Investigations of NASH and hepatic fibrosis in the guinea pig model

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Josephine Skat-Rørdam

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Name of department:  Department of Veterinary and Animal Sciences, Section of Experimental Animal Models

Principal Supervisor:  Pernille Tveden-Nyborg, DVM, PhD, DMSc, Associate Professor. Department of Veterinary and Animal Sciences, Section for Experimental Animal Models, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark.

Co-supervisors  Jens Lykkesfeldt, PhD, DMSc, Professor Department of Veterinary and Animal Sciences, Section for Experimental Animal Models, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

David Højland Ipsen, PhD, Senior Research Scientist Integrated Physiology Research, Obesity and NASH Pharmacology, Novo Nordisk A/S, Denmark

Markus Latta, PhD, Senior Scientific Director Integrated Physiology Research, Global Drug Discovery Novo Nordisk A/S, Denmark

Assessment committee  Axel Kornerup Hansen, Professor (Chair) Department of Veterinary and Animal Sciences, University of Copenhagen, Denmark

Peter Olinga, Professor Faculty of Science and Engineering, University of Groningen, Groningen, Netherlands

Aage Kristian Olsen Alstrup, Associate Professor Department of Clinical Medicine – Nuclear Medicine and PET, Århus University, Denmark

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Front page illustration  The histological image, and the image of guinea pigs is from study V and I of this thesis. The protein-protein association network is from study III.
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Preface
The work presented in this PhD was conducted from July 1st 2018 to June 1st 2022 (including a maternity leave from August 2021-May 2022). The project was supported by the Lifepharm centre of in vivo pharmacology, which also granted an additional three months due to COVID-19 related delays. The primary part of the experimental work was carried out at the University of Copenhagen, and the remaining work was carried out at Novo Nordisk.
This thesis is based on the following papers, referred to in the text by their roman numerals.


List of abbreviations adhering to the main text document

ASA: Acetylsalicylic acid
APOB: apolipoprotein B
ASK-1: apoptosis signal regulating kinase 1
CCL2/5: C-C motif chemokine ligand 2/5
CCL4: Carbon tetrachloride
CCR2/5: C-C motif chemokine receptor 2/5
CDAA: Choline deficient L-amino acid
CD68: Cluster of differentiation 68
CT: Computed tomography
CVC: Cenicriviroc
CVD: Cardiovascular disease
CXCR1: C-X-C motif chemokine receptor 1
Cyp7a1: Cytochrome p450 7a1
Cyp2e1: Cytochrome p450 2e1
DEG: Differentially expressed gene
DIAMOND: Diet-induced animal model of non-alcoholic fatty liver disease
DNL: De novo lipogenesis
ECM: Extracellular matrix
ELF: Enhanced liver fibrosis
ER: Endoplasmic reticulum
FFA: Free fatty acid
FIB-4: Fibrosis 4
FXR: Farnesoid X receptor
HCC: Hepatocellular carcinoma
HF: High fat
HMGCR: 3-Hydroxy-3-Methylglutaryl-CoA Reductase
HSC: Hepatic stellate cell
HU: Hounsfield unit
IL8: Interleukin 8
LDL: Low density lipoprotein
LF: Low fat
MCD: Methionine choline deficient
MMP: Matrix metalloproteinase
MRE: Magnetic metalloproteinase
MRI-PDFF: Magnetic resonance imaging proton density fat fraction
MTP: Microsomal triglyceride transfer protein
MRE: Magnetic resonance elastography
NAS: NAFLD activity score
NASH: Non-alcoholic steatohepatitis
NFS: NAFLD fibrosis score
PPARα/γ: Peroxisome proliferator activated receptor α/γ
PTX: Pentoxifylline
RNA: Ribonucleic acid
ROS: Reactive oxygen species
SERPINB9: Serpin family B member 9
SREBP2/1c: Sterol regulatory element binding protein 2/1c
TG: Triglyceride
THBS2: Thrombospondin 2
THRSP: Thyroid hormone responsive
TIMP: Tissue inhibitor of metalloproteinases
TNFα: Tumor necrosis factor α
VCTE: Vibration controlled transient elastography
VitC: Vitamin C
VitE: Vitamin E
VLDL: Very low density lipoprotein
VWF: Von willebrand factor
Summary in English
Reflecting the ongoing obesity and type 2 diabetes pandemics, non-alcoholic fatty liver disease (NAFLD) is estimated to affect a quarter of the global population. Progressing to steatohepatitis (NASH) with fibrosis, NAFLD can lead to chronic and debilitating liver disease and is linked to serious co-morbidities and increased mortality. Importantly, there is currently no approved pharmacotherapy, and diagnosis relies primarily on an invasive hepatic biopsy, rendering disease monitoring challenging. Furthermore, many of the preclinical models exhibit limited predictive validity, resulting in discordance between preclinical and clinical research findings. In view of these issues, this PhD aimed to elucidate the translational potential of the guinea pig as a model for NASH, and apply it for the identification of biomarkers and assessment of intervention.

Extending on previous findings confirming that guinea pigs develop NASH with a hepatic phenotype resembling humans, the studies of this PhD demonstrated a high degree of transcriptional similarity between guinea pig and human NASH. Moreover, correlation analysis of target genes and fibrosis severity identified SERPINB9 as a potential biomarker candidate for both guinea pig and human NASH. Investigating additional non-invasive modalities, CT scanning was applied in an intervention study assessing the effect of acetylsalicylic acid and pentoxifylline treatment. Though intervention did not improve histopathological hallmarks of NASH during the relatively short intervention time, CT scanning could be used to detect steatosis.

Exploring other potential treatment strategies, the effect of vitamin C (vitC) deficiency in combination with a high fat diet on NASH progression, was assessed. VitC deficiency did not exacerbate disease progression. However, the high fat NASH-inducing diet resulted in decreased glucose tolerance and weight gain compared to lean animals subjected to a low caloric/low starch diet, indicating metabolic dysregulation also reported in NASH patients. Supporting the role of diet, a change from high fat to a low fat diet appeared to be the primary driver of hepatic health improvement with minor additive effects of vitC. In this aspect, vitC deficiency appeared to delay recovery towards a healthy hepatic transcriptome compared to animals receiving a high vitC intake.

Collectively, the findings of this PhD thesis showed a high translational potential of the guinea pig NASH model, mimicking metabolic and hepatic aspects of human NASH. The disclosure of the NASH-associated transcriptome and subsequent response to intervention may promote the application of the guinea pig NASH model hereby paving the road for future discoveries of non-invasive disease markers and successful treatment modalities.
Resumé (Danish summary)
I takt med de voksende fedme og type 2 diabetes pandemier estimeres det, at en fjerdedel af verdens befolkning har non-alkoholisk fedtlever (NAFLD). Hvis det udvikler sig til steatohepatitis (NASH) med fibrose, kan NAFLD forårsage kronisk og invalidiserende leversyg med alvorlige følgesygdomme og øget dødelighed. Desværre er der i øjeblikket ingen godkendte lægemidler, og diagnosticering er primært baseret på en invasiv lever biopsi, der gør det svært at følge sygdomsudviklingen. Ydermere har mange af de prækliniske dyremodeller en begrænset prædiktiv validitet, hvilket resulterer i en uovrenstemmelse mellem prækliniske og kliniske forskningsresultater. I lyset af disse udfordringer, var målet med denne PhD at belyse den translationelle værdi af marsvinet som model for NASH, samt at bruge den til identifikation af biomarkører og til at teste intervention.


For at afsøge andre behandlingsmuligheder undersøgte vi effekten af vitamin C (vitC) mangel i kombination med en højfedtsdiæt på udviklingen af NASH. VitC mangel forværrede ikke sygdomsudviklingen. Dog resulterede den NASH fremkaldende højfedtsdiæt i en reduceret glukose tolerance og vægtøgning sammenlignet med slanke dyr på en lav kalorie/lav stivelsesdiæt, hvilket indikerede metabolisk dysregulering også set hos NASH patienter. Et skift fra højfedts- til lavfedtsdiæt understøtter disse resultater, da det var den primære årsag til forbedret lever status med en begrænset supplerende effekt af vitC. Det ser således ud til, at vitC mangel forsinker tilbagevenden til et sundt hepatisk transkriptom sammenlignet med dyr der fik tildelt højt vitC indtag.

Som helhed viser resultaterne i denne PhD et højt translationelt potentielle for marsvine modellen, der efterligner metaboliske og hepatiske aspekter af human NASH. Ved at kortlægge det NASH-
associerede transkriptom og dertilhørende reaktion på behandling for denne model, kan det være med til at promovere brugen af marsvinemodellen og derved bane vejen for opdagelsen af nye behandlingsmetoder og non-invasive sygdomsmarkører.
1. Introduction
Non-alcoholic fatty liver disease (NAFLD) affects ~25% of the population and is predicted to become the primary indication for liver transplant in the coming years (1). NAFLD is an umbrella term covering a range of disease states ranging from relatively benign hepatic steatosis (NAFL) to the more aggressive non-alcoholic steatohepatitis (NASH) defined by the presence of hepatocellular ballooning and inflammation with or without fibrosis (2, 3). An unhealthy diet coupled to a sedentary lifestyle are prime instigators of disease progression, and the worldwide obesity pandemic is generally believed to be the underlying driving force of increasing NAFLD prevalence (4, 5). Moreover, a large proportion of NAFLD patients suffer from related co-morbidities including dyslipidemia, hypertension and type 2 diabetes, emphasizing the need to view NAFLD as a multi-systems disease (2, 4-7). Despite intensive efforts to find a non-invasive alternative, diagnosis and disease monitoring is assessed by an invasive hepatic biopsy associated with a small albeit increased risk of fatal and non-fatal hemorrhaging, in addition to sampling, as well as intra- and inter observer variability (8, 9). Consequently, monitoring disease progression/regression in clinical and preclinical research with repeat biopsies, is suboptimal at best. Treatment options are limited as there is currently no pharmacotherapy approved by FDA or EMA, and development of new candidates is challenged by the lack of preclinical models reflecting the metabolic and hepatic phenotype of this disease (2, 3, 10). In this respect, the guinea pig offers several advantages as it is well established that this model develops diet-induced NASH with hepatic fibrosis bearing strong resemblance to human histopathology, and shares a low density lipoprotein (LDL) dominated lipoprotein profile (11-14). However, previous studies have failed to report insulin resistance and obesity in this model, and the molecular mechanisms driving disease progression have not been investigated in detail (13, 14). The overarching aim of this thesis was to study the molecular mechanisms of NASH disease progression and present a model with high translational potential, with the purpose to investigate intervention targeting inflammation and for the identification of novel biomarkers.

2. **Aims and Objectives**

This project aimed to identify biomarkers of fibrotic NASH and evaluate intervention targeting inflammation in an animal model closely mimicking human NASH. All studies applied the validated guinea pig model for NASH. To increase the translational potential of this model, study I aimed to induce insulin resistance and obesity by administering sugar water in addition to a high fat diet. Furthermore, the molecular mechanisms of guinea pig NASH were elucidated by targeted gene expression and transcriptome analysis, and the translational potential was assessed by comparison to two patient datasets and three murine models (II, III). As central disease mechanisms, inflammation and oxidative stress were targeted by intervention with acetylsalicylic acid and pentoxifylline (IV), and as one of the body’s major antioxidants, the effect of vitamin C deficiency on the progression and regression of NASH was assessed (V). To identify novel biomarkers with high translation potential, an in depth analysis of the guinea pig NASH transcriptome was performed (III). Additionally, as an established tool for detecting steatosis in patients, CT scanning was evaluated as a non-invasive measure to determine baseline disease stage and response to treatment in guinea pigs (IV).

Based on the outlined challenges in NAFLD/NASH research this PhD project investigated the following hypothesis:

I. **Guinea pig NASH resembles human NASH**  
   - Manuscript I, II, III

II. **NASH and associated fibrosis can be resolved through intervention targeting inflammation**  
   - Manuscript IV, V

III. **Determination of fibrotic NASH does not require a biopsy**  
   - Manuscript III, IV
3. NAFLD/NASH prevalence and etiology
NAFLD affects all age groups, and not only introduces a significant risk of detrimental consequences for liver function, but is also associated with serious co-morbidities and increased mortality (5, 15, 16) (figure 1). The importance of extrahepatic morbidities is substantiated by cardiovascular disease being the primary cause of death in NAFLD patients (17) (figure 1). Furthermore, a study of morbidly obese patients, identified NAFLD in 80.2% of the patients, and a meta-analysis estimated a NAFLD prevalence of 55.5% among type 2 diabetes patients, underscoring the multifactorial nature of this disease (6, 18) (figure 1). In consequence, the etiology of NAFLD is closely linked to obesity and insulin resistance, and is supported by studies demonstrating an inverse correlation between insulin sensitivity and the amount of intrahepatic triglycerides (19-21) (figure 1).

Figure 1. Metabolic co-morbidities
A diet high in simple sugars, fat and cholesterol may lead to obesity and adipocyte dysfunction (22). This can mediate an increase in circulating FFAs and cholesterol, which can be deposited in the arteries or the liver resulting in CVD and NAFLD respectively (22, 23). Likewise, a high intake of simple sugars can lead to fluctuating glucose levels putting a strain on pancreatic insulin production, in turn inducing insulin resistance and ultimately diabetes (24). Concomitantly, increased release of cytokines from the affected tissues may induce low-grade inflammation and deplete antioxidant reserves leading to oxidative stress (22, 24, 25). This illustrates the interrelated metabolic phenotype of NAFLD patients. FFA: Free fatty acids, NAFLD: Non-alcoholic fatty liver disease, CVD: Cardiovascular disease. Created with BioRender.com.
These mechanisms are in line with the multiple-hit hypothesis proposed by Tilg and Moschen, where steatosis arises as a result of increased free fatty acid supply, primed by insulin resistance, adipocyte dysfunction and excess dietary intake (25). The resulting steatosis is described as a hepatoprotective mechanism, which also explains why the majority of patients with simple steatosis do not progress to NASH (26, 27) (figure 2). Rather, insults leading to inflammation and fibrosis may even precede steatosis, or at least occur simultaneously, and include lipotoxicity, endoplasmic reticulum (ER) stress, adipocytokines, gut-derived endotoxins and genetics, which alone or in combination with steatosis lead NASH (25). NASH prevalence is estimated to 1.5%-6% and is expected to increase exponentially over the coming years (28, 29) (figure 2).

Although patients diagnosed with NASH are considered at increased risk of liver-related mortality, fibrosis alone is the number one predictor of liver- and all cause mortality in NAFLD patients (15, 28, 30, 31). Furthermore, whereas NAFL and even NASH with mild fibrosis is largely reversible, the vast architectural changes occurring with advanced fibrosis, and particularly cirrhosis and hepatocellular carcinoma, severely impact the chances of complete reversibility and disease resolution, although this is still a topic of debate (32, 33) (figure 2).
Figure 2. Disease spectrum and etiology
NAFLD is defined by the presence of >5% liver fat and encompasses several hepatic phenotypes (2). A high caloric intake coupled to a sedentary lifestyle and genetics can lead to increased release of FFAs from dysfunctional adipose tissue, which can be taken up by the liver resulting in steatosis (22). Simple steatosis or NAFL is the liver’s natural response to an increased lipid load, and is a reversible disease state without hepatocellular damage (2). However, endotoxins, adipocytokines and decreased antioxidant pool, provide further stimuli, driving progression towards NASH (25). Defined by inflammation and ballooning hepatocytes, NASH is still largely reversible, albeit advanced fibrosis and ultimately cirrhosis can be a permanent state, ultimately resulting in liver failure (2). NAFL: Non-alcoholic fatty liver, NASH: Non-alcoholic steatohepatitis, HCC: Hepatocellular carcinoma, FFAs: Free fatty acids. Adapted from “Non-alcoholic fatty liver disease (NAFLD) spectrum”, by BioRender.com (2020). Retrieved from https://app.biorender.com/biorender-templates.

4. Disease pathogenesis
4.1 Early NAFLD
The hepatic lipid build-up is a consequence of an imbalance between lipid uptake and de novo lipogenesis (DNL) on the one hand, and oxidation and lipid export on the other. In a study of obese NAFLD patients, results using stable isotopes to discern the sources of hepatic triglyceride (TG) accumulation in the liver, demonstrated that 59.9% of the TG came from peripheral tissues, 26.1% from DNL and 14.9% from the diet (34). Thus in obese patients, the primary source of TG is delivery from extrahepatic tissues (35-39) (figure 3). Upon entry, FFAs are esterified through a series of steps and ultimately stored as triglycerides (40). The other major contributor to the intracellular TG pool is DNL, which synthesizes fatty acids are from acetyl-CoA occurs in a setting of excess carbohydrates, and is increased in NAFLD patients (figure 3) (41). Thus while increased uptake and DNL may induce a hepatic lipid build-up, this is not necessarily a pathogenic mechanism in itself, but is actually hepatoprotective (25). It is generally accepted that TGs alone are not lipotoxic, rather the excess delivery and increased DNL coupled to a decreased antioxidant pool, may form lipotoxic derivatives and aggregation of intermediary products (26, 27, 42).
Histologically steatosis originates around the central veins and is predominantly in the form of large vesicles that decentralize the hepatocyte nucleus. Microvesicular steatosis is also commonly found, but lesions of purely diffuse microvesicular steatosis are not typically associated with adult NAFLD (43, 44).

Upon accumulation, lipids can leave the liver in two ways, export through very low density lipoprotein (VLDL) or oxidation (figure 3). The primary site for fatty acid oxidation is the
Upon mitochondrial dysfunction, or for oxidation of very long chain fatty acids (>C20), peroxisomal β-oxidation takes over. For branched and odd number FFAs, oxidation occurs through cytochrome p450 2e1 (CYP2e1) mediated cytochromal oxidation in the ER (27).

Collectively these pathways are positively regulated by peroxisome proliferator activated receptor α (PPARα) (45, 46).

**Figure 3. Major pathogenic mechanisms in NASH**

Multiple factors including a diet high in fat, simple sugars and cholesterol leads to obesity and insulin resistance (22). Adipocyte insulin resistance may lead to increased release of FFA, which can be taken up by the liver by FATP5 and CD36 (35–39). FFAs can then be stored in the liver as triglycerides. Lipids are also formed de novo (DNL) in the liver by ACC1 and FAS, which are activated by SREBP-1c which in turn is upregulated by PPARy, and simple carbohydrates such as fructose (35, 40, 47, 48). The accumulated lipids can leave the liver via VLDL mediated export, or oxidation. VLDL particles comprised of FFA, apolipoproteins, phospholipids and cholesterol are packaged in the ER by MTP (49). While oxidation is a natural process, increased lipid load may deplete the oxidative capacity leading to generation of toxic lipid intermediates and ROS (50). Furthermore, in some individuals an unhealthy diet and low-grade inflammation may further reduce the antioxidant pool increasing ROS, which induces hepatocyte injury (27). High cholesterol levels activate LXRα, which stimulates cholesterol excretion through bile (51). FXR is a major regulator of bile acids, and inhibits the rate limiting enzyme Cyp7a1, while promoting export through ABCGS/8 (51, 52). FXR also targets SREBP1c thereby lowering DNL (53). High hepatic cholesterol levels may directly promote...
hepatocyte apoptosis, which in turn activates resident Kupffer cells (54, 55). The activated Kupffer cells produce inflammatory and fibrotic cytokines which can promote recruitment of immune cells and activation of HSCs respectively (56, 57). HSCs deposit ECM leading to hepatic fibrosis, which in a positive feedback loop may further activate HSCs (57). Macrophages may adopt a resolution phenotype producing MMPs that can disintegrate the ECM (33, 58). MMPs are inhibited by TIMPs produced by HSCs and Kupffer cells (59). Upon cessation of stimuli, HSCs may revert to an inactive phenotype that remains primed waiting for the next insult (60, 61).


4.2 NASH
A multitude of factors drives the unhealthy progression of simple fatty liver to steatohepatitis. TG accumulation and lipid flux through mitochondrial, peroxisomal or ER oxidation is not in itself pathogenic, however, the perpetual lipid load may exhaust the oxidative capacities of these pathways, leading to the formation of reactive oxygen species (ROS), ER-stress and lipotoxic lipid metabolites (figure 3) (50). Furthermore, poor nutrition and lack of exercise drains the remaining antioxidant pool leading to further accumulation of ROS and lipid toxins inducing apoptosis, which may then activate inflammatory responses (27) (figure 3). Hepatocellular damage, including ballooning and apoptosis, is a major hallmark of NASH (62, 63). Ballooning hepatocytes are histologically described as enlarged cells with reticulated cytoplasm (64). Ballooning is generally regarded as a sign of active disease, and the NASH diagnosis is strictly confined to patients where ballooning is evident from histopathological stainings. Inflammation is typically presented as mild lobular infiltration of mononuclear cells such as T-cells and macrophages with the occasional finding of microgranulomas (43, 44). Free cholesterol is also directly damaging to hepatocytes, and free cholesterol was increased in patients with NASH compared to control livers (54, 55). Upon low levels of intracellular cholesterol, sterol regulatory element binding protein 2 (SREBP2) is activated and stimulates expression of the LDL receptor, which is increased in NAFLD patients and induces increased uptake of cholesterol-rich LDL (51, 52). Simultaneously SREBP2 stimulates expression of 3-hydroxyl-3-methylglutaryl-CoA (HMG-CoA) reductase, which is responsible for the rate limiting
step in cholesterol synthesis. Bile acids are the major site of excess cholesterol excretion from the body, which occurs through bile acid transporters (52). In mouse models omitting cholesterol from a high fat (HF) diet dampens the inflammatory response, and it is speculated that cholesterol, can accumulate in Kupffer cells leading to an inflammatory response (65). Collectively the damaged hepatocytes, increased ROS build-up, cholesterol and adipocytokine influx in the liver activates resident Kupffer cells, which release cytokines and chemokines, which further attracts infiltrating monocytes, and neutrophils (66) (figure 3). The cytokines released from the immune cells may, in turn, illicit damaging responses in hepatocytes thus propagating a vicious cycle of inflammation and hepatocellular damage. Finally, the activated Kupffer cells and infiltrating monocytes may release pro-fibrotic cytokines, which can fuel the differentiation of hepatic stellate cells (HSCs) from their quiescent to their activated state promoting fibrosis (56, 57) (figure 3).

4.3 Hepatic fibrosis
A core feature of the liver’s response to injury is fibrogenesis or scarring, characterized by excessive extracellular matrix (ECM) deposition and activation of HSCs (myofibroblasts) (57). Like steatosis, fibrosis begins around the central veins, and may extend to engulf individual hepatocytes creating a pattern of “chicken wire” fibrosis. Upon disease progression, the fibrotic tissue will range across a lobule forming bridges between two central veins or a central vein and a portal tract (43, 44). Under normal conditions, HSCs represent 10% of all liver cells and are located in the space of Disse (the subendothelial space between hepatocytes and endothelial sinusoidal cells), where they store retinoids (vitamin A) in their quiescent state (67). In contrast, activated HSCs (myofibroblasts) are the major ECM producing cells of the liver – and are contractile, proliferative cells characterized by α smooth muscle actin (α-SMA) expression (57, 60, 67). Macrophages and Kupffer cells are major instigators of HSC activation, and in mice ablation of macrophages severely dampens HSC activation (56, 68). Apart from infiltrating and resident immune cells, apoptotic bodies from hepatocytes have been shown to activate HSC by engulfment in vivo (69). Furthermore, dietary factors such as cholesterol may directly stimulate HSC activation (70). Upon activation, HSCs can produce a range of ECM proteins, the composition of which changes along with disease progression (58). Regardless of stage, the most abundant types are Collagen I and III while elastin, in particular, is primarily expressed in more advanced fibrosis (71). In a positive feedback loop HSCs receive differentiation and proliferative signals from ECM
components, creating a vicious cycle of fibrosis deposition and further activation of HSCs (57) (figure 3). Similar to the importance of HSC activation in fibrosis progression, inactivation or apoptosis of HSCs are cardinal mechanisms toward fibrosis regression. In contrast to quiescent HSCs, inactivated HSCs remain “primed” for activation, thus allowing them quick reversal to the activated phenotype, upon further insult (60, 61). Accordingly, complete neutralization of HSCs can only occur through apoptosis (59). True fibrosis regression is not only dependent on inactivation and apoptosis of HSCs, but also disintegration of the fibrotic tissue, which primarily occurs through matrix metalloproteinases (MMPs). MMPs are inhibited by tissue inhibitor of metalloproteinases (TIMPs), which are secreted by pro-fibrotic macrophages and HSCs themselves (figure 3) (59). Thus, fibrosis progression or regression is controlled by an intricate network of different cell types, growth factors, cytokines, and environmental cues, which collectively promote one direction over the other.

5. Diagnostics
Liver biopsy and assessment of patient histopathology remain the gold standard for several aspects of diagnosis and treatment options in the field of NAFLD/NASH. These include: distinguishing between NAFLD and NASH, fibrosis staging, and potential eligibility for pharmacological therapy such as inclusion criteria for clinical trials (2, 3). However, due to the invasive nature of the hepatic biopsy, the use for screening of NAFLD/NASH at a population level is not feasible. Consequently, researchers are heavily invested in the advancement of this field, and testing and development of biomarkers is overseen by two large consortia Liver Investigation: Testing Marker Utility in Steatohepatitis (LITMUS), and Non-invasive Biomarkers of Metabolic Liver Disease (NIMBLE) (72). Several non-invasive modalities are recommended for the assessment of “high risk” groups. These include circulating markers: NAFLD fibrosis score (NFS), Fibrosis 4 (Fib-4), enhanced liver fibrosis test ((ELF), by European guidelines only) and imaging modalities: ultrasonography ((US), by European guidelines only) and the FDA approved vibration controlled transient elastography ((VCTE), Fibroscan®) (2, 3) (table 1). In general, several serum markers and panels, including NFS and FIB-4 share a high negative predictive value and low positive predictive value, meaning that they perform well when excluding disease, but not well as diagnostic tools (table 1) (73, 74). Currently, VCTE is the most widely used noninvasive detection method for fibrosis, and a recent prospective study reported that VCTE distinguished advanced fibrosis (F3-F4)
from patients with mild or no fibrosis (F0-F2) (75). Magnetic resonance elastography (MRE) has been found superior to VCTE in assessing dichotomous and all stage fibrosis, in two head-to-head comparative studies and a meta-analysis (74, 76, 77). MRI-proton density fat fraction (MRI-PDFF) is the MRE equivalent for detection of steatosis. MRI-PDFF is FDA approved for the use as inclusion criteria in early phase (I-IIb) clinical trials for NASH and has a high diagnostic accuracy across the entire spectrum of fat infiltration (78-81). Assessment of liver steatosis can also be conducted using computed tomography (CT) scanning, which provides a quantitative measure of attenuation in Hounsfield units (HU) (82). CT scanning uses a cut-off for steatosis of <40 HU for the liver, or a liver-spleen difference of greater than 10 HU, and has been used as inclusion criteria for steatosis detection in clinical trials (82-85). Additionally, due to the metabolic co-morbidities, liver steatosis is often detected on CT scans obtained for other indications (86). However, due to the exposure to ionizing radiation, CT scanning is not routinely used to diagnose NAFLD (table 1). Thus, for the most reliable, safe and accurate diagnosis of either fibrosis or steatosis, MRE and MRI-PDFF are preferable. Nevertheless, due to the high cost and limited availability, these modalities are not feasible for population screening (table 1).

