after 2 and 4 years. In patients without NALFD at baseline, the treatment had no preventive effect. However, in patients with NAFLD, active treatment significantly reduced the prevalence of fatty liver [125]. This is contrary to the result of Ersöz *et al.*, who found no improvement in hepatic echogenicity after 6 months of treatment [124]. However, considerably lower vitamin doses, duration and different radiological methods were used by Ersöz *et al.*, which may explain the conflicting results. However, improvements in hepatic steatosis may of cause, in part be contributed to atorvastatin, as hepatic steatosis is associated with dyslipidemia [127]. Indeed, active treatment with VitC, VitE and atorvastatin did reduce plasma levels of LDL and cholesterol, while HDL and TG levels remained unchanged [125]. Furthermore, treatment with atorvastatin (20 mg/day) alone, improved ultrasound echo-patterns in NAFLD patients [128]. Unfortunately, radiological techniques are not able to detect small amounts of hepatic steatosis and can therefore not distinguish between the different forms of NAFLD [129]. Consequently, the effect of the treatment on more advanced stages of NAFLD is unknown.

4. Conclusion

Controlled animal experiments support a role of VitC deficiency in the elevation of plasma and hepatic lipids, alongside increased hepatic oxidative stress, fibrosis and inflammation. In agreement, VitC treatment of liver disease-induced animals has been shown to reduce hepatic markers of oxidative stress. Clinical studies of the relationship between VitC deficiency and NAFLD have not been conducted and studies of estimated dietary intakes of VitC in NAFLD patients are conflicting. Some studies show correlations between VitC intake and NAFLD progression, while others do not. Importantly, only a few studies have addressed VitC plasma levels in NAFLD patients [111–113]. The presently available RCTs have not found an effect of VitC superior to that of placebo. However, a number of studies do demonstrate hepatic improvements compared with baseline, but the use of treatment-cocktails and concurrent life-style interventions seriously confound the analysis of VitC specific effects. Additionally,

most RCTs have not recorded or reported baseline VitC concentrations and putative effects of VitC supplementation cannot be assessed in patients already saturated with VitC due to its non-linear absorption kinetics [16]. Therefore, the role of VitC in NAFLD should be investigated in future RCTs in which plasma VitC status is controlled for.

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Author Contributions

The manuscript was conceived by all authors. DHI wrote the draft manuscript which was subsequently edited by all authors.

Conflicts of Interest

The authors declare no conflict of interest.

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