Table 1. Advantages and disadvantages of commonly used non-invasive biomarkers

<table>
<thead>
<tr>
<th>Non-invasive marker (REF)</th>
<th>Detection</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultrasonography (87)</td>
<td>Steatosis</td>
<td>Readily available Low cost</td>
<td>Subject to inter – and intra observer variability, not quantitative, reliability influenced by obesity and diabetes</td>
</tr>
<tr>
<td>Computed tomography (82)</td>
<td>Steatosis</td>
<td>Semi-quantitative can be obtained from existing image</td>
<td>Exposure to radiation, high cost, not readily available</td>
</tr>
<tr>
<td>MRI-PDFF (79)</td>
<td>Steatosis</td>
<td>Quantitative, high diagnostic accuracy</td>
<td>Not readily available, high cost</td>
</tr>
<tr>
<td>MRE (88)</td>
<td>Fibrosis</td>
<td>Quantitative, high diagnostic accuracy</td>
<td>Not readily available high cost</td>
</tr>
<tr>
<td>VCTE (75)</td>
<td>Fibrosis</td>
<td>Quantitative, readily available (with fibroscan) FDA approved, low cost</td>
<td>Reliability influenced by obesity and diabetes</td>
</tr>
<tr>
<td>NFS (age, hyperglycemia, BMI, albumin, platelet counts, AST/ALT ratio) (89)</td>
<td>Fibrosis</td>
<td>Well validated, low cost, readily available, high negative predictive value</td>
<td>Low positive predictive value</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>----------</td>
<td>-----------------------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>FIB4 (age, ALT, AST and platelet count) (90)</td>
<td>Fibrosis</td>
<td>Well validated, readily available, low cost, high negative predictive value</td>
<td>Low positive predictive value</td>
</tr>
<tr>
<td>ELF (TIMP-1, HA, PIIINP) (91, 92)</td>
<td>Fibrosis</td>
<td>Fibrosis specific, high negative predictive value</td>
<td>Not readily available</td>
</tr>
</tbody>
</table>


5.1 Identification of novel biomarkers
To identify novel biomarkers and accommodate the increasing demand for screening and monitoring patient response to treatment, the omics and even multi-omics approach holds great promise. With high throughput methods and machine learning in rapid development, this field is expanding at an accelerated pace (93, 94). Transcriptomics in the form of circulating micro-RNAs may be used for diagnosis, and expression profiling is a valuable tool for the discovery of novel biomarkers (95-97) (III). Accordingly, in a recent study serum micro-RNAs were profiled in a patient cohort, and miR-193p-5a was shown to correlate with NAFLD activity score (NAS) and fibrosis stage (98). Another study used transcriptomics to identify secreted proteins demonstrating differential regulation between NAFL and NASH patients (n=98), and identified thrombospondin 2 (THBS2) as a potential biomarker candidate. To assess the value of THBS2 as a biomarker, this study also measured serum levels of the secreted protein (TSP-2) in NAFL and NASH patients (n=213), and reported that TSP-2 could predict NASH and advanced fibrosis to the same degree as NFS and FIB-4 (99). Furthermore, public databases such as geo expression omnibus has made the comparison between different patient groups and cross-species possible (100).

6. Current treatment options
Despite several drug candidates reaching phase III clinical trials, only the REGENERATE study (Obeticholic acid – a farnesoid X receptor (FXR) agonist see figure 3) has currently reported data
from the interim analysis meeting the primary endpoint (101). Several other candidates reaching phase III trials have been terminated (AURORA (Cenicriviroc (CVC)), RESOLVE-IT (Elafibranor), STELLAR-3 (Selonsertib) (102, 103)). This illustrates the inherent challenge in providing effective treatment to NASH patients. In lieu of FDA/EMA approved pharmacotherapy for the treatment of NASH, all patients are recommended lifestyle changes aiming at a weight loss. According to EASL and AASLD guidelines, patients are encouraged to restrict calorie intake (daily reduction of 500-1000 kcal) and exercise with moderate intensity, aiming at a 7-10% weight loss. Specifically, European guidelines suggest adherence to the Mediterranean diet (high in monounsaturated fatty acids), and excluding processed foods and products high in added fructose. Finally, vitamin E (vitE) alone or in combination with pioglitazone can be considered (2, 3, 47).

6.1 Targeting inflammation
When searching for potential drug targets three general aspects of NASH pathology can be targeted: steatosis, inflammation and fibrosis. Inflammation is what distinguishes NAFLD and NASH patients, and is believed to be a causal factor in the progression of fibrosis, and hence plays a key role in the progression from steatosis to active steatohepatitis (66, 104). Consequently, several drug candidates target inflammation, including CVC (a dual C-C motif chemokine receptor 2/5 (CCR2/5) inhibitor), Emricasan (a pan-caspase inhibitor), Selonsertib (an apoptosis signal regulating kinase 1 (ASK1) inhibitor), Pentoxifylline (PTX) and vitE (figure 4) (103).
Figure 4. Mechanism of action for drug candidates targeting inflammation

CCR2/CCR5 receptors are present on monocytes, Kupffer cells and HSCs. CVC is a dual CCR2/CCR5 receptor antagonist, and inhibits inflammation and fibrosis by halting monocyte recruitment and HSCs activation (105). Selonsertib is an ASK-1 inhibitor. ASK-1 is an oxidative stress activated kinase, which induces JNK mediated hepatocyte apoptosis, inflammation and activation of HSCs (106). Emricasan is a pan-caspase inhibitor, which decreases hepatocyte apoptosis, ballooning and ensuing inflammation (107). PTX has an antioxidative and anti-inflammatory effect, and has been shown to lower oxidized lipid products in patients (108). VitE is a free-radical scavenger with anti-apoptotic effects. Through inhibition of transcription factor NF-kB, VitE also suppresses expression of inflammatory cytokines TNF-α, IL-1 and IL-6 (109). CVC: Cenicriviroc, PTX: Pentoxifylline, VitE: Vitamin E, ROS: Reactive oxygen species, HSC: Hepatic stellate cell, ASK-1: Apoptosis signal-regulating kinase 1, TNF-α: Tumor necrosis factor α, IL: Interleukin. Created with BioRender.com.

6.1.1 Clinical research findings

CVC and Selonsertib, but also Emricasan have all been cancelled in costly late-phase trials (table 2). While neither study met its primary endpoint, the ENCORE-NF study was particularly devastating as nominally more patients in the treatment group had worsening of ballooning and fibrosis, suggesting the inability to halt cellular death signaling cascade once activated (107) (table 2, figure 4). PTX reduced ballooning or NAS score in two clinical trials, an effect which was corroborated by two meta-analyses (110, 111) (table 2). Likewise, antioxidant treatment with VitE reduced
inflammation in NASH patients without diabetes in a large phase III study, and vitE was found to improve all histological parameters in a recent meta-analysis (table 2) (112). As such PTX and vitE, which each have multiple targets, have generally been more successful than CVC, Emricasan and Selonsertib, which are more specific (figure 4). This is supported by a current trend using combination therapy, as an example CVC is currently being tested with Tropifexor (an FXR agonist) in a phase IIb trial (113).

Table 2. Major clinical trials for drugs targeting inflammation

<table>
<thead>
<tr>
<th>Clinical trial (REF)</th>
<th>Primary endpoint</th>
<th>Number of patients</th>
<th>Study duration</th>
<th>Reason for termination</th>
<th>Effect on inflammation</th>
<th>Effect on fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>AURORA (CVC) Phase III (NCT03028740) (114)</td>
<td>Fibrosis improvement without worsening of NASH</td>
<td>~1200 (part 1)</td>
<td>52 weeks (part 1)</td>
<td>Lack of efficacy</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>ENCORE-NF (Emricasan) (NCT02686762) Phase IIb (107)</td>
<td>Fibrosis improvement without worsening of NASH</td>
<td>320</td>
<td>72 weeks</td>
<td>Lack of efficacy</td>
<td>No. More patients in the treatment group had worsening of ballooning</td>
<td>No. Nominally more patients in the treatment group had worsening of fibrosis</td>
</tr>
<tr>
<td>STELLAR-3 Selonsertib (NCT03053050) Phase III (115)</td>
<td>Fibrosis improvement without worsening of NASH</td>
<td>802</td>
<td>48 weeks</td>
<td>Lack of efficacy</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>PTX (NCT00590161) Phase II (116)</td>
<td>Improvement of ≥2 points on NAS score</td>
<td>55</td>
<td>52 weeks</td>
<td>Study completed</td>
<td>Yes (no effect on ballooning)</td>
<td>Yes</td>
</tr>
<tr>
<td>PTX (NCT00267670) Phase II (117)</td>
<td>Improvement of ≥30% ALT from baseline</td>
<td>30</td>
<td>52 weeks</td>
<td>Study completed</td>
<td>No (only ballooning and NAS)</td>
<td>No</td>
</tr>
<tr>
<td>TONIC (VitE) (NCT00063635) Phase III (118)</td>
<td>Reduction in ALT to 50% of baseline or &lt;40 IU/L</td>
<td>173 (age 8-17)</td>
<td>96 weeks</td>
<td>Study completed</td>
<td>No (however more patients in VitE group had resolution of NASH, and</td>
<td>No</td>
</tr>
</tbody>
</table>
### Preclinical research findings

In response to the late-phase clinical trial failures, experts within the field have recently highlighted the need for more robust preclinical data before moving forward with human testing, supporting the need for animal models with greater translational potential (10, 102). Interestingly, in preclinical models, CVC did not affect inflammation, whereas CVC, Emricasan and Selonsertib all effectively reduced fibrosis, and thus, were considered successful (table 3). However, the translational potential of fibrosis is particularly challenging, as both the accumulation and disintegration of fibrotic tissue is believed to occur on a different timescale in mice compared to humans (10). This comparison is made increasingly difficult by the lack of non-invasive markers and standardized assessment of fibrosis regression, according to a recent panel discussion (121).

Unlike CVC, PTX and vitE both reduced inflammation scores in mice, albeit this was not the case in guinea pigs (table 3). Thus for CVC the lack of inflammatory response in the animal models also translated to negative results in the clinical setting. In contrast, PTX and vitE, which demonstrated inflammatory reduction in mice, showed more promise in clinical trials, underscoring the importance of preclinical testing. Finally, table 3 also highlights the variety of animal models used for initial drug screening. This makes inter-study comparison challenging, and potentiates advancement of candidate drugs based on a model with little relevance to human disease. Furthermore, the effectiveness of testing pharmacotherapy in animal models can be diminished by the heterogeneity of disease severity in individual animals. As such a baseline non-invasive test, to

<table>
<thead>
<tr>
<th>Study</th>
<th>Improvement in NAFLD disease activity (without diabetes)</th>
<th>Study completed</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIVENS (VitE) (NCT00063622) Phase III (119)</td>
<td>247 weeks</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>VitE (NCT01002547) Phase 4 (120)</td>
<td>2 point reduction in NAFLD disease activity score without worsening of fibrosis</td>
<td>78 weeks</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

*CVC: Cenicriviroc, PTX: Pentoxifylline, VitE: Vitamin E, NAFLD: Non-alcoholic fatty liver disease, NASH: Non-alcoholic steatohepatits, NAS: NAFLD activity score, ALT: Alanine aminotransferase, IU: International units, L: Liter, NA: Not applicable.*
determine disease stage and potential non-responders prior to pharmacological treatment, would increase the statistical power and reduce the number of animals in line with the 3R’s (122, 123).

Table 3. Major preclinical studies for drugs targeting inflammation

<table>
<thead>
<tr>
<th>Drug candidate (REF)</th>
<th>Species: Strain</th>
<th>Sex</th>
<th>Model</th>
<th>Effect on inflammation</th>
<th>Effect on fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVC (105)</td>
<td>Rat: Sprague dawley</td>
<td>Male</td>
<td>TAA</td>
<td>NA</td>
<td>Yes (Quantification)</td>
</tr>
<tr>
<td>Emricasan (125)</td>
<td>Mouse: C57BL6/J</td>
<td>Male</td>
<td>MCD</td>
<td>No (only on NAS, not inflammation) Reduction in number of infiltrating monocytes</td>
<td>Yes (Quantification)</td>
</tr>
<tr>
<td>Selonsertib (126, 127)</td>
<td>Mouse: C57BL/6</td>
<td>Male</td>
<td>HF</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>PTX (128)</td>
<td>Mouse: C57BL/6/J</td>
<td>Male and female</td>
<td>MCD</td>
<td>Yes</td>
<td>Mice did not develop fibrosis</td>
</tr>
<tr>
<td>PTX (IV)</td>
<td>Guinea pig: Hartley</td>
<td>Female</td>
<td>HF</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>VitE (129)</td>
<td>Mice: C57/B6</td>
<td>Male</td>
<td>MCD</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>VitE (130)</td>
<td>Guinea pig: Hartley</td>
<td>Female</td>
<td>HF</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>


7. Modelling NASH

There is an urgent need for animal models recapitulating human NASH disease pathology, allowing for higher predictive validity, increasing the success rate of drug candidates, and providing much needed insight into the complex pathological mechanisms. Furthermore, as NASH is a multi-
systems disease, diet-induced obesity, as well as accompanying dyslipidemia, adipose tissue dysfunction and insulin resistance, are important factors when assessing the translational potential of a given model (figure 1). As listed in table 4, HF diet models (HF diet, Western, diet-induced animal model of non-alcoholic fatty liver disease (DIAMOND)) share development of the metabolic phenotype, but lack the presence of advanced fibrosis (unless exposed to an HF diet >25 weeks). Typically, HF diet alone rarely induces advanced disease, rather addition of particularly cholesterol has been shown to induce hepatocellular damage and fibrosis (131). However, the very high cholesterol content sometimes seen in these diets (>0.5%) is toxic and cannot be compared to human consumption (132). To better reflect the macronutrient content in human diets, western diets (also named fast-food diets, cafeteria diets) often contain added simple sugars (fructose, glucose, sucrose). Furthermore, as soft drink consumption is linked to obesity, insulin resistance and NAFLD, several preclinical models include sugar in the drinking water (133, 134) (table 4). Particularly fructose has been shown to induce DNL and is believed to play a role in NAFLD pathogenesis, albeit large clinical studies to confirm these results are lacking (135, 136). In contrast to HF mice, DIAMOND and western diet mice both develop NASH with mild fibrosis (table 4). However, progression to a more advanced fibrosis stage required a lengthy feeding regime (36 weeks for DIAMOND mice) (137, 138). In contrast, choline deficient L-amino acid defined (CDAA), methionine choline deficient (MCD) and carbon tetrachloride (CCL4) mice displayed more progressive disease, all within a relatively short timeframe (12-16 weeks). Thus adding an additional insult to the HF/western diet mediates more severe disease, albeit as listed in table 4 this is at the expense of metabolic co-morbidities. Furthermore, opposite human NASH, DNL in MCD mice is downregulated, and the weight loss induced by this diet impacts its translational potential (139) (table 4). Finally, disease induction by VLDL accumulation (MCD, CDAA), or from exposure to toxins (CCL4) is far from human disease etiology and should be considered when choosing which animal model to use.

Table 4. Murine models of NAFLD

<table>
<thead>
<tr>
<th>Model (REF)</th>
<th>Diet</th>
<th>Pathology</th>
<th>Limitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>High fat diet</td>
<td>60% calories from fat, 20%</td>
<td>Obesity, hyperinsulinemia, glucose intolerance,</td>
<td>Fibrosis is not induced until week 50, and does</td>
</tr>
<tr>
<td>(140)</td>
<td>from fat, 20% from glucose</td>
<td>steatosis after 12 weeks. Mild</td>
<td>not progress from mild fibrosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet/Deficiency</td>
<td>Carbohydrates, 20% from proteins</td>
<td>Inflammation after 19 weeks. Mild fibrosis after 50 weeks. No ballooning reported</td>
<td>Obesity, increased insulin and glucose levels, NASH with mild fibrosis after 25 weeks</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------------------------</td>
<td>---------------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>Western diet (141)</td>
<td>40% calories from fat (high in saturated fats), 2% cholesterol, high in fructose, can also include access to HFCS in drinking water</td>
<td>Obesity, increased insulin and glucose levels, NASH with mild fibrosis after 25 weeks</td>
<td>Only mild fibrosis after 16-24 weeks</td>
</tr>
<tr>
<td>DIAMOND (137)</td>
<td>42% calories from fat, 0.1% cholesterol, HFCS in the drinking water</td>
<td>Obesity, insulin resistance, mild NASH with mild fibrosis after 16-24 weeks. Bridging fibrosis after 36 weeks</td>
<td>Only mild fibrosis after 16-24 weeks</td>
</tr>
<tr>
<td>CDAA + High fat diet (142)</td>
<td>Deficient in choline, defined L-amino acid, 0.1% methionine</td>
<td>No obesity or insulin resistance, mild NASH with mild to advanced fibrosis after 12 weeks</td>
<td>Metabolic co-morbidities are lacking, micronutrient deficient</td>
</tr>
<tr>
<td>MCD (143)</td>
<td>Deficient in choline and methionine</td>
<td>No obesity (weight loss), or insulin resistance, NASH with advanced fibrosis after 16 weeks</td>
<td>Metabolic co-morbidities are lacking, micronutrient deficient</td>
</tr>
<tr>
<td>CCL₄ + Western diet (144)</td>
<td>As described for western diet</td>
<td>No obesity or insulin resistance, NASH with advanced fibrosis after 16 weeks</td>
<td>Metabolic co-morbidities are lacking, toxin-induced</td>
</tr>
<tr>
<td>ob/ob + Western diet (145)</td>
<td>As described for western diet</td>
<td>Obesity and insulin resistance with mild inflammation and fibrosis and no ballooning after 3 months</td>
<td>Only mild disease, no ballooning</td>
</tr>
<tr>
<td>db/db + Western diet (145)</td>
<td>As described for western diet</td>
<td>Obesity and insulin resistance with mild inflammation and fibrosis and no ballooning after 3 months</td>
<td>Only mild disease, no ballooning</td>
</tr>
</tbody>
</table>

For the sake of simplicity only mouse model were included. Mild fibrosis is defined as grade 1-2, advanced as grade 3-4. Mild NASH defined as <5 average NAS score. NASH: Non-alcoholic steatohepatitis, HFCS: High fructose corn syrup, DIAMOND: Diet-induced animal model of non-alcoholic fatty liver disease, CDAA: Choline deficient L-amino acid defined, MCD: Methionine and choline deficient, CCL₄: Carbon tetrachloride.

7.1 The guinea pig NASH model
The studies included in this PhD are based on the previously established guinea pig model for NASH, which develops NASH with bridging fibrosis within 25 weeks on an HF diet (20% fat, 0.35% cholesterol, 15% sucrose) (13, 14, 146-149). Given the central role of the hepatic biopsy and subsequent histopathological scoring in diagnosis and disease monitoring, the hepatic read-out in
the ideal animal model should share the histological features of human NASH (132, 138). In this respect, the guinea pig has strong translational potential, mimicking all individual histopathological components of human NASH apart from hepatocellular carcinoma (figure 5).

Moreover, considering the vital role of inflammation in the progression and classification of NASH, the choice of this model is supported by several immunologic similarities between guinea pigs and humans. This includes the complement system, presence of interleukin 8 (IL8) and its receptor c-x-c motif chemokine receptor 1 (CXCR1), greater sequence similarity of cluster of differentiation 8 (CD8) and c-c motif chemokine ligand 5 (CCL5) and homologous major histocompatibility complex (150). Transcriptionally, guinea pigs and NASH patients shared 45.2% of differentially expressed genes (DEGs), and 60% of the top 100 genes associated with human NASH were differentially regulated in guinea pigs (III). Of these, genes involved in major pathogenic mechanisms and their regulation are listed in table 5. Table 5 illustrates similarities in guinea pig and human lipid, inflammatory and fibrotic processes, but also highlights differences in cholesterol metabolism. Humans and guinea pigs share increased DNL induced by sterol regulatory element binding factor 1 (SREBP1) and peroxisome proliferator activated receptor γ (PPARG) and driven by increased fatty acid synthase (FASN) expression (41, 151). This is accompanied by decreased VLDL export represented by lowered apolipoprotein b (APOB) and microsomal triglyceride transfer protein (MTP) expression, as well as decreased oxidation (only in guinea pigs) ultimately resulting in a net increase in hepatic lipid build-up (152, 153). Despite dissimilar hepatic cholesterol regulation,
guinea pigs mimic human lipid and cholesterol metabolism more closely than mice and rats, with regard to their lipoprotein profile which is dominated by LDL (11, 149). Finally, unlike most other mammals, guinea pigs and humans lack the ability to produce vitamin C (vitC) and are completely reliant on dietary supplementation, making it an excellent model to study the effect of vitC in a wide range of diseases (154-159).

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Genes associated with human NASH with increased expression in NASH guinea pigs</th>
<th>Genes associated with human NASH with decreased expression in NASH guinea pigs</th>
<th>Genes associated with human NASH with unaltered expression between NASH and control guinea pigs</th>
<th>Overall regulation in guinea pigs</th>
<th>Overall regulation in humans (REF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid import</td>
<td>CD36, LPL</td>
<td>LIPE/HSL</td>
<td>FABP1</td>
<td>↑</td>
<td>↑ (160, 161)</td>
</tr>
<tr>
<td>Lipid synthesis</td>
<td>SREBF1†, FASN†, PPARG†</td>
<td>DGAT2, MLXIPL†</td>
<td>ELOVL6, DGAT1, FGF21, SCD5, ACACA†</td>
<td>↑</td>
<td>↑ (41, 151)</td>
</tr>
<tr>
<td>Lipid oxidation</td>
<td>CYP2E1†, ACOX1†</td>
<td>PPARA†</td>
<td></td>
<td>↓</td>
<td>↔ (162-164)</td>
</tr>
<tr>
<td>Lipid export</td>
<td>APOB, MTP</td>
<td></td>
<td></td>
<td>↓</td>
<td>↓ (152, 153)</td>
</tr>
<tr>
<td>Cholesterol import</td>
<td>SLC10A1, LDLR†</td>
<td></td>
<td></td>
<td>↓</td>
<td>↓ (52, 165)</td>
</tr>
<tr>
<td>Cholesterol synthesis</td>
<td>NROB2/SHP, CYP8B1, SREBP2†</td>
<td></td>
<td></td>
<td>↓</td>
<td>↑ (52, 165)</td>
</tr>
<tr>
<td>Cholesterol export</td>
<td>NR1H4/FXR, ABCB11</td>
<td>CYP7A1†</td>
<td>(↑)</td>
<td>↓</td>
<td>(52)</td>
</tr>
<tr>
<td>Inflammation</td>
<td>NLRP3, CXCL8†, IL18, TLR4, TLR9, CCR2, CCL2†, TNF†, CD68</td>
<td>XBP1, CRP,</td>
<td>TLR2, IL10, CASP1, IL1B</td>
<td>↑</td>
<td>↑ (166-168)</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>TIMP1, SERPINE1†, COL1A1†</td>
<td></td>
<td></td>
<td>↑</td>
<td>↑ (169-171)</td>
</tr>
</tbody>
</table>
Genetic variants | MBOAT7 | PNPLA3, ↓ | TM6SF2, ↓* | LYPLAL1
--- | --- | --- | --- | ---

*These are loss of function mutations, and hence listed as downregulated.
† Verified by qpcr (II).

8. Summary of main findings
Due to the lack of preclinical models mirroring the entire NAFLD disease spectrum, drug development is seriously affected and subsequent monitoring of patient response to treatment needs to be made possible through non-invasive markers. In an effort to overcome these challenges, this PhD aimed to assess the effect of treatment and identify non-invasive markers in a translational animal model.

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

8.1 Inducing obesity and insulin resistance
In humans consumption of soft drinks is linked to insulin resistance and NAFLD, thus to induce co-morbidities, obesity and insulin resistance in the guinea pig model, animals were supplemented
with high fructose corn syrup (HFCS) in the drinking water in addition to an HF diet (133, 134) (I). Furthermore, previous studies have indicated increased plasma triglyceride levels and decreased glucose tolerance in guinea pigs receiving low fat (LF) diet (13, 14). In consequence, a new pair-fed control group receiving a low calorie/low starch diet was included in line with other reports (175). Results from this study revealed a clear effect of the new LF diet on body weights and glucose tolerance compared to HF, but also revealed no additional effect of sugar water. This led us to continue with the low calorie/low starch diet as the LF diet, and results from study V replicated our findings of increased body weights and altered glucose tolerance in HF compared to LF animals.

8.2 Comparing NASH transcriptomes
Having established a model now encompassing glucose intolerance and weight gain, further exploration of the molecular mechanisms behind guinea pig NASH was assessed through targeted gene expression. To this end, expression profiles of genes involved in major pathways: lipid metabolism, cholesterol metabolism, inflammation and fibrosis were reviewed, and revealed upregulation of DNL, inflammation and fibrosis in NASH guinea pigs similar to patients (II).

With these promising results, a more comprehensive expression profile was obtained from RNA-sequencing on samples from the same study (III). Furthermore, to put the results in a translational context the guinea pig NASH transcriptome was compared to two human datasets and three murine NAFLD/NASH models. This showed extensive overlap between human and guinea pig transcriptomes, on a single gene and pathway level. Particularly, fibrotic expression profiles were highly similar in humans and guinea pigs, and also indicated a higher similarity compared to the included murine models.

8.3 Investigating the effects of intervention targeting inflammation
As described above humans and guinea pigs share a vast overlap in their transcriptomic profiles, in particular with regards to inflammation and fibrosis. Thus the guinea pig seemed a suitable model to investigate the effects of acetylsalicylic acid (ASA) and PTX (IV) and vitC. In line with several clinical trials combination therapy of two anti-inflammatory compounds (ASA/PTX) was applied, in an attempt to increase the target range and optimize the chances of success (176). After a 16-week HF diet preloading period, animals were switched to an HF diet containing ASA/PTX either alone or in combination for 8 weeks, but neither had any effects on histological outcomes.
Upon reflection of these results, dietary intervention and a prolonged intervention period from 8 to 16 weeks, were included in study V. Furthermore, to induce an aggravated state of metabolic stress, animals were preloaded with a vitC deficient HF diet for 16 weeks and the effect of vitC intervention was assessed. While dietary intervention showed a clear effect on steatosis, inflammation, ballooning and fibrosis quantification, the effect of vitC was not as clear.

8.4 Non-invasive detection of NASH
In both ASA/PTX and vitC intervention studies, animals were euthanized prior to intervention start to establish a baseline measure of disease progression. While this is representative of the average disease status in the animals, it does not allow for within-subject comparison, and potential non-responders cannot be excluded from analysis. Thus to establish a protocol for future non-invasive assessment of disease, CT scanning before and after intervention, was performed on all animals in the ASA/PTX study (IV). CT scanning was found to correlate with steatosis degree and liver triglycerides, and enabled identification of non-responders. Liver densities also showed significant correlation to fibrosis, although this was at a lower degree than steatosis, and likely driven by lipid accumulation (figure 6A, B).

Figure 6. Liver densities do not reflect fibrosis
A Correlation of fibrosis grades and liver densities shows poor, but significant correlation. Data were analyzed by a Spearman’s rank correlation test, n=52 (6 animals euthanized at baseline, and two animals with missing data, were excluded). B Plotting of fibrosis and steatosis grades for each animal reveals a linear relationship, indicating that the correlation identified in figure A is likely driven by steatosis. Each circle represents the number of animals with the indicated steatosis and fibrosis grades. The number of animals represented by circles ranges from 1 (smallest circles) – 17 (largest circles) n=60.
8.4.1 Identification of novel biomarkers of fibrosis
Although CT scanning was able to detect steatosis, this procedure was time-consuming and costly, and therefore not feasible for all future in vivo studies. Consequently, correlation analysis on RNA-sequencing data was performed, which identified 9 genes demonstrating high correlation to fibrosis and similar regulation in human datasets. Two of these serpin family B member 9 (SERPINB9) and von Willebrand factor (VWF) were secreted factors and are interesting for future assessment as biomarker candidates.

9. Preliminary findings
In support of the main findings described above, a series of smaller experiments were performed. To investigate if the expression of SERPINB9 was also increased in similar studies, qPCR and western blotting analysis were performed on samples from study V. As indicated in figure 7, SERPINB9 was significantly upregulated in HF animals on an mRNA and protein level, supporting findings from (III).

![Figure 7. SERPINB9 expression](image)

**Figure 7. SERPINB9 expression**

A Gene expression analysis revealed increased expression of SERPINB9 in HF compared to LF guinea pigs. The graph depicts fold change with ranges, and results were analyzed by a student’s t-test, n=6. B Likewise protein expression analysis revealed upregulation of SERPINB9 in HF compared to LF. The graph depicts means with SDs, and results were analyzed by a Welch’s t-test, n=8. *p<0.05, ***p<0.001.

To validate the accuracy of non-invasive detection methods, any novel biomarkers should be compared to the gold standard. As the gold standard for diagnosing NASH and staging fibrosis is a hepatic biopsy, a protocol for obtaining an ultrasound-guided biopsy on anesthetized guinea pigs
was established. Although all guinea pigs recovered, the procedure resulted in weight loss of ≥ 10% (figure 8). One animal was euthanized due to general signs of malaise, albeit there was no indication of peritonitis when performing the necropsy. The resulting biopsies were fragmented, and encompassed several lobes, which was problematic, as hepatic triglyceride content has demonstrated inter- and intra lobular variation in guinea pigs (177). Consequently, this method is currently not feasible.

Figure 8. Weight loss after biopsy procedure
Ultrasound-guided fine-needle biopsy was performed on five anesthetized guinea pigs. Animals were weighed each day for the first seven days and then every second day. A Individual weights in grams. B Individual weights depicted as % of start weight.

10. Discussion
This thesis presents data from several in vivo studies applying the guinea pig NASH model. Main findings show high similarity between diet-induced guinea pig NASH and human patients, supporting the translational value of this model. Results also show CT scanning as an applicable methodology for non-invasive detection of hepatic steatosis in this species. Lastly, disclosure of the disease-associated transcriptome revealed potential circulating markers (i.e. SERPINB9) of hepatic fibrosis.

As a relatively new model in preclinical NASH research, one of the main objectives of this thesis was to explore the translational potential of the guinea pig model. RNA-sequencing of the guinea pig NASH transcriptome not only revealed a high degree of similarity to humans, but also compared to the murine models included in study III, specifically inflammatory and
fibrotic signaling pathways. Gene set enrichment analysis revealed upregulation of all pathways associated with inflammation in guinea pigs. This was in line with expression patterns in patients with advanced NASH, though not reflected in patients with mild disease (96, 171) (III). Moreover, the expression of tumor necrosis factor α (TNF-α), interleukin 8 (IL-8) and c-c motif chemokine ligand 2 (CCL2) which are among the top 100 genes associated with human NASH, was increased in this and other datasets assessing gene expression in the guinea pig NASH model (130) (II,III). Likewise, fibrotic gene expression was clearly upregulated in guinea pig NASH, and fibrotic pathways overlapped with those identified in humans with advanced NASH (ECM proteoglycans, extracellular matrix organization, elastic fiber formation) (III). However, detection of pro-fibrotic gene expression is not necessarily indicative of disease stage. In study II fibrotic markers were upregulated regardless of fibrosis stage, and in murine models with mild fibrosis (<2 grade fibrosis) expression of major ECM component COL1A1 is often increased in response to an HF diet (140, 141, 178). Thus, as single genes may be upregulated in early disease stages, such expression patterns should be interpreted with caution and be supported by additional data. In the guinea pig model, the pro-fibrotic expression profile was supported by histological endpoints mimicking human disease, and in combination these results advocate for the use of the guinea pig as a preclinical model for NASH. Despite these advantages, as mice dominate preclinical NASH research, development of immunologic assays, genome annotation and genome editing tools for guinea pigs is not prioritized, and must be made available in order to fully exploit the benefits that this model presents (150, 179, 180).

Apart from inflammatory and fibrotic signaling, study III also corroborated lipid-related expression patterns from study II, denoting increased DNL and lower lipid export as major contributors to intrahepatic fat accumulation in guinea pigs corresponding to findings in humans (41, 151-153). Likewise, in line with NAFLD/NASH patients, total cholesterol was increased in guinea pig livers in all of the included studies I, IV, V (52, 55). However SREBP2 and HMGCR expression was decreased in guinea pigs as opposed to findings in patients, and diet alone influenced hepatic and plasma cholesterol levels in guinea pigs fed either HF or LF diet with or without atorvastatin (HMGCR inhibitor) and vitE, indicating little or no contribution of endogenous cholesterol production (52, 147, 165). Nevertheless, despite a
difference in the primary source of cholesterol accumulation in guinea pigs and humans, the net result is comparable, and cholesterol-induced Kupffer cell, HSC, and hepatocellular damage is likely to occur in both species. In relation to this, the effects of a diet without cholesterol has not been assessed in the guinea pig NASH model, albeit studies in mice have repeatedly demonstrated that addition of cholesterol to an HF diet is necessary to progress from simple steatosis to NASH (131). Lowering the amount of dietary cholesterol to a level more relevant for humans (0.35% in the current guinea pig diet compared to 0.1-0.2% for humans), might also alleviate suppression of cholesterol biosynthesis (138). However, similar to guinea pigs cholesterol synthesis was also suppressed in DIAMOND mice, despite a diet with 0.1% cholesterol (137).

The results outlined above indicate close transcriptional resemblance to humans, however, co-morbidities insulin resistance and obesity have not been shown in guinea pigs (13, 14). Thus to mimic human dietary habits of a high carbohydrate intake e.g. in the form of sweetened beverages and increasing the construct validity, animals were given access to sugar water in combination with an HF diet. In addition, the effect of an LF diet with lower caloric and starch content was assessed. Consumption of additional calories through the drinking water decreased food intake in guinea pigs in a dose-dependent manner, resulting in similar energy intake and unchanged metabolic phenotype compared to HF (I). Consequently, it is not likely that this will be included in future studies. Despite the lack of sugar water effect, all HF animals displayed decreased glucose tolerance and weight gain compared to the pair-fed LF low starch group, but not the ad libitum fed LF high starch group in study I. The choice to implement a new LF diet with lower starch content and higher fiber was based on previous observations from our group, as studies have indicated increased TG, increased fat deposition and decreased glucose tolerance in LF compared to HF guinea pigs (13, 14, 130). This is reflective of a general concern that “control” animals may be overfed and sedentary, resulting in a metabolically compromised (e.g. obese and glucose-intolerant) animal model with little relevance to normal weight active humans (181). Caloric restriction has been shown to extend the life of laboratory animals, and reduce fat mass of C57BL/6 mice compared to ad libitum-fed mice (182, 183). This was supported by study I, where pair-fed animals demonstrated lower weights, TG-FFA- and total cholesterol plasma levels as well as
improved glucose tolerance compared to ad libitum-fed LF animals (I). The improved phenotype may not be directly attributable to caloric restriction, but could also be a result of lower starch content or higher fiber. Upon reflection of the results from study I, the LF low starch diet was selected as an appropriate control diet for future studies, including study V. HF diet guinea pigs in this study displayed weight gain and glucose intolerance compared to LF animals, similar to study I, and collectively these data show an animal model of HF-induced NASH with a metabolic phenotype more in line with NASH patients. Though OGTT responses support a disrupted ability to maintain glucose homeostasis, the presence of insulin resistance is less clear. As measurements of guinea pig insulin levels has unfortunately – and despite several attempts – proven unsuccessful, this remains to be confirmed. Nevertheless, findings from study V revealed increased hba1c plasma levels in HF compared to LF animals, indicating reduced glycemic control and corresponding to findings in NAFLD patients (184).

In line with the translational competency, the guinea pig model was applied to assess the effect of ASA/PTX intervention and vitC deficiency on NASH progression and regression (IV, V). In study V, vitC deficiency was induced in HF animals hereby mimicking lower plasma vitC levels reported in NAFLD patients, and emphasizing the uniqueness of the guinea pig as a natural model of diet-induced vitC deficiency as opposed to almost all other mammalian species (154, 185, 186). Overall, neither ASA/PTX nor vitC had any significant effects on the investigated histological hallmarks. However, as vitC deficiency and ASA/PTX intervention were induced/supplied concomitant to an HF diet, it could be speculated that the limited effects were due to an overpowering HF diet, masking more subtle effects on disease outcomes e.g. vitC deficiency. In support of this theory, study V indicated a slight effect of vitC when switching animals to an LF diet, as nominally more animals had improvements in inflammation, ballooning and fibrosis scores, compared to animals receiving LF and low vitC. Moreover, comparing transcriptomes between LF groups with or without vitC deficiency, revealed upregulation of inflammatory pathways (e.g. inflammatory response, interferon gamma response, TNFα signaling via NFκB) in the LF vitC deficient group. Hence, in the absence of an HF diet positive, albeit small, effects of vitC supplementation to deficient animals were evident. Likewise, an effect of vitE and atorvastatin on ballooning scores could be detected in combination with an LF diet, but not when combined with an HF diet (147).
addition, vitC also had a beneficial effect on Insulin tolerance. This could occur through increased levels of the insulin sensitizer, adiponectin, as previous results have demonstrated vitC stimulated release of adiponectin in human adipocytes (187). Moreover, a recent randomized clinical trial assessing the effect of oral vitC supplementation in 93 NAFLD patients, demonstrated decreased glucose and insulin levels as well as increased total adiponectin levels in response to vitC supplementation (188). Thus, vitC may have beneficial effects on the metabolic co-morbidities often presented in NAFLD patients.

In contrast to ASA/PTX and vitC, diet intervention significantly decreased steatosis, inflammation and ballooning scores in study V, in HF compared to LF diet groups. This is in line with a study assessing the effect of diet and exercise (lifestyle) intervention, measured by weight loss, in 293 NASH patients. In this study, 90% of patients who lost ≥10% body weight had resolution of NASH (189). Similarly, in study V, guinea pigs in the LF diet groups displayed 6-10% lower body weight compared to the HF groups, and only 10% (2/20) still had definite NASH following the 16-week intervention period (NAS score ≥5 (190)). In the same study mentioned above, 45% of patients who lost ≥10% body weight showed regression of fibrosis, indicating that lifestyle intervention was less effective on fibrosis (189). Likewise, we observed no effect on histological grading of fibrosis in study V. Consequently, in guinea pigs like humans, fibrosis regression is slow and varies between individuals. Thus, it could be speculated, that prolonging the intervention period would have shown an effect on fibrosis. In support of this, fibrosis quantification was decreased in LF compared to HF groups, and it is likely that delta values of fibrosis scores for each animal would have demonstrated an effect of diet, and potentially also of vitC. These observations illustrate the critical need for non-invasive markers allowing for disease monitoring at several time points as well as inter-individual comparison, reducing the chance of missing actual treatment effects.

As inflammation and oxidative stress are central to progression from NAFL to NASH, and the guinea pig displays inflammatory transcriptional and histopathological hallmarks resembling humans, the intervention strategies in the work presented here were centered around these mechanisms (II,III, IV, V) (66, 104). However, in recent reviews it is argued that targeting late-stage characteristics in general, such as inflammation and fibrosis leaves too much room for alternate
pathways to cancel out any beneficial effects, suggesting that targeting upstream disease
genesis may be preferable (10, 103). In support of this theory, the effect of liraglutide, a
agonist with antidiabetic effects, demonstrated weight loss, as well as reduced inflammation
in NASH guinea pigs, within 4 weeks of treatment (191). Moreover, results from a recent phase II randomized control clinical trial
with semaglutide (also a GLP-1 agonist), reported significantly more patients in the treatment
group achieving NASH resolution (192). In addition, the current landscape of phase III clinical
trials consists of Resmetirom (MAESTRO, NCT03900429), Aramcol (ARMOR, NCT04104321) and
Obeticholic acid (REGENERATE, NCT02548351) which all target early disease processes.

Resmetirom is a thyroid hormone receptor β agonist, which increases β-oxidation and lowers the
amounts of triglycerides stored in the liver (193). Interestingly, thyroid hormone responsive
 THRSP which is a target of thyroid hormone receptor β, was identified as a gene of interest in the
guinea pig NASH transcriptome analysis (194) (III). THRSP expression was severely decreased (log2
fold change: -7.28 (III), -8.7 (V)) in NASH guinea pigs compared to controls, and the translational
relevance was supported by a similar downregulation of THRSP in patients with advanced NASH
(III). Thus, the guinea pig may be a relevant model for investigation of thyroid hormone based
therapies.

In an effort to increase the statistical power of study IV and future intervention studies, we
assessed CT scanning as a tool for non-invasive detection of disease. In study IV, CT scanning
worked well for detecting steatosis, but showed poor, albeit significant, correlation with
fibrosis scores. The inverse relationship between HU and fibrosis grade was in contrast to our
expectations as fibrosis mediates increased HU (195). However, as steatosis grade also
correlated with fibrosis, it is likely that increased fat deposition rather than fibrosis itself
was the underlying driving force behind this observation. In NAFLD patients, CT scanning is
primarily used as a tool to detect steatosis, alongside US and MRI-PDFF. Of note a ≥30%
decline in MRI-PDFF relative to baseline, is also used as a biomarker for treatment response in
early-phase trials of anti-steatotic therapy (78). This has recently been criticized as intrahepatic
triglyceride levels were unable to predict histological outcomes of NASH (inflammation,
ballooning, fibrosis) in patients from pioglitazone trials (196). Furthermore, general concerns have
been raised as steatosis is known to decrease upon progression to more advanced disease stages,
particularly cirrhosis (78). Although this was not relevant in the current study, as neither TG nor steatosis scores decreased in response to HF diet with or without ASA/PTX, it is important to be aware of for the use of CT scanning as a biomarker for treatment response in future intervention studies.

In study III we aimed to identify biomarkers detectable by a simple blood test. This resulted in the identification of SERPINB9 and VWF as secreted markers correlating with fibrotic NASH. VWF has already been assessed as a marker in NAFLD patients, but little is known about SERPINB9 (197). In support of these findings, the expression of SERPINB9 was also increased in NASH guinea pigs from study V, on an mRNA and protein level and future studies will determine any potential correlation to fibrosis. However, as SERPINB9 is known to play a role in immune regulatory mechanisms it would be interesting to correlate SERPINB9 to a continuous measure of inflammation (198, 199) (III). Finally, despite having disclosed increased expression of SERPINB9 in NASH patients with advanced fibrosis (III), ultimately SERPINB9 must be validated in blood samples from a patient cohort.

11. Concluding remarks
This work elaborates on the translational potential of the guinea pig as an animal model of diet-induced NASH. A high degree of similarity to human patients could be shown on several parameters including histopathological hallmarks, metabolic co-morbidities, and the disease-associated hepatic transcriptome. In this way hypothesis I was accepted. Moreover, findings support the guinea pig as an underappreciated model, which could provide preclinical research data of high predictive validity. The primary driver of recovery from NASH appears to be a change from an HF to an LF diet, as the applied interventions (ASA/PTX combination therapy and vitC intervention in deficient animals) showed limited effects and no significant reduction of histopathological endpoints. In this aspect hypothesis II could not be confirmed. Hypothesis III was partially accepted, as CT scanning was able to detect steatosis, but did not correlate with fibrosis. Searching the transcriptome dataset, SERPINB9 was identified as a potential biomarker of fibrotic NASH. Validation of this as a putative biomarker for diagnostic purposes in humans should be pursued in future investigation of patient cohorts.
12. References


13. Papers I-V
**Paper I**

Differential Effects of Dietary Components on Glucose Intolerance and Non-Alcoholic Steatohepatitis

Josephine Skat-Rørdam 1, David Højland Ipsen 1, Patrick Duncan Hardam 1, Markus Latta 2, Jens Lykkesfeldt 1, and Pernille Tveden-Nyborg 1,2

1 Section of Experimental Animal Models, Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, 1870 Frederiksberg, Denmark; jsr@sund.ku.dk (J.S.-R.); dhi@sund.ku.dk (D.H.I.); patrickdh@sund.ku.dk (P.D.H.); jopl@sund.ku.dk (J.L.)
2 Liver Disease Research, Global Drug Discovery, Novo Nordisk A/S, 2880 Copenhagen, Denmark; mrlq@novonordisk.com

* Correspondence: ptn@sund.ku.dk; Tel.: +45-35333167

Abstract: Pharmacological treatment modalities for non-alcoholic fatty liver disease (NAFLD) and steatohepatitis (NASH) are scarce, and discoveries are challenged by lack of predictive animal models adequately reflecting severe human disease stages and co-morbidities such as obesity and type 2 diabetes. To mimic human NAFLD/NASH etiology, many preclinical models rely on specific dietary components, though metabolism may differ considerably between species, potentially affecting outcomes and limiting comparability between studies. Consequently, understanding the physiological effects of dietary components is critical for high translational validity. This study investigated the effects of high fat, cholesterol, and carbohydrate sources on NAFLD development and metabolic outcomes in guinea pigs. Diet groups (n = 8/group) included: low-fat low-starch (LF-LSt), low-fat high-starch (LF-HSt), high-fat (HF) or HF with 4.2%, or 8.4% sugar water supplementation. The results showed that calorific compensation in HF animals supplied with sugar water led to reduced feed intake and a milder NASH phenotype compared to HF. The HF group displayed advanced NASH, weight gain and glucose intolerance compared to LF-LSt animals, but not LF-HSt, indicating an undesirable effect of starch in the control diet. Our findings support the HF guinea pig as a model of advanced NASH and highlights the importance in considering carbohydrate sources in preclinical studies of NAFLD.

Keywords: NASH; glucose intolerance; diet; starch; soft drink

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) and the more advanced form steatohepatitis (NASH) are multifactorial and progressive diseases, and in humans are often accompanied by comorbidities such as obesity, insulin resistance and dyslipidemia [1]. Reflecting the global ‘obesity-pandemic’, the incidence of NAFLD is rapidly increasing to become the most common liver disease worldwide, estimated to affect up to 25% of the adult population [2]. The deposition of surplus fat in hepatocytes (hepatic steatosis) represents the initial hallmark of the disease and is accelerated by an excessive energy consumption. This is in line with the association with obesity and metabolic dysfunction reported for many human patients and commonly linked to a diet rich in fat, cholesterol and carbohydrates [3,4]. However, the complexity of human NAFLD/NASH has proven difficult to mirror experimentally and animal models accurately recapitulating the disease etiology and phenotype are scarce, reducing the predictive validity of findings and limiting research advances [5,6].

Despite recommendations of a maximum intake of 10%, the average American consumes around 17% of their calories from added simple carbohydrates (glucose, fructose, sucrose, high fructose corn-syrup (HFCS)), for example through soft drinks [7,8]. In addition, soft drink consumption has a limited effect on overall satiety often resulting in
additional caloric intake and subsequent ‘energy-overload’ in turn promoting metabolic stress [9,10]. This is corroborated by several studies in humans that have shown a link between soft drink consumption and obesity, insulin resistance and NAFLD [11–14]. More specifically, the addition of a fructose/corn-syrup based ‘soft drink component’ to a high fat diet has also been shown to accelerate NAFLD/NASH in some experimental animal models, consequently promoting the use of HFCS in preclinical disease modeling [15–20].

In contrast to simple carbohydrates, the physiological response to starch is underappreciated in many in vivo studies. Unlike fructose and glucose (the primary components of HFCS) starch is a polysaccharide and a major nutritional constituent of most human diets, as well as carbohydrate source in various rodent diets [8,21,22]. While starch has not been directly associated with the development of NAFLD, starches with a high glycemic index have been linked to obesity, diabetes and hyperlipidemia in humans, supporting an effect on metabolism [23–25]. Consequently, the starch content of control diets applied in preclinical modeling might induce a metabolic state with little relevance to a healthy human control and—unintentionally—reduce the value of comparisons. For the guinea pig NAFLD/NASH model, a low-fat high-starch (LF-HSt) diet promotes a healthy liver phenotype, however guinea pigs do not display differences in weight or glucose tolerance compared to a HFD [26–30]. To investigate putative differences in a more metabolically relevant control group, a diet low in fat and starch was included (LF-LSt). Moreover, as current clinical guidelines dictate a diet change to a low cholesterol/low saturated fat diet such as the Mediterranean diet or a hypocaloric diet in addition to therapeutic treatment, the potential use of improper controls in preclinical studies may overshadow findings of therapeautical effects, seriously limiting the ability to identify new drug candidates [31,32].

Here, we investigate the role of different carbohydrate sources by including three HF diet groups of which two were given free access to HFCS in their drinking water. The effects of the HF diets with or without sugar water were compared to two low-fat groups (LF) one with high amounts of starch (LF-HSt) and one with low amounts of starch (LF-LSt), the latter hereby targeting a low-fat/low-carbohydrate diet with proposedly minimal effect on metabolism.

2. Materials and Methods

2.1. Animals

All animal experimentation was approved by the Animal Experimentation Inspectorate under the Danish Ministry of Environment and Food, and in accordance with European legislation of Animal Experimentation 2010/63/EU. The study was conducted in accordance with the ARRIVE guidelines [33].

Forty female Hartley guinea pigs weighing 401–450 g (Charles River Laboratory, Lyon, France) were tagged with an XS 1.4 mm subcutaneous microchip (Evet, Haderslev, Denmark) upon arrival. In coherence with previous studies, this experiment was performed in female guinea pigs, as hierarchical fighting between males poses serious concerns for animal welfare. Following one week of acclimatization animals were weight-stratified into 5 groups (n = 8/group): low-fat low-starch (LF-LSt): 3.8% fat, 0% sucrose, 0% cholesterol, 13.4% starch, low-fat high-starch (LF-HSt): 4.3% fat, 0% sucrose, 0% cholesterol, 28.4% starch, or a high-fat sucrose diet (20% fat, 15% sucrose, 0.35% cholesterol, 7.9% starch) and receiving either tap water (HF) or HFCS supplemented drinking water (4.2% + HF): 4.2% (45% d-glucose, 55% d-fructose (Sigma Aldrich, St. Louis, MO, USA), or (8.4% + HF): 8.4% (19% d-glucose sugar, 23% d-fructose, 58% sucrose (Sigma Aldrich, St. Louis, MO, USA) (for more details on diet composition see Tables S1 and S2). To allow comparison to the 4.2% group, and as humans only consume around 50% of their added sugars as HFCS and 50% as sucrose [34], 42% of the sugar in the 8.4% group was HFCS while sucrose constituted the remaining 58%.

Group sizes were based on power calculations (power of 80%, p < 0.05), using variances based on our previous studies and the ability to observe a difference in OGTT AUC of
30%. All diets were chow based and produced by Ssniff Spezialdiäten (Soest, Germany. See Table S2 for detailed diet information).

The switch from acclimatization standard chow to experimental diets was achieved gradually over a 5-day period. Likewise, sugar water was introduced gradually following the diet change, with tap water being substituted with either 4.2 or 8.4% sugar water over a period of 7 days after which animals in the sugar water groups had ad libitum access to sugar water, and restricted access to normal water (150 mL/group/day) to ensure animal welfare. The LF-LSt group was pair-fed to the LF-HSt group ensuring lower calorie-intake in the LF-LSt group, whereas HF fed groups had ad libitum access to food. In an effort to limit additional starch intake, all animals had restricted access to hay. Daily food intake was calculated by subtracting the amount of food remaining with the amount given the previous day. Water intake was calculated in the same manner, also on a daily basis (due to daily fluctuations calculations of food, water and caloric intake is based on weekly averages). Body weights were measured once weekly.

2.2. Oral Glucose Tolerance Test (OGTT)

All animals were semi-fasted (allowing access to hay and water) for 12 h prior to testing. Testing was performed over the course of two days. To ensure unbiased sampling, animals were block randomized within groups based on weight, testing half the animals on day one and the other half the second day. On the day of testing, animals were administered 2 g/kg of a 150% glucose solution by oral dosing. In case of administration exceeding 3 min, the animal was excluded from the test. Blood samples were collected at 0 (baseline, prior to glucose administration), 30, 60, 90 and 120 min by puncturing the ear vein with a 27G needle and subsequently, glucose measurement was achieved on an Aviva Accu-check Glucometer (Roche A/S Diagnostics, Hvidovre, Denmark), as described previously [35]. All sampling was carried out in duplicates. At week 16, three animals (two from the 8.4%, and one animal from the HF group), were excluded based on the 3 min administration criteria.

2.3. Insulin Tolerance Test (ITT)

All animals were semi-fasted and randomized as described for OGTT. A dose of 0.5 U/kg insulin (Actrapid® (Novo Nordisk A/S, Bagsværd, Denmark) was injected subcutaneously in the neck (27G needle). Blood samples were collected at time points 0 (baseline, prior to insulin administration), 15, 25, 50, 75 and 120 min and glucose measured as described for OGTT. All sampling was carried out in duplicate. For week 8, three animals were excluded (one animal from 8.4% + HF, one from HF and one from LF-LSt group). One animal was excluded from the ITT due to hypoglycemia (glucose levels below 2 mM) and was treated with oral glucose supplement. The two remaining animals were excluded due to inaccurate dosing (due to handling issues when fixing the animals, a small amount of insulin was unfortunately not injected).

2.4. Euthanasia and Sampling

At euthanasia animals were semi-fasted overnight before being pre-anaesthetized with 1.25 mL/kg Zoletil-mix (125 mg Tiletamin, 125 mg Zoletapam (Zoletil 50 Virbac Laboratories, Carros, France) + 200 mg xylazin (Narcoyl vet 20 mg/mL; Intervet International, Boxmeer, Holland) + 7.5 mg butorphanol (Torbegesic vet 10 mg/mL; Scanvet, Fredensborg, Denmark). To ensure accurate dosing and isotonicity, the anesthetic mix was diluted 1:10 in isotonic NaCl and animals were dosed with 1.25 mL/kg body weight. Once anaesthetized, animals were placed on isoﬂuorane (3–5%) inhalation through a mask. Upon disappearance of inter-digital reflexes, intra-cardial blood was collected; for vitamin C, vitamin E, uric acid, malondialdehyde (MDA) analysis blood was collected in an EDTA coated 10 mL syringe, whereas samples for free fatty acids (FFA) and alkaline phosphatase (ALP) analysis were collected in sodium fluoride (NaF) and heparin coated microvetttes (Sarstedt, Nürnberg, Germany) respectively. For triglyceride (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total cholesterol (TC) blood samples were
collected in K3 EDTA coated microvettles (Sarstedt, Nümbrecht, Germany). Plasma was isolated by centrifugation at 2000 × g for 4 min at 4 °C. The isolated plasma for FFA, ALP, TG, TC, ALT, AST was stored in Cobas cups (Sample cup micro 13/16, Roche Diagnostics, Mannheim, Germany) at 20 °C, until analysis on a Cobas 6000 (Roche Diagnostics, Berne, Switzerland), according to manufacturer’s instructions.

Plasma for vitamin C measurement was immediately stabilized in metaphosphoric acid before storing at 80 °C. Plasma samples for vitamin E, uric acid, and MDA were transferred to 1.5 mL Eppendorf® tubes and stored at 80 °C, until analysis by high performance liquid chromatography as previously described [36–38].

2.5. Liver Samples

To retrieve liver samples, the whole liver was excised and briefly rinsed in ice-cold PBS. Six liver sections were then obtained from lobus sinister lateralis to limit variation, as previously described [39]. Four sections were immediately frozen on dry ice and stored at 80 °C for TG, TC, MDA, vitamin C, vitamin E, tetrahydrobiopterin (BH4), dihydrobiopterin (BH2) determination. The remaining two sections were fixed in 10% formalin in separate containers, one for histological staining and one for other purposes (not used in this study). Liver tissue for TG and TC analysis was prepared as previously described [40], and analyzed on a Cobas 6000 (Roche Diagnostics, Berne, Switzerland), according to manufacturer’s instructions. MDA, vitamin C, vitamin E, BH2 and BH4 were all determined by high performance liquid chromatography, as previously described [36–38,41,42].

2.6. Histology

Paraffin-embedded liver sections were sliced in sections of 2–4 µm and stained with hematoxylin and eosin (H&E) or Pico Sirius Red (PSR) with Weigert’s hematoxylin solution. To ensure reliable scoring, 10 random sections were selected for calculation of Cohen’s Kappa index [43–45]. The 10 sections were scored in a blinded manner, re-blinded and then re-scored. Cohen’s Kappa values were then calculated, and the observer was only allowed to proceed with the actual scoring if Kappa values > 0.8 ( steatosis, inflammation, ballooning, fibrosis). Scoring was performed in a blinded and randomized manner as previously described for the guinea pig model, and based on Kleiner et al. [28,46]. In short, steatosis, inflammation and hepatocellular ballooning was evaluated on H&E stained sections, fibrosis was evaluated on PSR stained sections. Steatosis was evaluated across the entire liver section and scored as 0 (<5%), 1 (5–33%), 2 (>33–66%), 3 (>66%). Lobular inflammation was scored in five separate lobules dispersed across the entire section. A lobule was defined as two portal areas and one central vein, with an inflammatory focus defined as three or more inflammatory cells in close proximity and scored as 0 (no foci), 1 (<2), 2 (2–4), or 3 (>4). Hepatocyte ballooning was evaluated across the entire section and scored as 0 (none), 1 (few), 2 (many). Fibrosis was evaluated across the entire section and scored as 0 (none), 1 (perisinusoidal or perportal), 2 (perisinusoidal and perportal), 3 (bridging), 4 (cirrhosis). NAFLD activity score (NAS) was derived from the cumulative sum of steatosis, inflammation and ballooning ranging from 0 to 8 [46].

2.7. Statistics

All statistical analyses were performed in the GraphPad Prism version 9.0.1 (GraphPad Prism software, La Jolla, CA, USA). All continuous normally distributed data with equal variances among groups were analyzed by a parametric test (either one-way ANOVA, two-way ANOVA with repeated measures, or mixed effects model), with Tukey’s test for multiple comparisons, and presented as means with standard deviation (SD). Upon deviations from normality, data were log transformed and re-analyzed, these data are presented as medians with 25th and 75th quartiles. If the data continued to deviate or were categorical, they were analyzed by a non-parametric Kruskal–Wallis with a Dunn’s test for multiple comparisons and presented as medians with 25th and 75th quartiles. If there were unequal variances between groups data were analyzed by Welch-ANOVA with
a Dunnett’s test for multiple comparisons and presented as means with SD. Repeated measures two-way ANOVA with a Tukey’s test for multiple comparisons were performed for body weights OGTT and ITT. In case of missing values, a repeated measures mixed-effects analysis with Tukey’s test for multiple comparisons was performed instead.

3. Results

3.1. Diet Composition, Calorie Intake and Body Weight

Body weights and energy intake was monitored for all groups throughout the study period (Figure 1a,c). The main differences in the basic dietary components of the LF-LSt, LF-HSt and HF groups were crude fat, crude fiber, starch and sugar content (Figure 1b). Although the metabolizable energy in the HF diet (16.8 MJ/kg) is greater than in LF-HSt (12.6 MJ/kg) and LF-LSt (11.2 MJ/kg) diets (Table S1), the total energy intake of the HF animals was only higher compared to LF-LSt (Figure 1c). The total energy and sugar water intake of the sugar water groups (SW; 4.2% and 8.4%) was similar (Figure 1c and Figure S1a). Therefore, it is not surprising that the 8.4% group ingests ~50% more calories from sugar water compared to the 4.2% group (0.70 MJ and 0.36 MJ, respectively (Figure 1c)). As shown in Figure 1c, the increased calorie intake from the sugar water was accompanied by a concomitant decrease in food intake, in the 8.4% group compared to the 4.2% group corresponding to the surplus calories received from the sugar water (0.37 MJ). The calorie intake was poorly reflected in the body weights of the various groups (Figure 1a). From week 10 and on LF-HSt showed higher body weight compared to LF-LSt, despite similar calorie intake (Figure 1a,c). At week 15, the LF-HSt and the HF groups showed significantly higher body weights compared to LF-LSt.

Figure 1. Body Weight and Energy Intake. (a) Body weights for each group are presented as means with SD, for each week. Data was analyzed using a mixed effects model with repeated measures with a Tukey’s test for multiple comparisons. n = 8 (b) Major dietary components presented in % (c) Energy intake in MJ for each group is presented as means with SD and analyzed using a one-way ANOVA with a Dunnett’s test for multiple comparisons. n = 13 average weekly intake pr. group during the study period. For LF-LSt vs. LF-HSt * p < 0.05, ** p < 0.01, for LF-LSt vs. HF * p < 0.05. LF: Low Fat, HSt: High Starch, LSt: Low Starch, HF: High Fat, MJ: Mega Joule.
3.2. Oral Glucose Tolerance Testing

To assess effects on glucose homeostasis (as a measure of metabolic state), OGTT and ITT was performed after 8 and 16 weeks on diets. All HF fed animals displayed altered glucose tolerance after 30 min compared to LF-LSt animals on both investigated time-points (Figure 2a,c). In contrast, at the 8 week time-point the LF-HSt group displayed altered glucose tolerance compared to LF-LSt at the 90 min time point and compared to LF-LSt and HF at 120 min (Figure 2a). This was not as clear after 16 weeks, as the LF-HSt group differed from all groups except HF already at the 60 min time point, and differed from all groups at the 90 min time point. The increased glucose levels in the LF-HSt group were also reflected in the AUCs for week 16 (Figure 2d), where only LF-HSt was different from LF-LSt. In contrast, the AUCs from week 8 revealed differences between LF-LSt and all HF fed groups, but no difference between any of the HF diet groups, or the LF-LSt and LF-HSt. This might seem counterintuitive as the LF-HSt group displayed the highest overall mean, however due to the large SD in this particular group, results did not reach statistical significance (p-value = 0.075). Insulin tolerance tests revealed decreased insulin sensitivity in the LF-HSt only after 8 weeks. No differences were observed after 16 weeks (Figure S2).

![OGTT - week 8](image1)

![AUC OGTT - week 8](image2)

![OGTT - week 16](image3)

![AUC OGTT - week 16](image4)

**Figure 2.** Oral Glucose Tolerance Tests. (a) Oral glucose tolerance test at week 8. Data are presented as means with SD and square root transformed data were analyzed by a repeated measures two-way ANOVA, and a Tukey’s test for multiple comparisons. n = 8 (b) Area under the curve for each group. Data are represented as means with SD, and analyzed by Welch ANOVA with a Dunnett’s test for multiple comparisons. n = 8 (c) Oral Glucose tolerance test week 16. Data are represented as means with SD, and analyzed by mixed effects model with repeated measures, and a Tukey’s test for multiple comparisons. n = 6–8 (d) Area under the curve for each group. Data are presented as means with SD and analyzed by a one-way ANOVA, with a Dunnett’s test for multiple comparisons. n = 6–8. For (a,c): * LF-LSt different from all, LF-LSt different from all except LF-HSt, ** LF-LSt different from HF and LF-HSt, † LF-HSt different from HF and LF-LSt, ‡ LF-HSt different from all except HF, § LF-HSt different from all. p < 0.05. For (b,d): * p < 0.05, ** p < 0.01. LF: Low Fat, HSt: High Starch, LSt: Low Starch, HF: High Fat, AUC: Area Under Curve, OGTT: oral glucose tolerance test, Min: Minutes.
3.3. Plasma Biochemical Markers

In support of the dietary cholesterol supplementation (Table S1), plasma TC levels were decreased in the LF-LSt group compared to all other groups (p < 0.001), which was already evident after 8 weeks of HF feeding (Table S5). Likewise, all HF diet groups displayed higher levels compared to LF-HSt (p < 0.001) (Table 1). FFA and TG showed a slightly different picture with higher levels in LF-HSt compared to LF-LSt and 8.4% + HF (p < 0.05). Liver damage marker AST was increased in all HF groups compared to both LF groups (p < 0.05), at both 8 and 16 weeks (Table S5 and Table 1). This was also seen for ALT in the HF and the 8.4% group at 16 weeks, whereas ALT in 4.2% animals only differed from LF-Lst (p < 0.05). Plasma α-Tocopherol levels were increased in all HF groups compared to both LF groups, except for 4.2%, which was only increased compared to LF-LSt. Vitamin C and Uric acid levels were decreased in the HF group (p < 0.05, Uric acid and vitamin C), and in sugar water groups (p < 0.001, vitamin C) compared to LF-LSt. Plasma ALP levels were lower in the HF group compared to LF-LSt and both sugar water groups (LF-LSt vs. HF p < 0.01, HF vs. sugar water groups p < 0.05). There was no effect on MDA and α-Tocopherol levels between groups (Table 1).

### Table 1. Plasma biochemical markers.

<table>
<thead>
<tr>
<th></th>
<th>LF-LSt</th>
<th>LF-HSt</th>
<th>HF</th>
<th>42% + HF</th>
<th>84% + HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFA mol/L</td>
<td>0.57 (0.6-0.65)</td>
<td>0.77 (0.67-0.98) *</td>
<td>0.58 (0.51-0.65)</td>
<td>0.74 (0.67-0.75)</td>
<td>0.74 (0.65-0.75) *</td>
</tr>
<tr>
<td>TC mmol/L</td>
<td>1.21 (1.02-1.42) ***</td>
<td>1.22 (1.03-1.45) ***</td>
<td>0.79 (0.66-0.97)</td>
<td>0.83 (0.74-0.92)</td>
<td>0.84 (0.72-0.92)</td>
</tr>
<tr>
<td>MDA μmol/L</td>
<td>0.42 ± 0.15</td>
<td>0.46 ± 0.19</td>
<td>0.57 ± 0.19</td>
<td>0.58 ± 0.15</td>
<td>0.51 ± 0.2</td>
</tr>
<tr>
<td>α-Tocopherol μmol/L</td>
<td>1.52 (1.34-1.61)</td>
<td>1.52 (1.34-1.61)</td>
<td>1.52 (1.34-1.61)</td>
<td>1.52 (1.34-1.61)</td>
<td>1.52 (1.34-1.61)</td>
</tr>
<tr>
<td>Uric acid μmol/L</td>
<td>21.70 (18.29-27.80) ***</td>
<td>21.70 (18.29-27.80) ***</td>
<td>21.70 (18.29-27.80) ***</td>
<td>21.70 (18.29-27.80) ***</td>
<td>21.70 (18.29-27.80) ***</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD and Q25-Q75 values in brackets. Data was analyzed using a one-way ANOVA. 1 Log transformed data were analyzed by one-way ANOVA with a Dunnett’s test for multiple comparisons, and are presented as medians with quartiles in brackets. 2 Data was analyzed using a Welch ANOVA. 3 Data was analyzed using a Kruskal-Wallis test. Different from LF-LSt *p < 0.05, ** p < 0.01, *** p < 0.001, different from LF-HSt #p < 0.001, ##p < 0.001, different from HF $p < 0.05, n = 8. LF: Low Fat, HF: High Fat, FFA: Free Fatty Acids, TC: Triglycerides, TC: Total Cholesterol, AST: Aspartate Aminotransferase, ALP: Alkaline Phosphatase, MDA: Malondialdehyde.

3.4. Liver Status

To determine liver health status, biochemical markers and histopathology was assessed for all animals at week 16 (Table 2, Figures 3 and 4). Liver TG and TC content was lower in the LF-LSt group compared to all other groups (p < 0.001, TC LF-LSt vs. LF-HSt p < 0.01). Liver TG was also significantly lower in LF-HSt compared to all HF groups (p < 0.001), whereas there was no difference between liver TG in LF-HSt and HF groups (Table 2). This was reflected in the steatosis score, as two animals in the LF-HSt group had a grade 1 steatosis, compared to LF-LSt where all animals displayed a grade 0. The steatosis score also revealed less advanced steatosis in both sugar water groups (4.2%: median grade 1.5, 8.4%: median grade 2), compared to the HF group (grade 3) (Figure 3); representative histological images depicted in Figure 4. Additionally, there was no difference in inflammation, ballooning or fibrosis between LF groups and the sugar water groups. This was in contrast to the HF group, which displayed higher scores in inflammation (p < 0.05), ballooning (p < 0.001) and fibrosis (p < 0.001) compared to both LF groups. There were no differences among markers of oxidative stress, apart from vitamin C levels, which were reduced in all groups compared to LF-LSt (p < 0.01) (Table 2).
Table 2. Liver biochemical markers.

<table>
<thead>
<tr>
<th></th>
<th>LF-LSt</th>
<th>LF-HSt</th>
<th>HF</th>
<th>4.2% ± HF</th>
<th>8.4% ± HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG µmol/g</td>
<td>6.64± (4.00–8.95)</td>
<td>23.74± (19.35–71.76) ***</td>
<td>53.45± (36.67–58.90) ***</td>
<td>46.21± (41.52–49.21) ***</td>
<td>38.58± (30.24–56) ***</td>
</tr>
<tr>
<td>TC µmol/g,2</td>
<td>5.61± (4.66–6.38)</td>
<td>8.26± (7.61–8.56) **</td>
<td>35.03± (32.92–37.17) ***</td>
<td>29.62± (23.46–36.28)</td>
<td>27.33</td>
</tr>
<tr>
<td>α-Tocopherol µmol/g</td>
<td>3.35± (2.85–6.13)</td>
<td>3.1± (1.4–4.93)</td>
<td>2.1± (1.6–3.3)</td>
<td>2.25± (0.95–7.75)</td>
<td>2.05± (1.13–3.13)</td>
</tr>
<tr>
<td>BH2/BH4 µmol/g</td>
<td>0.2± ± 0.19</td>
<td>0.28± ± 0.16</td>
<td>0.19± ± 0.12</td>
<td>0.2± ± 0.11</td>
<td>0.29± ± 0.14</td>
</tr>
<tr>
<td>MDA µmol/g</td>
<td>140.3± 43.92</td>
<td>149.4± 64.83</td>
<td>130± 56.14</td>
<td>135± 59.47</td>
<td>180.3± 70.66</td>
</tr>
<tr>
<td>Total Vitamin C,1</td>
<td>1810± (1567–2290)</td>
<td>1255± (1053–1471) ***</td>
<td>912.5± (829.9–1017) ***</td>
<td>962.3± (831.4–1023) ***</td>
<td>926.67</td>
</tr>
<tr>
<td>μM</td>
<td>1 (0.95–7.75)</td>
<td>2 (23.46–36.28)</td>
<td>3 (23.46–36.28)</td>
<td>4 (23.46–36.28)</td>
<td>5 (23.46–36.28)</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD and Q25-Q75 values in brackets. Data was analyzed using a one-way ANOVA. Log transformed data were analyzed by one-way ANOVA with a Dunnett’s test for multiple comparisons and presented as medians with quartiles in brackets.

2 Data was analyzed using a Welch ANOVA. Different from LF-LSt ** p < 0.01, *** p < 0.001, different from LF-HSt *** p < 0.001. n = 8.

LF: Low Fat; HSt: High Starch; LSt: Low Starch; HF: High Fat; TG: Triglycerides; TC: Total Cholesterol; BH2: Dihydrobiopterin, BH4: tetrahydrobiopterin, MDA: Malondialdehyde.

Figure 3. Histopathological scoring. Data are represented as individual scores with medians. (a) Steatosis scores for all groups on a scale of 0–3 (b) Inflammation scores for all groups on a scale of 0–3 (c) Ballooning scores for all groups on a scale of 0–2 (d) Fibrosis scores for all groups on a scale of 0–3 (e) Cumulative NAFLD activity score on a scale of 0–8. Scoring was performed as previously described [28]. The data was analyzed using a non-parametric Kruskal–Wallis with a Dunn’s multiple comparisons test. * p < 0.05, ** p < 0.01, *** p < 0.001. n = 8. LF: Low Fat; HSt: High Starch, LSt: Low Starch, HF: High Fat.
Figure 4. Representative histological images. (a,c,e,g,i) Hematoxylin and eosin stain. Scale bar shows 200 µm. (b,d,f,h,j) Picro Sirius Red stain. Scale bar shows 200 µm. Solid arrows indicate lipid vacuoles, and open arrows indicate fibrosis (in red). CV: central vein, PA: portal area, HF: high fat, LF: low fat, HSt: high starch, LSt: low starch.
4. Discussion

The present study demonstrates significant differences in the response to various calorie sources in the guinea pig NAFLD/NASH model. Sugar water did not increase overall energy intake significantly and, consequently, led to a different hepatic phenotype and disease severity compared to high fat counterparts. Moreover, though curves progressed differently, all high fat diets and the high starch diet had a significant effect on the ability to maintain glucose homeostasis. Considering the variability of experimental diets also with regards to the composition of control diets, these findings raise awareness towards an important translational aspect of diet-induced NAFLD/NASH modeling.

Contrary to expectations, increased weight gain in sugar water groups was not observed, but is readily explained by the total energy intake matching HF counterparts. It should be kept in mind that values of metabolizable energy levels in feed constituents are not available for guinea pigs and the calculated levels of metabolizable energy are therefore derived from studies in rats and mice, hence might not be completely accurate (communication with ssniff [47]). In response to an increased energy intake from sugar water, guinea pigs in the 8.4% and 4.2% + HF groups decreased their caloric intake from food in a dose dependent manner. This highlights the ability of the guinea pig to fine tune their caloric intake, in line with previous findings in this species [28], and is also corroborated by Mock et al., who showed decreased food intake of Sprague-Dawley rats in response to either fructose, sucrose or HFCS addition to the drinking water [48]. Furthermore, Kohli et al. reported similar energy intake in C57Bl/6 mice fed a HF diet and mice fed a HF diet with 4.2% HFCS supplemented drinking water [17]. Although data specifying food and water intake was not available for this study, the lack of additional consumption of calories in the HF + HFCS group clearly indicates caloric compensation in this strain of mice [17]. The caloric compensation is in contrast to several human studies, where caloric intake in liquid form has a limited effect on satiety and food intake, hereby advancing excessive energy intake [9,10,49]. Thus, in rats, guinea pigs and mice, the effects of an added soft drink component on satiety and food intake are not directly comparable to what is seen in humans, potentially affecting the translational value of studies including these types of dietary regimes.

More than 50% of patients with type 2 diabetes also have NAFLD, making insulin resistance an important co-morbidity of NAFLD pathogenesis. In a study by Podell et al. using a high fat high carbohydrate diet (30% calories from fat, 52% calories from carbohydrates in the form of refined sugar), guinea pigs showed glucose and insulin intolerance as well as increased insulin levels compared to controls (3% fat, 18% protein, 55% complex carbohydrates, 10.5 MJ/kg) [50]. In contrast to these findings, we have not previously been able to measure insulin resistance in the guinea pig model for NAFLD/NASH [28,40]. However, the control diet in the study of Podell and coworkers had a lower energy content (10.5 MJ/kg) compared to the LF-HSt (12.6 MJ/kg, Table S1) diet used as a control diet for this and previous studies in our group [26,28–30,40,51]. Considering the lower energy content as well as the higher amount of refined sugar in the high fat diet, the current study explored if insulin resistance could be induced by HFCS supplements in the drinking water while ensuring comparison to a low-calorie low-starch control group (LF-LSt, 11.2 MJ/kg). The OGTT displayed increased glucose response in all HF groups at the 30 min time point compared to LF-LSt, indicating a significantly altered glucose homeostasis, though not a direct measure of insulin resistance. These findings were not supported by insulin tolerance testing that revealed no differences between LF-LSt and HF groups. The recorded discrepancies could indicate a decreased insulin response in HF animals upon subjection to an oral glucose load, rather than peripheral insulin resistance.

While all HF groups resemble the general pattern of the LF-LSt group, the LF-HSt group displayed a different concourse with a delayed peak time and prolonged glucose clearance. A delayed peak time in OGTT in humans is associated with diabetes, increased HbA1c and decreased insulin sensitivity and secretion [52,53]. Delayed glucose absorption might contribute to a delay in peak-time. However, a previous study in diabetic
rats demonstrated increased glucose transport, likely as a result of intestinal hypertrophy with a concomitant increase in SGLT1 expression in intestinal epithelial cells, compared to non-diabetic controls [54]. Another study assessing human intestinal glucose absorption in patients with type 2 diabetes, observed no difference in intestinal absorption between patients with diabetes and healthy controls [55]. This supports that the recorded delayed glucose peak in the LF-HSt group could be due to decreased insulin sensitivity and secretion. In addition, insulin tolerance testing revealed decreased insulin response in the LF-HSt group after 8 weeks, albeit this finding was not replicated at the 16-week time point (Figure S2). Accordingly, in this study high fat with or without sugar water supplementation and high starch diets lead to glucose intolerance, and only high starch appears to affect insulin tolerance. This finding could be attributed to the increased content of high glycemic index starch in the LF-HSt diet compared to the LF-LSt diet (Table S2), indicating that the amount of starch in control diets, either high or low glycemic, should be carefully considered. However, to establish a clear connection between starch and insulin resistance, further studies are needed. Regrettably, despite intensive efforts, attempts to measure insulin concentrations in guinea pig plasma samples have not been successful due to an absence of reliable commercially available detection methods for this species, thus limiting the ability to confirm insulin resistance in the current study. In addition, assessment of cellular signaling at a gene or protein level, e.g., the insulin receptor (INSR), insulin receptor substrate (IRS1) or GLUT4 (SLC2A4) in adipose and hepatic tissues, will be interesting for future studies investigating the cellular mechanisms underlying the current findings of metabolic dysregulation.

Corresponding to previous findings in the guinea pig NAFLD/NASH model, plasma TG and FFA levels (p < 0.05) were increased in the LF-HSt group, compared to the LF-LSt and the 8.4% + HF group. In line with these findings, a study with Sprague-Dawley rats assessing the effect of high or low glycemic index starches, demonstrated higher TG and FFA plasma levels in animals fed the high glycemic index starch compared to low glycemic index starches [56]. In a Chinese population study, the dietary habits and association with metabolic disorders were assessed for 4,154 randomly sampled participants (excluding individuals with cancer, type 1 diabetes, metabolic syndrome, or pregnant women) revealed high carbohydrate intake from starchy foods to be associated with increased risk of hyperlipidemia (RR: 1.73, 95% CI: 1.05–3.35) [23]. In the present study, plasma TG levels were positively correlated with body weight in the LF-HSt group, whereas hepatic TG levels showed an inverse correlation (Figure S3a,b), indicating that intake of high glycemic index starch is not stored in the liver but rather enters the circulation, and becomes stored in extrahepatic tissues. Consequently, this study shows the importance of considering the amount of starch used in the “control” diet and indicates a possible effect on hyperlipidemia, which may result in inclusion of a control group with little relevance to healthy individuals. The diets included in the current study all contain starch and animals were allowed access to hay in accordance with animal welfare legislation, preventing the evaluation of an isolated effect of starch. Though an inclusion of a non-starch group would be helpful in this aspect, an abstinence of dietary starch holds little relevance for both animal and human diets, and would not possess any significant translational value.

Ascorbate is a major free radical scavenger in the blood. Like humans, guinea pigs are unable to synthesize vitamin C, and are dependent on dietary supplementation. All groups displayed lower plasma and liver vitamin C levels compared to LF-LSt. This was in direct contrast to plasma α-Tocopherol levels, which were increased in all HF diet fed groups, in line with previous findings [30]. This increase is most likely due to the lipophilic nature of α-tocopherol and its facilitated uptake with dietary fat [57]. Low plasma vitamin C and a concomitant decrease in liver vitamin C levels could be an indication of increased oxidative stress and antioxidant usage in these animals. However, apart from its antioxidative capacity vitamin C is involved in a number of other processes, such as collagen synthesis cholesterol metabolism and proinflammatory signaling, which may contribute to disease progression [58]. Lower vitamin C status has previously been observed in both individuals
with obesity and in guinea pigs on high fat diet [59,60]. It could also be argued that this is due to the higher food intake in the LF-LSt group. However, the observed differences persisted when accounting for food intake in each group (Figure S1b, Table S3).

While the role of simple carbohydrates in the development of NAFLD is well documented [61–63] the role of other carbohydrate sources, such as starch is less clear. In a study of obese children, isocaloric replacement of fructose with starch for 9 days improved liver steatosis, visceral fat and de novo lipogenesis, suggesting starch as a preferred carbohydrate source compared to fructose, albeit this was not compared to a low carbohydrate replacement diet [64]. A western/cafeteria diet, like the HF diet used in our study, has been shown to be a superior inducer of steatosis compared to HF diet alone, in C57BL/6 mice [65]. Furthermore, fructose alone or in combination with glucose (as HFCS) (13% w/v, administered through the drinking water) induced steatosis in rats on a control diet [48]. In the current study, increasing the amount of calories derived from simple sugars, did not seem to have an added effect on hepatic lipid accumulation in NAFLD as neither steatosis score nor liver triglycerides were increased in the sugar water groups compared to the HF group. However, liver TG was increased in the LF-HSt group compared to the LF-LSt, and two animals in the LF-HSt group displayed a mild degree of hepatic steatosis, a phenomenon that has been described previously for the guinea pig model and likely due to biological variation [29]. Regardless, excluding the two steatotic animals from the LF-HSt group did not affect the statistical conclusions of increased hepatic TG levels compared to LF-LSt, but resulted in significantly lower TG levels compared to all HF groups (Table S4). The findings from this study indicates a small increased risk of hepatic fat accumulation in guinea pigs subjected to a high starch diet. However, since the LF-LSt group and LF-HSt diets were not isocaloric, it cannot be ruled out that calorie excess itself may contribute to the increased hepatic lipid deposition.

Hepatic histology revealed a milder phenotype in the sugar water groups, where ballooning, inflammation and fibrosis was not significantly different from low fat groups, indicating that HF alone was more potent in inducing advanced NASH. This reduction in disease phenotype is likely due to the recorded ‘energy-calibration’ in the guinea pigs, leading to a reduced feed intake in both sugar-water groups, hence reducing the hepatic lipid and cholesterol burden and consequent NASH progression. This is in contrast to a study in C57BL/6 mice, where HFD + HFCS was found to be more effective in inducing inflammation and fibrosis compared to HFD alone [17]. In addition, increased liver weights were recorded in mice fed an ALIOS diet (a diet high in trans-fat supplemented with 4.2% HFCS in the drinking water) with HFCS supplement rather than ALIOS diet alone, and liver triglycerides showed a tendency to be increased when omitting HFCS from the diet, albeit this difference was not significant [19]. However, in both studies, only mild (3/6 had no fibrosis, 2/6 had a grade 1 fibrosis, and 1 animal had a grade 2 fibrosis) or no fibrosis was present in the animals [17,19]. This may be explained by the lack of cholesterol in their experimental diets, as cholesterol is generally considered a critical factor in the development of advanced liver pathology [5,6,66].

5. Conclusions

In this study, we show the limited translational value of adding a soft drink component to an experimental diet and highlight potential undesirable effects of using a high starch control diet. Supplementation of HFCS in the drinking water in addition to a HF, did not exacerbate NAFLD progression but instead reduced feed intake in a dose-dependent manner, consequently reducing inflammation, hepatocyte ballooning and fibrosis and indicating that fat and cholesterol, were superior inducers of advanced NAFLD. In addition, consideration should be paid to putative effects on metabolic phenotype e.g. when choosing a low-fat control diet. Finally, this study shows that guinea pigs on a HF develop increased weight gain, glucose intolerance and NASH with fibrosis within 16 weeks compared to a LF-LSt diet, presenting an animal model recapitulating a large part of the NAFLD disease spectrum observed in humans.
Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nu13082523/s1, Figure S1: Water and food intake pr. group, Figure S2: Insulin tolerance test, Figure S3: Correlation of body weight and triglyceride content in the LF-HSt group, Table S1: Major dietary components listed in %, Table S2: Detailed list of dietary components, Table S3: Total plasma and liver vitamin C levels corrected for food intake, Table S4: Liver TG levels upon exclusion of steatotic animals, Table S5: Plasma biochemical markers at week 8.


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Institutional Review Board Statement: All animal experimentation was approved by the Animal Experimentation Inspectorate under the Danish Ministry of Environment and Food, and in accordance with European legislation of Animal Experimentation 2010/63/EU.

Data Availability Statement: The data presented in this article are available upon request from the corresponding author.

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Molecular drivers of non-alcoholic steatohepatitis are sustained in mild-to-late fibrosis progression in a guinea pig model

David Højland Ipsen1 · Josephine Skat-Rørdam1 · Maria Malvina Tsamouri1 · Markus Latta2 · Jens Lykkesfeldt1 · Pernille Tveden-Nyborg1

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Abstract
Hepatic fibrosis increases mortality in humans with non-alcoholic steatohepatitis (NASH), but it remains unclear how fibrosis stage and progression affect the pathogenic mechanisms of NASH. This study investigates the transcriptional regulation and the impact of fibrosis stage, of pathways relating to hepatic lipid and cholesterol homeostasis, inflammation and fibrosis using RT-qPCR in the guinea pig NASH model. Animals were fed a chow (4% fat), a high-fat (20% fat, 0.35% cholesterol) or high-fat/high-sucrose (20% fat, 15% sucrose, 0.35% cholesterol) diet for 16 or 25 weeks (n = 7/group/time point). High-fat diets induced NASH. In NASH, markers of hepatic de novo lipogenesis were enhanced (e.g. FASN, > twofold, p < 0.05) while markers of mitochondrial, peroxisomal and cytochrome fatty acid oxidation were reduced (e.g. CPT1A > twofold, p < 0.05). Markers of fatty acid uptake were unaltered or decreased. Likewise, expression of cholesterol uptake and synthesis markers were decreased, whereas genes relating to lipid and cholesterol export were unaltered. Inflammatory and chemotactic cytokines were enhanced alongside fibrogenic pathways including increased hepatic stellate cell activation and migration, matrix deposition (e.g. MCP1, TNFα, β-PDGf and Col1a1, > threefold, p < 0.05) and decreased matrix degradation. Fibrosis stage (mild vs. severe) and progression did generally not affect the expression of the investigated pathways. This suggests that liver dysfunction at the transcriptional level is induced early and maintained throughout fibrosis progression, allowing potential treatments to target dysregulated pathways already at early disease stages. As the guinea pig NASH model mimics several aspects of human molecular pathophysiology, these results may be used to increase the current understanding of NASH pathology and explore future treatment targets.

Keywords Non-alcoholic fatty liver disease · Non-alcoholic steatohepatitis · Animal model · Guinea pig · Fibrosis · Molecular mechanisms

Introduction
Affecting 25% of the adult population, non-alcoholic fatty liver disease (NAFLD) is now the world’s most prevalent liver disease with considerable clinical and economic implications (Younossi et al. 2016). NAFLD describes a range of hepatic conditions, from liver steatosis to steatohepatitis (NASH) accompanied by varying degrees of fibrosis (Lau et al. 2017). Although current understanding of the molecular mechanisms of NASH development is incomplete, its multifactorial complexity is well recognized. In NASH, the liver is characterized by disruption of lipid and cholesterol metabolism, as well as increased inflammation and fibrogenesis (Berlanga et al. 2014) (Fig. 1). Development and progression of hepatic fibrosis is associated with increased mortality, in particular...
when advanced, and is accepted as a primary prognostic factor in clinical NASH (Angulo et al. 2015; European Association for the Study of the Liver 2016; Hagstrom et al. 2017).

Understanding the molecular mechanisms of NASH and how they may be altered by fibrosis stage is critical for the development of effective treatment options. In this regard, animal models have been indispensable (Lau et al. 2017). The development of robust and translatable pre-clinical models are, therefore, an essential step in improving the outcome of clinical trials (Santhekadur et al. 2018). Notably, guinea pigs display dyslipidemia and liver histopathology with a close similarity to what is reported in humans in addition to developing bridging (severe) fibrosis when fed a human-like Western diet (Ipsen et al. 2016; Tveden-Nyborg et al. 2016). In contrast to other rodents, low-density lipoprotein is the predominant lipoprotein of guinea pigs and humans, and various enzymes related to cholesterol and lipid metabolism are shared between the two species, accentuating the high degree of face and construct validity of the model (Fernandez 2001). However, knowledge regarding the molecular mechanisms of NASH in the guinea pig model and how these mechanisms compare to the human disease are limited. Here, the guinea pig NASH model was used to investigate the molecular mechanisms driving disease development and explore how fibrosis stage (mild vs. severe) and progression affected the primary cellular pathways related to hepatic lipid and cholesterol metabolism, inflammation and fibrogenesis in NASH. The results show a NASH-induced transcriptional change of multiple metabolic, inflammatory and fibrogenic pathways and suggest that hepatic molecular dysregulation is induced already at the stage of mild fibrosis and maintained as the disease progresses.

Materials and methods

Animals

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

This study is based on extended analysis of tissues from an in vivo study in guinea pigs, of which some results have been published previously (Ipsen et al. 2016). In short, 10–12-week-old female Hartley guinea pigs (Cavia porcellus) were block randomized based on weight and subjected to either a chow (control, 4% fat, 0% sucrose and 0% cholesterol), high-fat (HF, 20% fat, 0% sucrose and 0.35% cholesterol) or high-fat/high-sucrose (HFHS, 20% fat, 15% sucrose and 0.35% cholesterol) diet for 16 or 25 weeks (n = 7 in each group at each time point). All animals were group housed in floor pens and allowed free access to hay, feed and water. At study termination, animals were anesthetized and then killed by intra-cardial desanguination and subsequent decapitation, as previously described (Ipsen et al. 2016). Basic hepatic characteristics are provided in Online Resource 1—at both time points HF and HFHS displayed hepatomegaly with a 2–2.5-fold increase in relative liver weight. Histopathological grading of liver sections (H&E and Masson’s Trichrome stains) were done in accordance to Kleiner et al. (2005) and revealed normal liver histology at both time points in the chow-fed control group. In contrast, HF and HFHS had NASH after 16 (a single animal in the HFHS group did not display hepatic inflammation at week 16 and was consequently not classified as having NASH) and 25 weeks (Online Resource
After 16 weeks, median fibrosis grade was 1 in HF and progressed to a median fibrosis grade of 3 after 25 weeks. HFHS had a median fibrosis grade of 3 at both weeks 16 and 25 (Ipsen et al. 2016). Exact frequencies of the histopathological grading are shown in Online Resource 2.

**RNA extraction and reverse transcription**

Following euthanasia, liver samples were collected from the left lateral lobe (lobus hepatitis sinister lateralis), flash frozen in liquid nitrogen and stored at −80 °C. Approximately 50 mg of liver tissue was homogenized in 1000 µl MagMax Lysis/Binding Solution Concentrate (Thermo Fisher, Waltham, MA, USA) using glass beads (G4649-100G, Sigma, St. Louis, MO, USA) according to the manufacturer’s specifications. Quantity and purity (A260/A280 and A260/A230 ratios) were determined with a NanoDrop 2000 (Thermo Fisher, Waltham, MA, USA). cDNA synthesis was achieved by subjecting 500 ng RNA to reverse transcription (High-Capacity cDNA Reverse Transcription Kit; Thermo Fisher, Waltham, MA, USA) on a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 s. The cDNA was then diluted with RNAse-free water to a final concentration of 3 ng/µl. The absence of genomic DNA contamination was confirmed in all samples prior to inclusion, using an intron-spanning primer set as previously reported (Paidi et al. 2014).

**qPCR**

Standard curves were produced for all primer pairs to confirm primer efficiency and formed the basis for determining optimal cDNA dilution. 2 µl of fivefold diluted cDNA was mixed with 8 µl Master Mix consisting of 5 µl PowerUp SYBR Green Master Mix (Thermo Fisher, Waltham, MA, USA), 1 µl Primer mix (containing 5 µM of both forward and reverse primer) and 2 µl RNAse-free water. RNAse-free water was added instead of sample as a negative control. All samples were run in triplicates using the StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) under the following conditions: 50 °C for 2 min followed by 95 °C for 5 min and then 40 cycles consisting of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 20 s. For tumor necrosis factor α (TNFα)/interleukin 8 (IL-8) and acetyl-CoA carboxylase 1 (ACC1), the annealing step was carried out at 62 °C and 56 °C, respectively. Melting curves were produced for each primer set. Seven candidate reference genes were tested for homogeneous expression. Ultimately, HPRT1 was selected as a reference gene, since this gene displayed the most stable and homogeneous expression between groups. Primers were obtained from previously published sequences or designed with the help of Primer3 (Kores-saar and Remm 2007; Untergasser et al. 2012). Details are provided in Table 1. PCR products from not previously published primers were sequenced by Eurofins (Ebersberg, Germany) and target specificity confirmed by NCBI BLAST sequence alignment.

**Statistics**

Data were processed using StepOne Software version 2.3 (Thermo Fisher, Waltham, MA USA), analyzed by the ΔΔCT-method and presented as fold-changes with ranges (Livak and Schmittgen 2001). Graphs were made and statistical analyses were performed on ΔΔCT-values and their standard deviations using a two-way ANOVA in GraphPad Prism version 7 (GraphPad Software, La Jolla, CA, USA). If “time” was not a significant factor, multiple comparisons were done by Sidak’s test; otherwise Tukey’s test was applied.

**Results**

Detailed overviews of the targeted cellular pathways relating to specific findings are provided in Figs. 2, 3 and 4.

**Triglyceride metabolism**

Lipid accumulation in liver cells is caused by an imbalance between acquisition and clearance of lipids, and is controlled by four key molecular pathways (Fig. 1). The liver can acquire lipids by removing lipids from circulation or by synthesizing them via de novo lipogenesis (DNL). Conversely, the liver disposes of lipids by exporting them as very low density lipoproteins (VLDL) or oxidizing them in the mitochondria, peroxisomes and cytochromes (Fig. 2). Expression of key regulators and genes directly related to these processes were investigated to clarify which mechanisms were disrupted in the applied NASH model.

Grade 3 hepatic steatosis was present in all HF and HFHS animals at weeks 16 and 25, except one HFHS animal presenting grade 2 steatosis at week 16 (Online Resource 2). Hepatic lipid uptake is, in part, controlled by peroxisome proliferator (PPAR) γ, which induces the expression of specific transporters required for the uptake of fatty acids. Expression of PPARγ was only increased at week 16 in HFHS (p < 0.05) (Fig. 5a). The fatty acid transporters,
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<th>Reverse (5′–3′)</th>
<th>Product (bp)</th>
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Commonly used synonyms are shown in parentheses. Previously published primer pairs are indicated as <sup>a</sup>(Sarr et al. 2014), <sup>b</sup>(Podszun et al. 2014), <sup>c</sup>(Xu et al. 2015), <sup>d</sup>(Zhang et al. 2013), <sup>e</sup>(Zhang et al. 2013), <sup>f</sup>(Lyons et al. 2002), <sup>g</sup>(Balgobin et al. 2013), <sup>h</sup>(Sarr et al. 2016), <sup>i</sup>(Cho et al. 2005)
transcription factors: sterol regulatory element-binding protein 1 (SREBP1) and carbohydrate response element-binding protein (ChREBP), which both induce the transcription of DNL-genes. SREBP1 was increased four- to sevenfold in both groups at week 16 and in HFHS at week 25 (p < 0.01) (Fig. 5a). In contrast, ChREBP was decreased in HFHS after 25 weeks (p < 0.01), and in HF after 16 weeks (p < 0.05), and non-significantly at week 25 (p = 0.0721). Fatty acid synthase (FASN) catalyzes the last step in DNL, and is positively regulated by SREBP1. FASN was increased two- to fourfold in HF and HFHS at both time points (p < 0.05). No differences were found in the expression of ACC1, also known to be regulated by SREBP1.

Fatty acid oxidation preferentially occurs in the mitochondria, but may also take place in the peroxisomes or cytochromes, and is transcriptionally regulated by PPARα (Rao and Reddy 2001). PPARα levels were not different between groups in the current study, but PPARα target genes—carnitine palmitoyltransferase 1 (CPT1A) and acyl-coenzyme A oxidase (ACOX1)—were decreased (Fig. 5c). Transport of fatty acids across the mitochondrial membrane is a prerequisite to mitochondrial oxidation. CPT1A is essential in facilitating this transport, and was decreased in HF and HFHS at weeks 16 (p < 0.01) and 25 (p < 0.05). Following entry into the mitochondria, the
first step of β-oxidation is catalyzed by medium-chain and long-chain acyl-CoA dehydrogenase (MCAD and LCAD, respectively). Only LCAD was found to be increased and only in HFHS after 25 weeks (p < 0.05). Compromised fatty acid oxidation in peroxisomes and cytochromes was suggested by the decreased ACOX1 expression after 16 and 25 weeks in HF (p < 0.05) and decreased cytochrome P450 2E1 (CYP2E1) expression after 16 weeks in HF and 25 weeks in both HF and HFHS (p < 0.05).

To be cleared via export from the liver, fatty acids must be packed into water-soluble VLDL particles. The formation of VLDL particles requires apoB100 and is catalyzed by microsomal triglyceride transfer protein (MTTP). Expression of apoB100 and MTTP was similar between groups (Fig. 5d).

**Cholesterol metabolism**

Similar to triglycerides, cholesterol metabolism is regulated by uptake, synthesis and excretion. These pathways are managed by transcriptional regulators, which tightly control the expression of transport molecules and enzymes involved in these processes (Fig. 3).

SREBP2, the main regulator of cholesterol metabolism, is negatively regulated by cholesterol, controlling cholesterol metabolism by inducing genes related to cholesterol uptake and synthesis. SREBP2 was decreased in HF after 16 (p < 0.001) and 25 (p < 0.05) weeks, but not statistically significant in HFHS animals (Fig. 6). Expression of the low-density lipoprotein receptor (LDLR), responsible for uptake of circulating cholesterol was decreased in both groups at week 16 (p < 0.001) and in HF at week 25 (p < 0.01).

Endogenous cholesterol synthesis was decreased in HF, evident by lower expression of the rate-limiting enzyme HMG-CoA reductase (HMGR) after 16 (p < 0.01) and 25 weeks (p < 0.05). Similar regulation of HMGR was noted in HFHS, although not reaching statistical significance. High cholesterol levels usually activate the ‘cholesterol sensor’ liver X receptor α (LXRA), in turn promoting expression of genes associated with the conversion of cholesterol to bile acids and the subsequent bile excretion. In the current study, LXRA expression was similar between groups at both time points, and no alterations were detected in the expression of genes related to bile synthesis (cytochrome P450 7A1 (CYP7A1)) or export (ATP-binding cassette sub-family G member (ABCG) 5 and ABCG8. Additionally, farnesoid X receptor (FXR) expression—a bile acid sensor regulating bile acid synthesis in a negative feedback loop as well as DNL by inhibiting SREBP1—did not differ between groups.

**Inflammation and fibrogenesis**

After 16 weeks, inflammation in HF and HFHS (grade 2) was not significantly different from control animals (grade 1). At week 25, all HF and HFHS animals had grade 3 inflammation, which was significantly higher than controls (grade 0) (Online Resource 2). Inflammation is part of the pathophysiology of NASH and is linked to the development of fibrosis (Fig. 1). Propagation of dyslipidemia and hepatic steatosis leads to the secretion of inflammatory and pro-fibrotic cytokines and growth factors, resulting in infiltration of circulating immune cells, activation of hepatic stellate cells (HSC) and enhanced production of extracellular matrix components such as collagen (col), laminin and elastin (Fig. 4).
Fig. 5 Hepatic triglyceride metabolism in NASH. a Hepatic lipid transporters were unaltered or downregulated in NASH compared to control (b) while DNL, mediated by SREBP1, was increased. c Concomitant with decreased fatty acid oxidation and similar (d) export of lipids, this facilitates hepatic lipid accumulation in NASH. Means with ranges expressed as fold-changes relative to control. *p < 0.05, **p < 0.01, ***p < 0.001 vs. control. n = 6–7 (n = 6 for HF at week 25 for all genes due to technical difficulties). Data were analyzed using a two-way ANOVA with Tukey’s or Sidak’s post hoc test where appropriate. ACC1 acetyl-CoA carboxylase, ACOX1 acyl-coenzyme A oxidase 1, ApoB100 apolipoprotein B 100, CD36 cluster of differentiation 36, ChREBP carbohydrate regulatory element-binding protein, CPT1A carnitine palmitoyl transferase 1A, CYP2E1 cytochrome P450 2E1, FASN fatty acid synthase, FATP fatty acid transport protein, LCAD long-chain acyl-coenzyme A dehydrogenase, MCAD medium-chain acyl-coenzyme A dehydrogenase, MTTP microsomal triglyceride transfer protein, PPAR peroxisome proliferator-activated receptors, SREBP1 sterol regulatory element-binding protein 1

Fig. 6 Hepatic cholesterol metabolism in NASH. Hepatic LDLR-mediated cholesterol uptake and endogenous cholesterol synthesis were downregulated in NASH, likely due to decreased SREBP2 expression. However, in the face of high intrahepatic cholesterol levels conversion of cholesterol to and export as bile acids was not increased, potentially due to disruption of the LXRα-axis. Means with ranges expressed as fold-changes relative to control. *p < 0.05, **p < 0.01, ***p < 0.001 vs. control. n = 6–7 (n = 6 for HF at week 25 for all genes due to technical difficulties). Data were analyzed using a two-way ANOVA with Tukey’s or Sidak’s post hoc test where appropriate. ABCG5 ATP-binding cassette sub-family G member 5, ABCG8 ATP-binding cassette sub-family G member 8, CYP7A1 cytochrome P450 7A1/cholesterol 7α-hydroxylase, FXR farnesoid X receptor, HMGCR 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, LXRα liver X receptor α, LDLR low-density lipoprotein receptor, SREBP2 sterol regulatory element-binding protein 2
A strong 32–39-fold increase in monocyte chemotactic protein 1 (MCP-1/CCL2) expression was observed after 16 weeks in HF and HFHS (p < 0.001), progressing to 75–88-fold after 25 weeks in both groups (p < 0.001). After 16 and 25 weeks, IL-8 expression was increased 5–13-fold in HF and HFHS (p < 0.001), with IL-8 expression levels significantly higher in HF compared to HFHS at week 16 (p < 0.05).

Besides promoting recruitment of circulating immune cells, these inflammatory cytokines activate the HSC, which are normally present in the liver in a quiescent state. HSC are also activated by a range of pro-fibrotic growth factors such as transforming growth factor β1 (TGFβ1), β-platelet-derived growth factor (β-PDGF) and connective tissue growth factor (CTGF). TGFβ1 expression was increased in HFHS at 16 weeks (p < 0.05), and week 25 (p < 0.001) (Fig. 7b). Expression of β-PDGF was increased ~fivefold in both HF and HFHS after 16 and 25 weeks (p < 0.001), whereas CTGF levels did not differ between groups. HSC activation can be assessed by α smooth muscle actin (αSMA), which was increased in HFHS after 25 weeks (p < 0.001), and tended to be increased in HF animals after 25 weeks (p = 0.056). Activated HSC promotes fibrosis by increasing the synthesis of different collagens and other components of the extracellular matrix (e.g. laminin and elastin). Col1α1 expression was increased ~15-fold in HF and HFHS after 16 and 25 weeks (p < 0.001) (Fig. 7c). Col3a1 levels were higher in HF and HFHS at week 16 (p < 0.05) and only in HFHS at week 25 (p < 0.01). In HFHS, Col3a1 was upregulated at weeks 16 and 25 compared to both control and HF (p < 0.01), but Col4a1 expression did not differ between HF and controls on either time points. Laminin was increased in HF and HFHS after 16 weeks (p < 0.01) and in HF after 25 weeks (p < 0.05). Elastin expression was elevated after 25 weeks in

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**Fig. 7** Hepatic inflammation and fibrogenesis in NASH. a Markers of hepatic inflammation and b pro-fibrotic growth factors were increased in guinea pigs with NASH (HF and HFHS) compared to controls. c Accordingly, genes related to extracellular matrix composition, fibrosis and d the inhibition of extracellular matrix degradation were also increased. Overall, gene expression did not differ between grade 1 (HF weeks 16) and grade 3 (HF week 16 and HFHS week 16 and 25) fibrosis. Means with ranges expressed as fold-changes relative to control. *p<0.05, **p<0.01, ***p<0.001 vs. control. †p<0.05, ††p<0.01, †††p<0.001 vs. HFHS. n=6–7 (n=6 for HF at week 25 for all genes, n=5 for control at week 25 for IL-8. n=5 for control at week 25 for PAI-1 due to technical difficulties). Data were analyzed using a two-way ANOVA with Tukey’s or Sidak’s post hoc test where appropriate. αSMA α smooth muscle actin, β-PDGF β-platelet-derived growth factor, Col1α1 collagen 1α1, Col3a1 collagen 3α1, Col4a1 collagen 4α1, CTGF connective tissue growth factor, IL-8 interleukin 8, MCP-1: monocyte chemoattractant protein 1, PAI-1 plasminogen activator inhibitor 1, TGFβ1 transforming growth factor β1, TIMP tissue inhibitor of metalloproteinase, TLR4 toll-like receptor 4, TNFα tumor necrosis factor α
both HF and HFHS ($p < 0.05$). Besides enhanced formation of extracellular matrix components, decreased degradation also contributes to the development of fibrosis. Lysyl oxidase (LOX) promotes fibrosis and limits fibrosis regression by cross-linking and stabilizing collagen (Liu et al. 2016). After 16 weeks, LOX expression was higher in HFHS compared to HF and control ($p < 0.05$) and tended to be higher in HFHS after 25 weeks compared to control ($p = 0.086$) (Fig. 7d). Tissue inhibitor of metalloproteinase (TIMP) may promote fibrosis by inhibiting the enzymes responsible for breaking down the extracellular matrix. TIMP1 expression did not differ between groups, but TIMP2 was increased in HFHS after 16 ($p < 0.05$) and 25 ($p < 0.001$) weeks. Similarly, PAI-1 promotes fibrosis by indirectly inhibiting its degradation. PAI-1 expression was induced in HF and HFHS at both time points ($p < 0.01$) and was higher in HFHS compared to HF at week 25 ($p < 0.05$).

**Discussion**

This study reports transcriptional changes of multiple metabolic, inflammatory and fibrogenic pathways as result of NASH and fibrosis progression in the dyslipidemic guinea pig. The results support that development of NASH, in this model, is associated with enhanced DNL, while also indicating impaired fatty acid oxidation as a potential contributor to hepatic lipid accumulation. Furthermore, hepatic cholesterol overload appears to occur despite decreased uptake and biosynthesis, due to insufficient clearance of cholesterol. Lastly, markers of inflammation and fibrogenesis are elevated irrespective of fibrosis stage, suggesting that these pathways are induced in the first stages of fibrosis and then maintained throughout disease progression.

The liver acquires lipids in two principal ways: through uptake of circulating lipids and synthesis via DNL (Fig. 2). The current findings suggest unaltered (CD36 and FATP2) or even suppressed (FATP5) hepatic lipid uptake in NASH, in agreement with previous reports describing unaltered CD36 expression in patients with steatosis or NASH compared to controls (Auguet et al. 2014). In contrast, others have reported an upregulation of CD36 and FATP5 in patients with steatosis or NASH (Miquilena-Colina et al. 2011; Westerbacka et al. 2007). Hepatic lipid accumulation appears to be facilitated by enhanced DNL as SREBP1 and its downstream target FASN were upregulated in guinea pigs with NASH, supporting findings from patients with NAFLD, in which expression of lipogenic genes and hepatic lipid synthesis are elevated (Donnelly et al. 2005; Kohjima et al. 2007; Zhu et al. 2011). Overall, the other central regulator of DNL, ChREBP, was decreased in NASH but was not affected by fibrosis stage. This may be due to the relatively lower total carbohydrate content of the high-fat diets (36.3–37.9% carbohydrates) compared to the chow diet (47.1% carbohydrates), as a carbohydrate-rich diet (75.2% carbohydrates) more potently induces ChREBP than a high-fat diet (30.5% carbohydrates) in rats (Yamashita et al. 2001). ChREBP has been found to mitigate fructose-induced liver damage and dissociate hepatic steatosis from insulin resistance (Benhamed et al. 2012; Zhang et al. 2017), proposing that decreased ChREBP expression in NASH might play a role in promoting disease progression. Collectively, our results support findings from humans (Higuchi et al. 2008) and indicate that SREBP1, rather than ChREBP, mediated DNL which is enhanced in NASH.

The liver may attempt to alleviate the development of steatosis by increasing the export of lipids as VLDL particles or by oxidation. In humans, hepatic triglyceride secretion increases with hepatic lipid content until the intrahepatic lipid levels reaches 10% where after secretion reaches a plateau (Fabbrini et al. 2008). However, secretion may also decrease as the disease progresses from steatosis to NASH (Fujita et al. 2009). In the current study, markers of hepatic lipid outflow (apoB100 and MTTP) were not different between groups. However, levels of circulating triglycerides were decreased in HF and HFHS compared to control as has previously been reported for the guinea pig NASH model (Ipsen et al. 2016; Tveden-Nyborg et al. 2016). This indicates that hepatic triglyceride export is indeed compromised in NASH and may contribute to the advancing liver steatosis, albeit our current data suggest that this is not regulated at the transcriptional level. Hepatic steatosis is further substantiated through the suppression of hepatic fatty acid oxidation and accordingly, markers representing all three major organelles involved, i.e. mitochondria (CPT1A), peroxisomal (ACOX1) and cytochrome (CYP2E1), were decreased in the present study. Expression of the fatty acid oxidation master regulator PPARα was diminished in NASH patients and PPARα decreased progressively with increasing NAFLD activity score (Francque et al. 2015). Failure to increase PPARα in guinea pigs with NASH could contribute to the impaired fatty acid oxidation resulting in hepatic steatosis (Mari et al. 2006; Savard et al. 2013). Similar steatosis grade at weeks 16 and 25 in HF and HFHS indicates that once maximal steatosis grade is reached (week 16), lipid metabolism does not deteriorate further, at least in this time-span. Furthermore, these results also suggest that the propagating hepatic fibrosis did not induce further alterations in genes relating to lipid metabolism. PPARα expression is suppressed by TNFα in vitro, and in vivo cholesterol induces impaired β-oxidation and sensitizes to TNF-α-mediated steatohepatitis (Lim et al. 2013). Consequently, impaired oxidation of fatty acids in NASH may be connected to enhanced levels of cytotoxic cholesterol due to faulty cholesterol clearance and hepatic inflammation.
In agreement with previous findings in guinea pigs (Fernandez 2001), cholesterol uptake and synthesis were downregulated in response to high dietary cholesterol intake. The current study further suggests that this downregulation is likely mediated by suppressed SREBP2 expression (Fig. 3). Thus, regulation of cholesterol import and synthesis appear to function correctly in this NASH model. However, high levels of circulating cholesterol in guinea pigs with NASH compared to control could result in a relatively larger uptake of cholesterol, despite LDLR downregulation and may, thereby, account for the high intraparenchymal cholesterol levels.

Despite high intrahepatic cholesterol levels in guinea pigs with NASH, expression of LXRx and downstream targets, i.e. the rate-limiting enzyme in bile acid synthesis (CYP7A1) and bile acid transporters (ABCG5 and -8), were not enhanced. This suggests that cholesterol accumulation may, in part, be due to an absent upregulation of cholesterol clearance pathways. The findings are in agreement with molecular analysis of liver samples from NASH patients, exhibiting a downregulation of LDLR, CYP7A1 and ABCG8 proteins (Min et al. 2012). Potentially mediated by TNFα, inflammation may disrupt bile acid production and export in mice by decreasing LXRx and CYP7A1 (Chen et al. 2012). These findings support a role of disrupted cholesterol clearance in NASH, possibly precipitated by inflammation while also finding that the transcriptional regulation of hepatic cholesterol metabolism is equally altered in the presence of grade 1 and 3 fibrosis.

Patients with NASH have increased SREBP2-mediated expression of HMGCR, suggesting exaggerated cholesterol synthesis to be a feature of NAFLD/NASH (Min et al. 2012). These results contrast our in vivo findings from guinea pigs, but may be explained by differential intake of dietary cholesterol as HMGCR is negatively regulated by dietary cholesterol (Hojland Ipsen et al. 2016; Ness and Chambers 2000). Despite being used in moderately low amounts compared to other pre-clinical models of NASH, the dietary cholesterol consumption in the present study (0.35%) is still high when compared to reported daily cholesterol intake in NAFLD patients (Ipsen et al. 2016; Lau et al. 2017; Schattenberg and Galle 2010; Tveden-Nyborg et al. 2016). Accumulation of cytotoxic cholesterol directly injures hepatocytes, activates Kupffer cells and HSC, hereby, promoting inflammation and fibrogenesis and linking these pathways to cholesterol metabolism (Musso et al. 2013). Hepatic inflammation was histologically confirmed in guinea pigs with NASH (Ipsen et al. 2016) and is in agreement with elevated levels of TNFα, MCP-1 and IL-8 (Fig. 4). Most notably was the extensive upregulation of MCP-1, a chemokine which is also increased in NASH patients (Bertola et al. 2010). Recently, a dual CCR2/CCR5 receptor antagonist was found to improve hepatic fibrosis after 1 year compared to placebo, supporting a key role of MCP-1 in disease progression (Friedman et al. 2018). IL-8 is the principal recruiter of neutrophils, but data regarding its role in NASH are limited (Remick 2005). Similar to MCP-1, IL-8 was increased in patients with NASH, but not steatosis, suggesting that these chemokines may contribute to the progression of bland steatosis to steatohepatitis and liver injury (Bertola et al. 2010). Indeed, in patients with alcoholic steatohepatitis, IL-8 was an independent predictor of 90-day mortality, associated with both a deteriorating prognosis and the degree of neutrophil infiltration (Dominguez et al. 2009). Furthermore, expression of IL-8 highlights a similarity in the immunological response of NASH in humans and guinea pigs, which would be absent in other models such as the rat and mouse as these species do not express IL-8 (Remick 2005). In NASH-associated hepatic fibrosis, the deposition of fibrogenic extracellular matrix is promoted by the enhanced production of inflammatory cytokines (e.g. TNFα, and IL-8) and pro-fibrotic growth factors (e.g. TGFβ1) that activate the HSC (Tsuchida and Friedman 2017). These cells are essential drivers of hepatic fibrosis in both experimental and human liver injury (Tsuchida and Friedman 2017) (Fig. 4).

Increased expression of TGFβ1 has been found in the liver of patients with NASH (Bertola et al. 2010). Expression of TGFβ1 was not pronounced in HF animals with mild fibrosis (grade 1) and was only increased in HFHS in which severe fibrosis (grade 3) was evident at week 16. HSC activation (assessed by αSMA expression) followed TGFβ1 expression, whereas β-PDGF, one of the most potent mitogens for HSC, was rapidly induced and highly expressed at both fibrosis stages, whereas CTGF was not. This could be due to an absence of hyperglycemia in guinea pigs (Ipsen et al. 2016), which has been found to induce CTGF (Paradis et al. 2001). Following activation, HSC initiates collagen deposition. Accordingly, Col3a1 and in particular Col1a1 were highly expressed in the liver of high-fat-fed animals resembling the extracellular matrix composition of human liver fibrosis (Lee and Friedman 2011). This enhanced expression occurred irrespective of fibrosis stage, suggesting collagen transcription to peak already at the stage of mild (grade 1) fibrosis and then be maintained throughout fibrogenesis. PAI-1 was increased in HF and HFHS indicating reduced degradation of the extracellular matrix hereby contributing to the progression of fibrosis. PAI-1 indirectly promotes fibrosis by inhibiting the formation of plasmin and the subsequent activation of MMP-8, which predominantly degrades collagen (Benyon and Arthur 2001). Increased PAI-1 expression has also been reported in human studies (Bertola et al. 2010) and could potentially contribute to the augmented Coll1 levels observed in the present study. TIMP2 was only elevated in HFHS with grade 3 fibrosis and tended to be elevated as HF reached grade 3 fibrosis after 25 weeks. Excess TIMP2 blocks MMP-2 activity and thereby the degradation of Col4a1 (Benyon and Arthur 2001). Enhanced expression...
of TIMP2 in HFHS may account for the higher levels of Col4a1 in HFHS compared to HF and control, possibly contributing to fibrosis progression. Enhanced TGFβ1, Col4a1 and TIMP2 expression were only found in severe fibrosis (grade 3) potentially playing a role in advanced fibrosis. However, induction of inflammatory cytokines, fibrogenic growth factors, collagen synthesis and inhibition of extracellular matrix degradation were largely unaffected by fibrosis stage and NASH-duration in the present study. This suggests that most of the transcriptional machinery relating to these pathways are already maximized at the stage of mild hepatic fibrosis (grade 1) and subsequently maintained as more severe fibrosis (grade 3) develops. Therefore, it is possible that treatment may be successfully implemented at the stage of mild fibrosis, thereby, facilitating regression and/or preventing progression of more advanced fibrosis known to be associated with increased mortality (Angulo et al. 2015; Hagstrom et al. 2017).

In conclusion, our findings suggest that the underlying mechanisms of NASH in the guinea pig model are characterized by enhanced DNL, decreased fatty acid oxidation and failure to upregulate the export of lipids and cholesterol resulting in hepatic steatosis and accumulation of cholesterol. In turn, hepatocellular injury induces inflammation and fibrogenesis, mimicking several aspects of the human NASH pathogenesis and accentuating the translational value of this pre-clinical model. These pathways were generally not modulated by mild-to-late fibrosis stage, suggesting that liver dysfunction at the transcriptional level is maintained throughout fibrosis progression and identifying targets that may be affected prior to the development of advanced hepatic fibrosis.

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Compliance with ethical standards

Conflict of interest  The authors declare no competing interests in relation to the present work.

Ethical approval  All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Article

Modelling Nonalcoholic Steatohepatitis In Vivo—A Close Transcriptomic Similarity Supports the Guinea Pig Disease Model

Josephine Skat-Rorholm 1,†, David H. Ipsen 1,‡, Stefan E. Seemann 2,*, Markus Latta 3, Jens Lykkesfeldt 1,‡ and Pernille Tveden-Nyborg 1,‡

1 Section of Experimental Animal Models, Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, DK-1870 Frederiksberg, Denmark; jsr@sund.ku.dk (J.S.-R.); jpl@sund.ku.dk (J.L.)
2 Center for non-coding RNA in Technology and Health, Department of Veterinary and Animal Sciences, Section for Animal Genetics, Bioinformatics and Breeding, University of Copenhagen, DK-1871 Frederiksberg, Denmark; seemann@rh.dk
3 Liver Disease Research, Global Drug Discovery, Novo Nordisk A/S, DK-2760 Måløv, Denmark; mrl@novonordisk.com
* Correspondence: ptn@sund.ku.dk; Tel.: +45-35-33-31-67
† Shared first-authorship.

Abstract: The successful development of effective treatments against nonalcoholic steatohepatitis (NASH) is significantly set back by the limited availability of predictive preclinical models, thereby delaying and reducing patient recovery. Uniquely, the guinea pig NASH model develops hepatic histopathology and fibrosis resembling that of human patients, supported by similarities in selected cellular pathways. The high-throughput sequencing of guinea pig livers with fibrotic NASH (n = 6) and matched controls (n = 6) showed a clear separation of the transcriptomic profile between NASH and control animals. A comparison to NASH patients with mild disease (GSE126848) revealed a 45.2% overlap in differentially expressed genes, while pathway analysis showed a 34% match between the top 50 enriched pathways in patients with advanced NASH (GSE49541) and guinea pigs. Gene set enrichment analysis highlighted the similarity to human patients (GSE49541), also when compared to three murine models (GSE52748, GSE38141, GSE67680), and leading edge genes THRSP, CCL20 and CD44 were highly expressed in both guinea pigs and NASH patients. Nine candidate genes were identified as highly correlated with hepatic fibrosis (correlation coefficient > 0.8), and showed a similar pathway pattern in NASH patients. Of these, two candidate genes (VWF and SERPINB9) encode secreted factors, warranting further investigations as potential biomarkers of human NASH progression. This study demonstrates key similarities in guinea pig and human NASH, supporting increased predictability when translating research findings to human patients.

Keywords: nonalcoholic steatohepatitis; fibrosis; transcriptome; animal model; guinea pig; biomarkers

1. Introduction

Hepatic fibrosis is the primary prognostic marker of mortality in nonalcoholic steatohepatitis (NASH) globally affecting millions [1]. However, treatment options are scarce, and drug development is hampered by the lack of animal models reflecting the disease spectrum and etiology. Though frequently applied, many mouse and rat models do not develop NASH with advanced fibrosis (bridging fibrosis and cirrhosis) without the use of hepatotoxins and micronutrient-deficient diets, consequently compromising construct and, by extension, predictive validity [2–4].

Uniquely, guinea pigs develop NASH with advanced fibrosis when subjected to a westernised diet [5–7]. Similar to human NASH, lesions originate from the hepatic central veins and sequentially progress from mild fibrosis to advanced (bridging) fibrosis...
within 25 weeks [6,7]. Furthermore, guinea pigs and humans share an LDL-dominant lipoprotein profile in contrast to the HDL-dominant profile of rats and mice, and targeted analysis of genes related to hepatic lipid metabolism, inflammation, and fibrogenesis further supports a high degree of similarity between the guinea pig disease model and human NASH [8,9]. However, unbiased systematic analysis of the guinea pig NASH transcriptome has not previously been undertaken, and is impeded by the incompletely annotated guinea pig genome. Using RNA sequencing and human orthologue mapping to improve annotation, this paper investigates the translational validity of the guinea pig NASH transcriptome and directly compares transcriptome remodelling to profiles from two patient datasets representing either mild or advanced disease, and three frequently used mouse models. Lastly, we propose a selection of candidate genes present in both human and guinea pig fibrotic NASH that may prove valuable in future drug discovery and biomarker development.

2. Materials and Methods

2.1. Animals

Animal experiments were approved by the Animal Experimentation Inspectorate under the Danish Ministry of the Environment and Food, and in accordance with the European Legislation of Animal Experimentation 2010/63/EU.

This study utilises a subset of liver samples, collected from intact nonperfused livers, from a previously published study [6]. Briefly, 10-week-old female Hartley guinea pigs (Charles River Laboratory, Lyon, France) were allowed one week of acclimatisation before being block-randomised into groups on the basis of body weight. Consistent with previous studies, this study was only performed on female guinea pigs, as hierarchical fighting in males is a critical welfare concern in long-term studies of this kind. The guinea pigs were fed a high-fat (20% fat, 15% sucrose, and 0.35% cholesterol) or chow (4% fat, 0% sucrose, 0% cholesterol) diet for 25 weeks. The exact dietary compositions were published elsewhere [6]. Six high-fat-fed animals with grade 3 (bridging) fibrosis and six randomly selected control animals without NASH and fibrosis were used for RNA sequencing.

At termination, the animals were preanaesthetised with 0.8 mL/kg body weight Zoletil mix (125 mg tiletamine (0.93 mg/kg), 125 mg zolazepam (0.93 mg/kg, Zoletil 50 Virbac Laboratories, Carros, France) + 200 mg xylazine (1.49 mg/kg, Narcoxyll vet 20 mg/mL; Intervet International, Boxmeer, Holland) + 7.5 mg butorphanol (0.06 mg/kg; Torbugesic vet 10 mg/mL; Scanvet, Fredensborg, Denmark) diluted 1:10 in isotonic NaCl), placed on isoflurane (3–5%) and, following the disappearance of intradigital reflexes, euthanised by decapitation as previously described [6].

2.2. Liver Samples and Histology

Liver samples were collected from the left lateral lobe (lobus hepatitis sinister lateralis), snap-frozen in liquid nitrogen, stored at −80°C or fixed in paraformaldehyde, and subsequently embedded in paraffin and stained with haematoxylin and eosin or Masson’s trichrome for histological evaluation as previously published [6]. Liver histology was scored in accordance with guidelines by Kleiner et al. [10]. Steatosis was scored as 0 (5%), 1 (5–33%), 2 (>33–66%), or 3 (>66%) of the overall parenchymal tissue. Ballooning was scored as 0 (none), 1 (few/minimal), or 2 (many/prominent). Inflammation was scored in three lobuli, defined by two portal areas and one central vein as 0 (no foci), 1 (1 foci), 2 (2–4 foci), or 3 (>4 foci) per ×200 field. A focus was defined as 3 inflammatory cells in close proximity. Fibrosis was scored as 0 (none), 1A (mild, zone 3 perisinusoidal), 1B (moderate, zone 3 perisinusoidal), 1C (periportal), 2 (perisinusoidal and periportal), 3 (bridging), or 4 (cirrhosis). All scorings were performed in a randomised and blinded manner, and were previously published [6]. For the quantification of the relative fibrosis area, sections were stained with Picro Sirius Red and Weigert’s haematoxylin. Total collagen area was analysed on digital images of the entire liver section using VisioPharm Image analysis (version 2020.01.3.7887, VisioPharm, Hørsholm, Denmark) in accordance with quantifi-
cations of picrosirius red staining in liver samples from preclinical models and human patients [11,12]. Fibrosis area is shown in Supplementary Table S1, Additional File S1.

2.3. Guinea Pig Hepatic RNA Sequencing

RNA sequencing was performed on 12–24 mg of liver tissue from control (n = 6) and NASH animals with bridging fibrosis (n = 6). RNA extraction, purification, and sequencing were performed by QIAGEN Genomic Services (QIAGEN, Hilden, Germany). Briefly, library preparation was performed using a TruSeq® Stranded mRNA Sample preparation kit (Illumina Inc., San Diego, CA, USA), and library size distribution was validated and quality-inspected on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Subsequently, single-read 75-nucleotide read-length sequencing was performed on the Illumina NextSeq 500 platform with 30 million reads per sample according to the manufacturer’s specifications (Illumina Inc., San Diego, CA, USA). Sequencing data are available at GEO expression omnibus with accession number GSE158168.

2.4. Transcriptome Analyses

FASTQ files obtained from QIAGEN Genomic Services were processed in Trimmmomatic (version 0.38.0) [13]. Reads shorter than 50 bases were removed, and all reads were trimmed of the leading 9 bases due to base-call quality below a Phred score of 32 and biased sequence composition. Trimmed reads were mapped to the Ensembl Cavia Porcellus genome Cav.Por.3.0 (Ensembl release 98, September 2019) with Hisat2 (version 2.1.0); multimapped reads were ignored for further analysis [14]. Subsequent transcript assembly and quantification were performed using Stringtie and Stringtie merge (version 1.3.6) [15], using the Cav Por.3.0 gene annotation as a guide. As the guinea pig genome is incompletely annotated, BioMart (version 2.4.20) [16] was used instead to obtain human orthologues (GRCh38.p13, Ensembl release 98, September 2019). Although the evolutionary distance of the guinea pig genome is marginally smaller to that of mice than to that of humans [17], many of the regulatory mechanisms of the immune system are more comparable between humans and guinea pigs [18]. Human orthologues were obtained with a sequence identity cut-off >50% using the Bioconductor package (version 3.10) [19]. Genes without annotation were excluded from analysis, resulting in a total of 17,332 unique genes. To diminish background noise, genes with total read count < 200 across all samples were excluded from subsequent analysis, producing a final list of genes containing 11,896 genes, for which differential expression was calculated using DESeq2 (version 1.26.0) [20].

2.5. Protein–Protein Association Network

A protein–protein association network was built using Cytoscape (version 3.8.0) [21] of the 100 proteins with the strongest association to NASH, as annotated by the DISEASES database [22,23]. Protein–protein associations were based on different confidence channels (e.g., physical association from experimental data and functional associations from curated pathways, automatic text mining, and prediction methods) provided by the STRING database [24,25]. A STRING confidence score of at least 0.7 was applied. For gene identifiers CBLC, CCN1, RACK1, PCDHGA6, and HCN2, no exact match was found by Cytoscape, and they were thus excluded from analysis.

2.6. Translational Aspects of the Guinea Pig Model

To investigate the translatability and benchmark the guinea pig as a model of NASH, the transcriptome was compared to two human datasets and three murine datasets (Table 1), which were all publicly available through the GEO expression omnibus [26]. Specifically, the guinea pig transcriptome was compared to human dataset (GSE126848) Human NASH1 (HNASH1) consisting of 14 healthy patients and 16 patients diagnosed with NASH (steatosis–activity–fibrosis 2) [27,28]. To determine if a core set of NASH-associated advanced-fibrosis genes could be identified for NASH irrespective of species, the guinea pig dataset was compared to human gene-expression data (GSE49541) Human NASH
2 (HNASH2), consisting of 40 patients with mild (F0–F1) nonalcoholic fatty liver disease (NAFLD) and 32 patients with advanced NAFLD and F3–F4 fibrosis [29]. In addition, guinea pig expression data were compared to two preclinical mouse NAFLD models, Western Diet 1 (WD1) (GSE52748) [30], Western Diet 2 (WD2) (GSE38141) [31], and one preclinical mouse NASH model, DIAMOND (GSE67680) [32] (Table 1). The mouse models were selected on the basis of similarity in dietary content (high fat, cholesterol, and sucrose), the availability of data, and the inclusion of a relevant control group without NASH and fibrosis.

Table 1. Overview of included preclinical models.

<table>
<thead>
<tr>
<th>Preclinical Model</th>
<th>Sex</th>
<th>Species: Strain</th>
<th>Weeks on Diet</th>
<th>Histological Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig</td>
<td>Female</td>
<td>Guinea pig: Dunkin-Hartley</td>
<td>25</td>
<td>NASH with fibrosis. Histological scoring: NASH CRN [10]</td>
</tr>
<tr>
<td>DIAMOND [32]</td>
<td>Male</td>
<td>Mouse: B6/129 (isogenic cross between C57BL/6j and 129S1/SvImJ)</td>
<td>52</td>
<td>NASH with fibrosis Histological scoring: NASH CRN [10]</td>
</tr>
<tr>
<td>WD1 [30]</td>
<td>Male</td>
<td>Mouse: C57BL/6N</td>
<td>12</td>
<td>NAFLD No histological scoring. Positive α-sm actin stain and picrosirius red indicative of activated hepatic stellate cells and fibrosis, respectively</td>
</tr>
<tr>
<td>WD2 [31]</td>
<td>Male</td>
<td>Mouse: C57BL/6j</td>
<td>20</td>
<td>NAFLD. No histological scoring</td>
</tr>
</tbody>
</table>

NAFLD: nonalcoholic fatty liver disease. NASH CRN: nonalcoholic steatohepatitis Clinical Research Network [10].

2.7. Correlation Analysis of Gene Expression and Fibrosis Quantification

Genes potentially linked to advanced fibrosis stages were identified by correlating the normalised log-transformed values [20] for each gene to the relative fibrosis area of each animal (Supplementary Table S1, Additional File S1). We selected genes with a Pearson correlation coefficient of 0.8 for both all animals (control and NASH) and NASH animals alone. Additionally, the direction (negative or positive) of the Pearson correlation coefficient had to be identical for both groups (NASH animals alone and all animals). Lastly, only genes with log2 fold change >1 in the guinea pig dataset and a similar expression pattern (up- or downregulated) in the human dataset (GSE49541) were selected. For the regression analysis of each gene, see Additional File S2.

2.8. Statistical Analysis

Guinea pig and human dataset HNASH1 (GSE126848) were analysed by high-throughput sequencing [28]. Raw counts from RNA sequencing analysis were used as input for differential expression analysis performed with the DEseq2 package [20]. In contrast, human dataset HNASH2 (GSE49541) [29] and murine datasets (WD1 [30], WD2 [31], DIAMOND [32]) are array datasets, and the limma package (version 3.42.2) [33] was used instead to identify differentially expressed genes (DEGs). For all datasets, DEGs were defined as genes with a Benjamin–Hochberg corrected p-value (q-value) < 0.05. For selection of the top 20 or top 200 DEGs in any dataset, the criterion for inclusion was q < 0.05, and genes were sorted by absolute log2 fold change. Gene set enrichment analysis (GSEA) was performed on log2 fold change pre-ranked values using the fgsea (version 1.12.0) package in R [34]. Pathways used as input were obtained from the Molecular Signature Database (MSigDB) [35–37]. Hallmark pathways [37] provided an overview
when comparing animal and human datasets. For the analysis of a selected set of pathways, the Reactome pathways were applied [38]. When identifying the top 50 enriched Reactome pathways, the top 25 downregulated and the top 25 downregulated pathways were selected on the basis of their absolute normalised enrichment score and \( q < 0.1 \). The full analysis of Hallmark gene sets and leading edge genes defined as the genes accounting for most of the enrichment signal are shown in Supplementary Table S3, Additional File S1 [36]. Principal-component analysis was performed on normalised transformed values that had been obtained using Deseq2 [20] (GP, HNASH1) or LIMMA [33] (DIAMOND, WD1, WD2) packages. All genes were included unless otherwise stated. For correlation analysis, Pearson’s correlation was used and \( p \) values < 0.05 were considered statistically significant.

3. Results

3.1. Guinea Pig NASH Development and Disease Stage

High-fat-fed guinea pigs developed NASH with advanced bridging fibrosis (F3) as previously reported (Figure 1) [6]. Steatosis (\( p < 0.01 \)), inflammation (\( p < 0.01 \)), hepatocyte ballooning (\( p < 0.05 \)), and fibrosis (\( p < 0.01 \)) were evident in all NASH animals compared to healthy controls (Table 2) as previously reported [6]. In accordance with advanced fibrosis (F3) stage, the relative fibrosis area was significantly increased in NASH animals compared to in the controls (\( p < 0.001 \)).

![Representative pictures of the distribution of hepatic fibrotic tissue in control and NASH (stage F3) guinea pigs in Picro Sirius red stained sections.](image)

**Figure 1.** Representative pictures of the distribution of hepatic fibrotic tissue in control and NASH (stage F3) guinea pigs in Picro Sirius red stained sections. (A) In control animals, fibrous tissue (red) surrounds the central veins and portal areas, but does not expand into the hepatic parenchyma. An isolated small area of fibroplasia (arrowhead) can be seen as a normal occasional finding. (B) Bridging fibrosis (arrows) (F3 grade) is clearly evident in animals with NASH after 25 weeks on a high-fat diet.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control (n = 6)</th>
<th>NASH (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steatosis (^1)</td>
<td>0</td>
<td>3 **</td>
</tr>
<tr>
<td>Ballooning (^1)</td>
<td>0</td>
<td>2 (1–2) *</td>
</tr>
<tr>
<td>Inflammation (^1)</td>
<td>0 (0–1)</td>
<td>3 **</td>
</tr>
<tr>
<td>Fibrosis (^1)</td>
<td>0</td>
<td>3 **</td>
</tr>
<tr>
<td>Relative fibrosis area (^2)</td>
<td>1.39 ± 0.24</td>
<td>7.48 ± 1.81 ***</td>
</tr>
</tbody>
</table>

Steatosis, Ballooning, inflammation, and fibrosis: medians with range (if applicable). Relative fibrosis area: means with standard deviations. Histopathological scoring data (steatosis, ballooning, inflammation, and fibrosis) is previously published [6]. \(^1\) Analysed using Mann–Whitney U test. \(^2\) Analysed by unpaired t-test with Welch’s correction. * \( p < 0.05 \), ** \( p < 0.01 \) and *** \( p < 0.001 \).

3.2. The Hepatic Transcriptome Clearly Distinguishes Guinea Pigs with NASH from Controls

A total of 6683 DEGs were identified between guinea pigs with NASH and controls (\( q < 0.05 \)). Principal-component analysis (Figure 2A) showed a clear separation of the two groups, corroborating distinct transcriptomic differences between NASH and control animals. The top 20 DEGs include genes involved in immune cell signalling (ADAMDEC1, CCL7, TNFSF18), cell-to-cell contact (SPTA1, PAK6, DSG4), cholesterol metabolism (STAR),
All pathways were selected on the basis of corrected Benjamin–Hochberg p-values and normalised enrichment score PC1 explaining 64.03% of the total variance. (b) Top 50 reaction pathways obtained from CSEA in guinea pigs with NASH vs. healthy animals clearly separated on the basis of all genes by normalised enrichment score. Normalised enrichment score (NES) component steatohepatitis, NES: normalised non-alcoholic NASH: component, enrichment NASH

Figure 2. Principal-component analysis and top 50 dysregulated pathways in guinea pigs with NASH (A) Principal-component normalised enrichment score of guinea pig samples.

NASH

Control

Group

Top 50 Reaction Pathways in Guinea Pig NASH

PC1 (63.94%)

PC2 (4.03%)
Table 3. Top 20 differentially expressed genes in guinea pigs.

<table>
<thead>
<tr>
<th>Gene Name (Full Name)</th>
<th>Log2 Fold Change</th>
<th>Adjusted ( p )-Value</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAMDEC1 (ADAM like decysin 1)</td>
<td>8.01</td>
<td>(4.94 \times 10^{-60})</td>
<td>Secreted protein involved in dendritic cell maturation.</td>
</tr>
<tr>
<td>ADGRG3 (adhesion G protein-coupled receptor 3)</td>
<td>7.66</td>
<td>(3.79 \times 10^{-40})</td>
<td>GPCR possibly involved in tumor angiogenesis.</td>
</tr>
<tr>
<td>KRT23 (keratin 23)</td>
<td>10.97</td>
<td>(1.53 \times 10^{-35})</td>
<td>Member of keratin family of intermediate filaments responsible for structural integrity of epithelial cells.</td>
</tr>
<tr>
<td>TMEM213 (transmembrane protein 213)</td>
<td>10.12</td>
<td>(1.28 \times 10^{-29})</td>
<td>Vacuolar ATPase mediating acidification of intracellular compartments necessary for protein sorting, zymogen activation, receptor-mediated endocytosis and synaptic vesicle protein gradient generation.</td>
</tr>
<tr>
<td>CIB4 (calcium and integrin binding family member 4)</td>
<td>9.70</td>
<td>(1.82 \times 10^{-26})</td>
<td>No listed function.</td>
</tr>
<tr>
<td>PAK6 (p21 (RAC1) activated kinase 6)</td>
<td>9.38</td>
<td>(5.69 \times 10^{-26})</td>
<td>p21 stimulated serine/threonine kinase involved in cytoskeleton rearrangement, apoptosis and MAP kinase signalling pathway.</td>
</tr>
<tr>
<td>TMCI (transmembrane channel like 1)</td>
<td>9.27</td>
<td>(1.82 \times 10^{-25})</td>
<td>No listed function.</td>
</tr>
<tr>
<td>CCL7 (C–C motif chemokine ligand 7)</td>
<td>9.09</td>
<td>(2.30 \times 10^{-25})</td>
<td>Encodes MCP3-a secreted chemokine recruiting macrophages during inflammation, and also a substrate of MMP2.</td>
</tr>
<tr>
<td>PTPRN (protein tyrosine phosphatase receptor type N)</td>
<td>9.23</td>
<td>(2.58 \times 10^{-24})</td>
<td>Signalling molecule regulating processes such as cell growth, differentiation, mitotic cycle, and oncogenic transformation.</td>
</tr>
<tr>
<td>VSIG1 (V-set and immunoglobulin domain containing 1)</td>
<td>8.26</td>
<td>(1.06 \times 10^{-22})</td>
<td>Encodes a member of the junctional adhesion molecule (JAM) family.</td>
</tr>
<tr>
<td>SLC34A2 (solute carrier family 34 member 2)</td>
<td>8.49</td>
<td>(1.13 \times 10^{-20})</td>
<td>pH-sensitive sodium-dependent phosphate transporter.</td>
</tr>
<tr>
<td>DSG4 (desmoglein 4)</td>
<td>8.15</td>
<td>(2.78 \times 10^{-19})</td>
<td>Desmosomal cadherin possibly playing a role in cell-cell adhesion in epithelial cells.</td>
</tr>
<tr>
<td>TNFSF18 (TNF super family member 18)</td>
<td>7.74</td>
<td>(1.92 \times 10^{-17})</td>
<td>Cytokine belonging to the TNF ligand family that plays a role in T-lymphocyte survival and the interaction between endothelial cells and T lymphocytes.</td>
</tr>
<tr>
<td>MTHFD2 (methylene tetrahydrofolate dehydrogenase (NADP + dependent) 2, methenyltetrahydrofolate cyclohydrolase)</td>
<td>7.63</td>
<td>(6.53 \times 10^{-17})</td>
<td>Nuclear encoded mitochondrial bifunctional enzyme with methylene tetrahydrofolate dehydrogenase and methenyltetrahydrofolate cyclohydrolase activities.</td>
</tr>
<tr>
<td>SPOCK1 (SPARC (osteonecic), ccvc and kazal-like domains proteoglycan 1)</td>
<td>7.72</td>
<td>(8.38 \times 10^{-17})</td>
<td>Seminal plasma proteoglycan containing chondroitin and heperan sulfate chains.</td>
</tr>
<tr>
<td>ECT2 (epithelial cell transforming 2)</td>
<td>8.43</td>
<td>(5.94 \times 10^{-16})</td>
<td>Guanine nucleotide exchange factor, expressed at high levels in mitotic cells in the regenerating liver.</td>
</tr>
</tbody>
</table>
Table 3. Cont.

<table>
<thead>
<tr>
<th>Gene Name (Full Name)</th>
<th>Log2 Fold Change</th>
<th>Adjusted p-Value</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPTA1 (spectrin alpha, erythrocytic 1)</td>
<td>7.99</td>
<td>$7.35 \times 10^{-15}$</td>
<td>Molecular scaffold protein that links the plasma membrane to the actin cytoskeleton and determines the cell shape.</td>
</tr>
<tr>
<td>STAR (steroidogenic acute regulatory protein)</td>
<td>8.36</td>
<td>$2.48 \times 10^{-14}$</td>
<td>Involved in the acute regulation of steroid hormone synthesis by enhancing the conversion of cholesterol into pregnolone.</td>
</tr>
<tr>
<td>KEL (Kell metalloendopeptidase (Kell blood group))</td>
<td>7.66</td>
<td>$1.34 \times 10^{-12}$</td>
<td>Encodes a type II transmembrane glycoprotein of the Kell blood group antigen.</td>
</tr>
</tbody>
</table>


3.3. The Guinea Pig NASH Transcriptome Is Similar to That of Humans with Early-Stage NASH

The translational value of the guinea pig DEGs was assessed by comparing these to the 100 human genes most strongly associated with NASH, as annotated in the DISEASE database [22,23]. This comparison revealed that 60% of the human genes were differentially expressed in guinea pigs, whereas 40% were not, and is visualised as a protein–protein association network in Figure 3. To further substantiate the translational findings, RNA sequencing results were also compared to a human dataset with early-stage NASH patients and healthy controls (HNASH1, GSE126848) [28]. Similar to the number of DEGs identified in guinea pigs, a total of 5964 DEGs ($q < 0.05$) were identified in the human dataset, and of these, 2697 (45.2%) genes were identical to the DEGs in the guinea pigs (Figure 4A). Moreover, the top 200 DEGs from the guinea pig dataset were sufficient to distinguish patients with early-stage NASH from healthy controls by using principal-component analysis (Figure 4B).

To benchmark these results in relation to other preclinical models of NAFLD/NASH (WD1, WD2, DIAMOND), the similarity of the animal models to the human dataset (GSE126848) was compared by investigating the comparability of enriched pathways. Figure 4C shows a heatmap based on normalised enrichment scores of all 50 hallmark pathways in the different animal and human datasets (GSE126848). The three murine models group together more closely compared to the guinea pig, and all four rodent models are more similar to each other than to the human dataset. Importantly, the GSE126848 dataset is derived from patients with early NASH as opposed to the more severe NASH in the guinea pigs, which may reduce comparability between groups. In contrast to the animal models, 14 pathways were uniquely downregulated in the human dataset. These 14 pathways include inflammatory signalling (e.g., inflammatory response ($q < 0.01$), complement ($q < 0.05$), and IL2 STAT5 signalling ($q < 0.1$)). Leading edge genes specifically revealed CD44 and CCL20 (C–C motif chemokine ligand 20) to be differentially regulated in human and guinea pig datasets (Supplementary Table S3, Additional File S1). The downregulation of adipogenesis ($q < 0.05$) and fatty acid metabolism ($q < 0.01$) was only found in guinea pigs.

3.4. Guinea Pig NASH Transcriptome Resembles Human Advanced NASH

The translatable value of the guinea pig transcriptome was also assessed in relation to patients with advanced disease. In contrast to the HNASH1 dataset, where most patients had ≤1 grade fibrosis (1 of 16 patients had grade 2 fibrosis), and all patients had ≤1 grade inflammation, the HNASH2 dataset compares NAFLD patients with either mild (grade 0–1) or severe (grade 3–4) fibrosis [28,29]. The top 200 DEGs from these patients distinguished guinea pigs and WD1 with NAFLD/NASH from the controls (Figure 5A,C). DIAMOND and WD2 animals could also be separated by these DEGs (Figure 5B,D), but their fraction...
of variance explained by first principal component PC1 was lower than that of guinea pigs and WD1.

Figure 3. Protein–protein association network showing top 100 genes most strongly associated with human NASH. Yellow nodes indicate 40 proteins that are not differentially expressed in guinea pig NASH. Remaining network nodes are coloured by log2 fold changes of differentially expressed genes in guinea pigs using the default Cytoscape colour gradient blue–white–red of log2 fold change from 3 to 3.

In the GSEA, murine (WD1, WD2 and DIAMOND) models clustered, while guinea pigs and the HNASH2 dataset formed a separate cluster (Figure 5E). To explore this relationship, the top 50 enriched Reactome pathways in the HNASH2 dataset were compared to the top 50 enriched Reactome pathways in each of the preclinical models. Guinea pig and HNASH2 datasets share 17/50 (34%) enriched pathways, whereas the WD2, WD1, DIAMOND mice and HNASH2 datasets share 17/50 (34%), 7/50 (14%), and 9/50 (18%), respectively (see Supplementary Table S2, Additional File S1 for a list of matching pathways). The 17 pathways shared between guinea pigs and the patients with advanced fibrosis were all regulated in the same direction with 12 downregulated and 5 upregulated pathways (Figure 5F). Consistent with advanced fibrosis in both guinea pigs and patients, the upregulated pathways (extracellular metabolism, ECM proteoglycans, elastic-fibre formation, molecules associated with elastic fibres) were linked to fibrosis. Downregulated pathways included mitochondrial processes such as the citric acid cycle and respiratory electron transport, peroxisomal protein import, mitochondrial translation, and fatty acid metabolism. Further evaluation of fatty acid metabolism leading edge genes revealed thyroid hormone
responsive (THRSP) to be differentially regulated in HNASH2 and guinea pig datasets, whereas it was upregulated in HNASH1 (Supplementary Table S4, Additional File S1).

**Figure 4.** Comparison of preclinical models and patients with early-stage NASH. (A) Venn diagram of differentially expressed genes from HNASH1 (patients with mild disease vs. healthy controls) and guinea pig datasets, and their overlapping genes. Both datasets were analysed by DESeq2, and differentially expressed genes were selected on the basis of q < 0.05. (B) Principal-component analysis using top 200 differentially expressed genes in guinea pigs clearly separated patients with NASH from healthy controls in the HNASH1 dataset. Top 200 differentially expressed genes were selected on the basis of q < 0.05 and highest absolute log2 fold-change values. (C) Heatmap demonstrating overlap in expression patterns of Hallmark pathways. The heatmap is based on normalised enrichment scores from gene set enrichment analysis of Hallmark pathways from each dataset, i.e., HNASH1, guinea pig, and the included murine datasets (WD1, WD2, and DIAMOND). Dendrogram depicts hierarchical clustering of groups according to normalised enrichment scores. Colour bar indicates normalised enrichment scores, blue indicates a downregulated gene set, and red indicates an upregulated gene set. WD1 refers to GSE52748, WD2 refers to GSE38141, HNASH1 refers to GSE126848, DIAMOND refers to GSE67680. GP: guinea pig, DEG: differentially expressed gene, PC: principal component, NASH: nonalcoholic steatohepatitis.
Figure 5. Comparison of preclinical models and patients with advanced NASH. (A–D) Principal-component analysis using top 200 differentially expressed genes from HNAS2 (NASH patients with advanced vs. mild fibrosis) could separate NASH/NAFLD from healthy controls in all included preclinical models. Differentially expressed genes from HNAS2 dataset selected on the basis of \( p < 0.05 \) and highest absolute \( \log_2 \) fold change. Principal-component analysis plots depict normalised and transformed values for the 200 genes in each of the animal datasets. (E) Heatmap demonstrating overlap in expression patterns of Hallmark pathways. Heatmap is based on normalised enrichment scores from the gene set enrichment analysis of Hallmark pathways from each dataset. Dendrogram depicts hierarchical clustering of groups according to normalised enrichment scores. Colour bar indicates normalised enrichment scores for each gene set. Blue indicates a downregulated gene set, whereas red indicates an upregulated gene set. (F) Overview of normalised enrichment scores of the 17 pathways in common between HNAS2 and guinea pigs. Top 50 pathways of the HNAS2 dataset were compared with the top 50 enriched pathways in guinea pigs. Top 50 pathways included the top 25 most upregulated and the top 25 most downregulated pathways. All pathways were selected on the basis of corrected Benjamini–Hochberg \( p \)-values and normalised enrichment scores. WD1 refers to GSE52748, WD2 refers to GSE38141, DIAMOND refers to GSE67680, HNAS2 refers to GSE49451. GP: guinea pig, PC: principal component, NAFLD: nonalcoholic fatty liver disease, NASH: nonalcoholic steatohepatitis.
3.5. Identification of Potential New Biomarkers of Fibrosis Deposition

To identify genes directly related to the amount of fibrosis in our NASH guinea pigs, the relative fibrosis amount was correlated to the expression of the 11,896 identified genes. Only genes with a correlation coefficient of 0.8 and a log2 fold change of 1 were included. Each gene was compared to the two human datasets HNASH1 and HNASH2, which consisted of NASH patients with mild disease and NASH patients with advanced fibrosis (grade 3–4), respectively. The final list comprises nine genes: ACKR3, BIRC3, CHST11, EMP3, FZD7, RGS14, RHBDLF1, SERPINB9, and VWF (Table 4; for regression analysis, see Additional File S2).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Pearson’s ρ</th>
<th>p Value</th>
<th>GPLog2FC</th>
<th>HLog2FC</th>
<th>Function 1</th>
<th>Secreted</th>
<th>Role in NASH</th>
<th>Cell-Specific Expression 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKR3</td>
<td>All: 0.91</td>
<td>NASH: 0.87</td>
<td>All: 4.54 × 10^-5</td>
<td>1.36</td>
<td>HNASH1: ND HNASH2: 0.69</td>
<td>GPCR, orphan receptor</td>
<td>NO</td>
<td>?</td>
</tr>
<tr>
<td>BIRC3</td>
<td>All: 0.88</td>
<td>NASH: 0.82</td>
<td>All: 1.77 × 10^-4</td>
<td>1.1</td>
<td>HNASH1: 1.9 HNASH2: 0.7</td>
<td>Inhibits apoptosis</td>
<td>NO</td>
<td>YES (hypoxia induced) [39]</td>
</tr>
<tr>
<td>CHST11</td>
<td>All: 0.95</td>
<td>NASH: 0.87</td>
<td>All: 1.42 × 10^-6</td>
<td>1.16</td>
<td>HNASH1: 0.35 HNASH2: 0.19</td>
<td>Promotes synthesis of chordin-like protein (Chordin-like) (ECM)</td>
<td>NO</td>
<td>?</td>
</tr>
<tr>
<td>EMP3</td>
<td>All: 0.93</td>
<td>NASH: 0.81</td>
<td>All: 9.41 × 10^-6</td>
<td>1.5</td>
<td>HNASH1: 0.98 HNASH2: 0.19</td>
<td>Membrane protein, cell proliferation</td>
<td>NO</td>
<td>?</td>
</tr>
<tr>
<td>FZD7</td>
<td>All: 0.87</td>
<td>NASH: 0.83</td>
<td>All: 2.22 × 10^-4</td>
<td>1.2</td>
<td>HNASH1: 0.98 HNASH2: 0.19</td>
<td>Wnt signalling Regulates GPCR (increases microtubule assembly)</td>
<td>NO</td>
<td>YES in HCC [40]</td>
</tr>
<tr>
<td>RGS14</td>
<td>All: 0.63</td>
<td>NASH: 0.80</td>
<td>All: 8.26 × 10^-4</td>
<td>1.58</td>
<td>HNASH1: 0.3 HNASH2: 0.2</td>
<td>Regulates ADAM17 and release of TNF-α</td>
<td>NO</td>
<td>?</td>
</tr>
<tr>
<td>RHBDLF1</td>
<td>All: 0.96</td>
<td>NASH: 0.92</td>
<td>All: 4.11 × 10^-7</td>
<td>1.17</td>
<td>HNASH1: 0.6 HNASH2: 0.03</td>
<td></td>
<td>NO</td>
<td>?</td>
</tr>
<tr>
<td>SERPINB9</td>
<td>All: 0.9</td>
<td>NASH: 0.84</td>
<td>All: 6.19 × 10^-5</td>
<td>1.4</td>
<td>HNASH1: 0.7 HNASH2: 0.5</td>
<td>Inhibits activity of granzyme B</td>
<td>YES</td>
<td>YES [41]</td>
</tr>
<tr>
<td>VWF</td>
<td>All: 0.97</td>
<td>NASH: 0.86</td>
<td>All: 7.52 × 10^-8</td>
<td>1.46</td>
<td>HNASH1: 0.03 HNASH2: 0.5</td>
<td>Platelet aggregation</td>
<td>YES</td>
<td>YES [42,43]</td>
</tr>
</tbody>
</table>

Table 4. Genes related to fibrosis quantification.

Genes listed in alphabetical order. In Pearson’s ρ column: All, correlation calculated using both control and NASH animals; NASH, correlation calculated using only NASH animals. 1 Description of function based on [44]. 2 Based on liver cell atlas [45,46]. ECM: extracellular matrix, GPCR: G-protein coupled receptor, GPLog2FC: guinea pig log2 fold change, HCC: hepatocellular carcinoma, HLog2FC: human log2 fold change, HSC: hepatic stellate cell, HNASH1: NASH patients with mild fibrosis vs. healthy controls. HNASH2: NASH patients with advanced fibrosis vs. NASH patients with no or mild fibrosis.

4. Discussion

This paper shows the first comparison of human and guinea pig NASH transcriptomes, and reveals the high translational potential of this model compared to the included murine models. DEGs clearly separated guinea pigs with NASH from healthy controls, and GSEA revealed an over-representation of fibrosis-related signalling, while energy-generating processes were downregulated. Importantly, guinea pigs with NASH and advanced fibrosis (F3–F4) recapitulate the transcriptional profile of NASH patients with advanced (F3–F4) fibrosis, emphasising that the guinea pig NASH model possesses high translational potential, which can be used in drug and biomarker discovery.
A comparison of the guinea pig transcriptome to the human dataset (GSE126848 (HNASH1)) demonstrated that the top 200 DEGs in NASH guinea pigs were able to separate patients with NASH from healthy individuals [28]. This similarity was further substantiated by the 60% overlap in guinea pig NASH DEGs and human genes from the DISEASE database [25], and a separate grouping of guinea pigs and patients from the HNASH2 dataset in the GSEA compared to the murine models. However, some pathways displayed altered regulation in guinea pigs compared to the HNASH2 dataset, including heme metabolism, cholesterol homeostasis, and the reactive-oxygen-species pathway. In this aspect, differences in pathways associated with cholesterol homeostasis are not surprising, as the guinea pig HF diet contains an excess (0.35%) of cholesterol. Differences in the content and composition of dietary fatty acids may also induce alterations in hepatic metabolism and associated pathways that may influence end points and limit comparisons between studies, underlining controlled dietary regimes as a central point of attention when modelling this disease [47]. Similar to humans with advanced NASH (HNASH2), adipogenesis and fatty acid metabolism gene sets were downregulated in the guinea pig, in contrast to humans with mild disease (HNASH1) and the murine datasets (WD1, WD2 and DIAMOND). Fatty acid metabolism was also among the 17 shared pathways between HNASH2 and guinea pigs, supporting effects on hepatic lipid turnover as a factor in disease development. To delineate specific genes for advanced disease, leading edge genes for each dataset were reviewed (Supplementary Table S4, Additional File S1). THRSP was highly downregulated in the guinea pig and HNASH2 datasets compared to HNASH1, indicating a specific role for THRSP in advanced disease. Decreased THRSP serum levels is reported in patients with metabolic syndrome (increased BMI, HbA1c, triglycerides, alanine-transaminase, and lower HDL-C) compared to healthy individuals, supporting a differential regulation of THRSP when (lipid) metabolism is altered; however, hepatic histopathological status was not recorded, preventing cross-referencing to NASH [48]. Thus, increased expression in the HNASH1 dataset and decreased expression in HNASH2 and guinea pigs, and lower serum THRSP levels in patients with metabolic syndrome could indicate that THRSP varies with disease state and stimuli, and might be upregulated in mild disease, but downregulated in advanced disease. Accordingly, THRSP could be a marker of advanced NASH and would be interesting to assess as a serum marker in patients with advanced NASH. Genes involved in, e.g., steatosis-promoting pathways may be overlooked in the HNASH2 dataset, as both patient groups displayed similar degrees of hepatic steatosis. Furthermore, both patients groups were obese (BMI > 30), whereas the guinea pigs analysed in this study did not differ in body weight, also differing from the murine models, in which all Western diet-fed groups had significantly higher body weight compared to that of the controls. Regarding data analysis, only this study considered human orthologues for gene annotation of the animal model, which might also account for some of the similarity between guinea pig and human datasets.

Mitochondrial -oxidation is central for hepatic lipid metabolism, and mitochondrial dysfunction is considered to be a symptom of advanced NASH [49,50]. Accordingly, complex 1 biogenesis and respiratory electron transport were downregulated in humans (HNASH2), guinea pigs and DIAMOND mice that also have advanced disease, indicating an overlap in late-stage pathogenesis between these models and humans [29,32]. The protein–protein association network demonstrated either the down- or no regulation of key genes involved in mitochondrial -oxidation, including CPTIA, PPARA, and ACOX1 in guinea pigs with NASH. Compared to HNASH1 patients, the advanced NASH guinea pig showed downregulation of other mitochondrial processes, i.e., peroxisome and oxidative phosphorylation. Increased mitochondrial activity was reported in patients with mild disease, similar to in the HNASH1 dataset [51]. This could be a compensatory mechanism to mitigate hepatic lipid overload by increasing fatty acid oxidation, which, over time, results in increased levels of oxidative stress and ultimately reduces mitochondrial oxidative capacity, as reported in patients with advanced disease [49]. Thus, the different stages of
disease (mild vs. advanced) are likely to account for differences between HNASH1 and guinea pig expression patterns.

Within the group of inflammatory response genes HNASH2, guinea pig and murine models showed upregulated expression patterns. Closer inspection of the genes in leading edge analysis (Supplementary Table S3, Additional File S1 (highlighted in yellow)) revealed that CCL20 and CD44 were upregulated in HNASH2 and guinea pigs, but not in HNASH1. CCL20 is a strong chemoattractant for lymphocytes and the main ligand of the chemokine receptor CCR6, and is expressed by cholangiocytes, Kupffer cells, hepatocytes, and hepatic stellate cells [52,53]. Differential expression analysis in healthy individuals and NASH patients with lobular inflammation showed CCL20 to be among the top 20 genes with the highest fold-change levels [54]. Furthermore, increased serum levels of CCL20 were found in NASH patients with severe fibrosis compared to those of healthy individuals [52]. In addition, the serum levels of soluble CD44 were increased in patients with NASH (n = 39) vs. non-NASH (n = 25) [55]. CD44 plays a major role in hepatic leukocyte recruitment and infiltration [56]. CD44 null mice showed markedly decreased hepatic macrophage and neutrophil infiltration compared to wild types in response to a methionine–choline-deficient diet, and were partially protected from inflammation compared to wild types in response to a lithogenic diet [55,57]. Thus, the increased expression of CCL20 and CD44 appears to be linked to inflammation in NASH. As both factors can be readily measured in serum, these proteins may be interesting as biomarker candidates.

With regards to hepatic fibrosis, 4/17 of the overlapping pathways between guinea pigs and advanced NASH patients (HNASH2) are exclusively related to fibrosis. None of the pathways overlapping between DIAMOND and advanced NASH patients are involved in fibrotic processes, whereas WD1 and WD2 showed 3/7 and 12/17 of overlapping pathways, respectively. In the principal-component analysis, guinea pigs and WD1 were clearly separated by the DEGs from patients with advanced vs. mild disease (HNASH2), whereas the WD2 and DIAMOND datasets did not separate as clearly. This analysis could well be confounded by factors within the individual experiments; however, these results may collectively indicate that the fibrotic signalling network in the DIAMOND model is different from the human network, or less regulated than that in guinea pigs or WD mice. Extracellular-matrix organisation is also upregulated in DIAMOND mice, though not included in the 50, as is the case for the human and guinea pig dataset. With regards to oxidative capacity and fibrosis signalling, the guinea pig model seems to mirror the human NASH transcriptome to a higher degree than the other included preclinical models. The two human datasets include either patients with mild disease (HNASH1) or more severe NASH (HNASH2), but no healthy controls. Thus, to confirm if these findings are consistent in more progressive NASH with increased fibrosis, a comparison is warranted between the guinea pig transcriptome and an advanced NASH patient group compared to a matched healthy control. In line with the ability to display several of the human histopathological hallmarks of NASH (including fibrosis), the current findings demonstrate a clear advantage of the guinea pig model. A relatively novel preclinical model of this disease, the currently disclosed transcriptome supports a high degree of translational validity, putatively enforcing increased predictability of findings between guinea pig NASH and human patients. In this aspect, potential challenges with applying the guinea pig model (e.g., different species preferences and requirements compared to mice and rats) are outweighed. We recently reported an impact of breeder-associated variation on guinea pig NASH development [58]. Consequently, there could be differences in the NASH transcriptome between animals from different breeders, rendering the presented findings limited to guinea pigs bred at Charles River (Lyon, France).

The above findings show high similarity between guinea pig and human fibrotic gene expression, prompting further investigation of specific targets, with high clinical potential. This yielded a list of nine fibrosis-related genes, of which two secreted factors, von Willebrand factor (VWF) and serpin family B member 9 (SERPINB9), showed high correlation with the relative fibrosis area. VWF is secreted from endothelial cells, and circulating levels
of vWF predicted mortality and risk of decompensation in patients with cirrhosis [59–61]. Furthermore, vWF increases with fibrosis stage in hepatitis C and NASH patients, supporting this as a potential marker of advancing hepatic fibrosis [42,62]. Increased SERPINB9 expression was also reported in patients with hepatocellular carcinoma [63]. SERPINB9 could be a circulating biomarker for cytomegalovirus infection, and immunostainings confirmed the hepatocyte expression of SERPINB9 in cirrhotic hepatitis C patients [64,65]. Several of the other identified genes encode proteins that indirectly regulate the release of soluble factors to the bloodstream. This includes rhomboid 5 homolog 1 (RHBDF1), which has the highest overall correlation coefficient and regulates the activity of ADAM metallopeptidase domain 17 (ADAM17), which in turn regulates the release of tumour necrosis factor-α (TNF-α) [66]. Thus, RHBDF1 indirectly mediates the detachment of surface molecules, including TNF-α, known to contribute NASH progression, which was also among the 60 genes in common between guinea pigs and NASH patients identified from the DISEASE database [66–68]. Only four of the nine genes correlating with relative fibrosis area have been investigated, to the best of our knowledge, in relation to NASH. Consequently, the remaining five genes sharing a high correlation to hepatic fibrosis area and a similar expression pattern in patients with advanced NASH may serve as putative biomarkers worthy of future investigation.

5. Conclusions

This study showed significant overlap between the transcriptomes of the guinea pig NASH model and NASH patients with advanced fibrosis on a pathway and single-gene level. In addition to similarities in liver histopathology, this further establishes the guinea pig as a model of fibrotic NASH with high translational validity. Moreover, several genes correlating with the amount of hepatic fibrosis in guinea pigs displayed a similar expression pattern in NASH patients, supporting the clinical potential of using the guinea pig as a model in the search for biomarkers of NASH and NASH-associated fibrosis.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.390/ biomedicines9091198/s1. Additional File S1: Word document docx. Supplementary Table S1. Table of relative fibrosis area based on Pico Sirius red staining for each animal. Supplementary Table S2. List of top 50 overlapping pathways between the preclinical murine models and HNAS2 dataset. Supplementary Table S3. List of all Hallmark gene sets for the guinea pig dataset, including leading edge genes. Supplementary Table S4. List of Reactome pathway gene set: fatty acid metabolism for HNAS1, HNAS2, and guinea pig. Additional File S2: PDF of linear regression on the normalised (normalised by DeSeq2 size factor) and rlog-transformed values, and the fibrosis fraction for each animal, for each of the nine genes identified in correlation analysis. R² and the linear equation are reported for each gene.


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Institutional Review Board Statement: 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.

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The effect of acetylsalicylic acid and pentoxifylline in guinea pigs with non-alcoholic steatohepatitis

David Højland Ipsen | Josephine Skat-Rørdam | Marianne Svenningsen | Mia Andersen | Markus Latta | Lene Elisabeth Buelund | Kristine Lintrup | René Skaarup | Jens Lykkesfeldt | Pernille Tveden-Nyborg

Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg C, Denmark
Liver Disease Research, Global Research, Novo Nordisk A/S, Måløv, Denmark
Section of Veterinary Imaging, Department of Veterinary Clinical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg C, Denmark

Correspondence
Pernille Tveden-Nyborg, Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg C, Denmark. Email: ptv@sund.ku.dk

Funding information
LifePharm Centre of In Vivo Pharmacology at University of Copenhagen

Abstract
Therapeutic options are urgently needed for non-alcoholic fatty liver disease (NAFLD), but development is time-consuming and costly. In contrast, drug repurposing offers the advantages of re-applying compounds that are already approved, thereby reducing cost. Acetylsalicylic acid (ASA) and pentoxifylline (PTX) have shown promise for treatment of NAFLD, but have not yet been tested in combination. Guinea pigs were fed a high-fat diet for 16 weeks and then continued on the diet while being treated with ASA, PTX or ASA+PTX for 8 weeks. Chow-fed animals served as healthy controls. Guinea pigs were CT scanned before intervention start and at intervention end. Animals without steatosis (ie NAFLD) at week 16 were excluded from the data analysis. ASA and PTX alone or in combination did not improve hepatic steatosis, ballooning, inflammation or fibrosis nor did the treatments affect liver enzymes (aminotransferases and alkaline phosphatase) or circulating lipids. Liver triglyceride levels, relative liver weight and hepatic mRNA expression of monocyte chemoattractant protein 1, interleukin 8 and platelet-derived growth factor b were nominally decreased. Thus, in the current study, treatment with ASA and PTX alone or in combination for 8 weeks did not ameliorate NASH or hepatic fibrosis in guinea pigs.

KEYWORDS
acetylsalicylic acid, guinea pig, non-alcoholic steatohepatitis, pentoxifylline, pharmacotherapy

INTRODUCTION

Non-alcoholic fatty liver disease affects approximately 25% of the world’s population and can progress to non-alcoholic steatohepatitis (NASH). The disease imposes a substantial economic burden with estimated annual healthcare costs exceeding 100 billion dollars in the USA alone. Current treatment options are scarce, but the multifactorial pathogenesis in NASH provides a clear basis for successful combination therapy. However, due to the substantial requirements in both time and costs associated with developing novel therapeutics, monotherapy will likely be expensive once drugs are approved, and combination therapy may not be possible for all. Consequently, drug repurposing might be a feasible option for NASH patients. This approach offers the advantage that drug candidates have already undergone the expensive and time-consuming evaluation related to pharmacokinetics, dynamics and toxicology. In this regard, acetylsalicylic acid (ASA) and pentoxifylline (PTX) are relevant to investigate
in NAFLD: Two meta-analyses have suggested that PTX can improve hepatic inflammation and disease activity in NAFLD, but findings regarding hepatic steatosis and fibrosis were less clear.\textsuperscript{4,5} PTX may decrease TNF\textsubscript{α}, production of reactive oxygen species and lipid peroxidation, all of which are mechanisms involved in NAFLD and, therefore, could prove to be beneficial in a NAFLD treatment strategy.\textsuperscript{6} ASA is a commonly used non-steroidal anti-inflammatory drug and could mediate its effect against NAFLD by targeting the inflammatory axis of the disease. In preclinical models, preventive treatment with aspirin (ASA) decreased NAFLD and fibrosis in mice fed a choline-deficient amino acid-defined diet and decreased CCl\textsubscript{4}-induced liver fibrosis in rats.\textsuperscript{7,8} Furthermore, anti-platelet drugs—ASA and/or P2Y12 receptor antagonists—were associated with decreased risk of liver fibrosis in a meta-analysis,\textsuperscript{9} and a cross-sectional analysis of NHANES III associated regular (>15 times/month) use of ASA with lower incidence of ultrasonography diagnosed NAFLD.\textsuperscript{10} ASA use was also associated with lower incidence of liver fibrosis in patients with chronic liver disease from the NHANES III.\textsuperscript{11} In addition, ASA is already used to manage cardiovascular disease and as cardiovascular mortality is one of the primary causes of deaths in NAFLD patients,\textsuperscript{12} the use of ASA in patients with NAFLD could potentially target two clinically relevant end-points. Thus, this study suggests that these two well-characterized compounds may have potential additive effects on NAFLD. Utilizing the well-established guinea pig NASH model, which develops steatosis, hepatocellular ballooning, inflammation and advanced fibrosis as well as gene expression patterns similar to patients, this study investigates the effect of ASA and PTX, alone and in combination.\textsuperscript{13–16}

2 | MATERIALS AND METHODS

2.1 | Animals

All animal experimentation was approved by the Animal Experimentation Inspectorate under the Danish Ministry of Environment and Food (permit number 2018-15-0201-01591), and in accordance with the European Legislation of Animal Experimentation 2010/63/EU. The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.\textsuperscript{17}

Female Hartley guinea pigs were housed in floor pens at 20-24°C with a 12-hours light/dark cycle and access to shelters, gnawing blocks, water and food. Following 1 week of acclimatization, guinea pigs were randomly assigned to chow (n = 5) or a high-fat diet (HFD) (n = 55, 20% fat, 15% sucrose and 0.35% cholesterol). Group sizes were based on a power analysis (power of 80%, \( P < .05 \)) with variances adopted from our previous studies and an effect size of 30% considered to be biologically relevant. Dietary compositions were similar to previously described.\textsuperscript{18} As previously shown, guinea pigs develop NASH with fibrosis following 16 weeks of high-fat feeding and proceed to develop advanced hepatic fibrosis by week 25-35.\textsuperscript{13,14,18,19} Thus, after 16 weeks, six animals from the high-fat diet group (HFD pre-intervention) were randomly selected for baseline euthanasia. The remaining high-fat diet animals were block-randomized into four groups based on weight: HFD (n = 10), acetylsalicylic acid (ASA, n = 13), pentoxifylline (PTX, n = 13) and acetylsalicylic acid + pentoxifylline (ASA+PTX, n = 13). ASA and PTX (Glostrup Apotek, Glostrup, Denmark) were formulated into the diets (Ssniff Spezialdiäten GmbH, Soest, Germany) to ensure continuous drug exposure. Food intake was calculated as the difference between the amount of food given to a floor box and the amount of feed remaining the next day. After 8 weeks of intervention, all animals were euthanized.

2.2 | Selection of doses

In most RCTs, patients were treated with 1200 mg PTX per day, which corresponds to \(-13.3 \text{ mg/kg} \) as calculated by the available data from the clinical trials\textsuperscript{20} and is equivalent to 51.7 mg/d in guinea pigs (weighing 840 g). The dose of ASA was based on previous preclinical studies assessing pharmacokinetics of dietary administered ASA in rats\textsuperscript{21} and doses of ASA that decreased liver enzymes, steatosis and/or fibrosis in mice.\textsuperscript{22,23} Based on food intake data from our previous studies,\textsuperscript{14,24} a concentration of 2174 mg/kg diet for both compounds was selected, aiming for an estimated daily intake of 50 mg of both ASA and PTX. In the current study, this resulted in a mean intake of approximately 46.3 and 45.6 mg/d for ASA and PTX, respectively.

2.3 | Blood samples

At euthanasia, animals were pre-anästhetized with 1.25 mL/kg Zoletil-mix (Zoletil 50 Vet (Virbac Laboratories, Carros, France) dissolved with 10 mL 20 mg/mL Xylazin (Rompun Vet (Bayer Animal Health, Copenhagen, Denmark) and 0.75 mL 10 mg/mL butorphanol (Torbugesic Vet, ScanVet, Fredensborg, Denmark) and then diluted 1:9 in isotonic saline) and placed on 3%-5% isoflurane before an intracardiac blood sample was collected in a K3-EDTA flushed syringe following disappearance of interdigital reflexes as previously described.\textsuperscript{13,14} Samples for free fatty acids (FFA) and alkaline phosphatase (ALP) were collected in NaF- and heparin-coated microvetttes (Sarstedt, Nünembrecht), respectively. Plasma was isolated by centrifuging samples at 2000 g for 4 minutes at 4°C and stored at \(-80°C \) until analysis. Plasma concentrations of triglycerides, total cholesterol, FFA, alanine
aminotransferase (ALT), aspartate aminotransferase (AST), ALP, bilirubin and albumin were measured on a Cobas 6000 (Roche Diagnostic Systems, Berne, Switzerland) according to the manufacturer’s specifications.

2.4 | Liver biochemistry and histology

Serial liver samples, approximately 2 mm thick transversal sections spanning the entire lobe, were collected from the left lateral lobe (lobus hepatitis sinister lateralis) and fixed in 10% formalin for histology, frozen at −80°C for biochemistry or snap frozen in liquid nitrogen for qPCR and subsequently stored at −80°C. Livers were stained with haematoxylin & eosin or Picro Sirius Red with Weigert’s haematoxylin solution. Liver sections were graded in a randomized and blinded manner according to Kleiner et al.25 as previously described for the guinea pig NASH model.25 Steatosis was graded as 0 (<5%), 1 (5%-33%), 2 (>33%-66%) or 3 (>66%). Lobular inflammation was graded as 0 (<2 foci per 200× field), 1 (>2 foci per 200× field) or 2 (>4 foci per 200× field). Portal inflammation was based on the scores from six portal areas as evaluated as 0 (absent, <2 foci per 200× field) or 1 (present, ≥2 foci per 200× field). For lobular inflammation and portal inflammation, a focus was defined as ≥3 and ≥5 inflammatory cells in close proximity, respectively. Hepatocyte ballooning was graded as 0 (none), 1 (few) or 2 (many). Fibrosis was graded as 0 (none), 1 (perisinusoidal or periportal), 2 (perisinusoidal and periportal), 3 (bridging) or 4 (cirrhosis). The NAFLD activity score was calculated as the unweighted sum of inflammation and ballooning and ranges from 0 to 8.

2.5 | Liver gene expression

RNA extraction, purification and qPCR were performed as previously described.15 Briefly, liver tissue (~50 mg) from the left lateral lobe was homogenized on a FastPrep-24 (Mpbio, Solon, OH, USA) in 1000 μL Mag-Max Lysis/Binding Solution Concentrate (Thermo Fisher, Waltham, MA, USA) with 0.7% β-mercaptoethanol (Sigma-Aldrich, MO, USA) using glass beads (G4649-100G, Sigma, St, Louis, MO, USA). Subsequently, samples were centrifuged at 9500 g for 1 minute at 4°C and the supernatant frozen at −20°C for 24 hours. RNA was purified using the MagMax-96 Total RNA Isolation Kit (Thermo Fisher, Waltham, MA, USA) according to the manufacturer’s specifications and purity measured using a NanoDrop 2000 (Thermo Fisher, Waltham, MA, USA).

cDNA was synthesized from 1000 ng RNA using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, Waltham, MA, USA) on a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using the following program: 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 seconds. The cDNA was diluted to a final concentration of 6 ng/μL.

Primer sequences for qPCR are provided in Table 1 and have been published previously.15

2.6 | Computed tomography (CT) scans

To assess longitudinal changes in liver fat and to exclude animals without fatty liver disease before intervention start, liver

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### Table 1: Primers for qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product (bp)</th>
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</thead>
<tbody>
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<td>CGAATGTTCAAGGCTTGAGT</td>
<td>75</td>
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<tr>
<td>Tnf</td>
<td>NM_001173025.1</td>
<td>GGCCTCTTCTACCCCGAA</td>
<td>TGAATGCGCCCCGAATCGC</td>
<td>203</td>
</tr>
<tr>
<td>Cxcl8</td>
<td>NM_001173399.2</td>
<td>GGAGCCTCTCTGTCCTCT</td>
<td>CAGCTCCGAGCACACTTGT</td>
<td>67</td>
</tr>
<tr>
<td>Tgfb1</td>
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<td>TGCTTTATAGATATGGCTTGTTG</td>
<td>78</td>
</tr>
<tr>
<td>Pdgb</td>
<td>XM_013153075.1</td>
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<td>GGTCTAATCAGGTTCCAA</td>
<td>199</td>
</tr>
<tr>
<td>Col1a1</td>
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<tr>
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<td>CATCAGACATAGGGGCATC</td>
<td>76</td>
</tr>
</tbody>
</table>

Note: Commonly used synonyms are shown in parentheses. Previously published primer pairs are indicated as a, b, c, d, e.
densities were assessed 1 week prior to intervention start and again 1 week prior to euthanasia. Liver densities were measured using a single slice CT scanner (Siemens Somatom Emotion, Erlangen, Germany) set to 110 kV and 140 mAs. Images were reconstructed using a standard soft-tissue kernel (B41s) and a pixel size of 0.24 mm × 0.24 mm and analysed using Horos Project (version 3.3.5) by selecting six regions of interest (1.0 cm² each), three in the left lateral lobe (lobus hepatitis sinister lateralis) and three in the right lateral lobe (lobus hepatitis dexter lateralis). The liver density in Hounsfield units (HU) was calculated as a mean of these six measurements. Change in HU (ΔHU) from baseline to study end was calculated as follows: \( \Delta HU = HU_{\text{end}} - HU_{\text{baseline}} \). It was not possible to calculate \( \Delta HU \) for two animals in the ASA group as CT scan data at week 25 were unfortunately lost due to file corruption.

2.7 | Exclusion of animals without NAFLD

Steatosis grade \( (\rho = -0.6962, \quad P < .0001) \) and hepatic triglyceride content \( (r = -0.7615, \quad P < .0001) \) correlated with liver densities (Figure 1). After termination, but prior to data interpretation, CT scans were used to identify animals without significant hepatic steatosis prior to the initiation of interventions. Thus, only HFD animals with a liver density <75% (<47.5 HU) of healthy chow-fed controls were included in the statistical analysis and presented data (except correlation analysis). Consequently, group sizes following exclusion were as follows: HFD (pre-intervention) n = 5, chow n = 5, HFD n = 7, ASA n = 12, PTX n = 10, ASA+PTX n = 11.

2.8 | Statistics

All statistical analyses were done in GraphPad Prism version 8.4.0 (GraphPad Software, La Jolla, CA, USA). The healthy chow-fed controls serve only as a reference to normal values, and the effect of the intervention on disease parameters was, therefore, assessed by comparing the intervention groups (ASA, PTX and ASA+PTX) to the HFD group. Continuous data were analysed with a one-way ANOVA followed by Dunn’s multiple comparisons test comparing to the HFD group if applicable. For qPCR data, the \( \Delta \Delta C_t \) values were used for the statistical analysis. Body weights were analysed by a two-way repeated measures ANOVA. Data not following a Gaussian distribution and/or with heterogeneous variance were log₁₀-transformed prior to analysis and subsequently presented as geometric means with 95% confidence intervals. Frequencies of fibrosis grades for each intervention group were compared to the HFD group by a chi-squared test and subsequently Bonferroni-corrected for multiple comparison. Liver histology was analysed by a Kruskall-Wallis test followed by Dunn’s multiple comparisons tests comparing to the HFD group and presented as medians.

3 | RESULTS

3.1 | Body weight, food intake, liver density, histology and biochemistry

Body weights and food intake were not different between groups (Figure 2A,B). Liver densities decreased in all high-fat-fed groups during the intervention period but did not differ between HFD and intervention groups \( (P = .1578) \) (Figure 3). Compared to HFD pre-intervention, all high-fat-fed groups progressed in steatosis, lobular inflammation, fibrosis, NAFLD activity score and SAF activity. A nominal reduction in steatosis grade was observed for ASA+PTX and in lobular inflammation for ASA and ASA+PTX. However, there was no statistically significant difference between HFD, ASA, PTX and
ASA+PTX in hepatic steatosis (P = .5759), hepatocyte ballooning (P = .6852), lobular inflammation (P = .3684), portal inflammation (P = .3066), NAFLD activity score (P = .6712) or SAF activity score (P = .6852) (Figure 4A-F and Figure 5). Fibrosis grade was not different between HFD and intervention groups (P = .5640) (Figure 5 Figure 6A). Seemingly, more animals had severe fibrosis (F3-4) in the HFD (71.4%) and ASA (75.0%) group than in PTX (40%) and ASA+PTX (54.5%) group (Figure 6B), but this was not statistically significant for any group compared to HFD. The relative fibrosis area was not significantly different between HFD, ASA, PTX and ASA+PTX (P = .7478), but a similar trend towards lower mean relative fibrosis area in the PTX and ASA+PTX was observed (Figure 6C).

Absolute and relative liver weights were decreased in all intervention groups by approximately 10 g (~18%) and 15%, respectively, compared to HFD, but this was not statistically significant (P = .1307 and P = .1440, respectively) (Table 2). Hypothesis-driven post hoc testing suggested that the reduction in relative liver weight approached statistical significance for ASA (P = .091) and ASA+PTX (P = .0874) compared to HFD. Similarly, liver triglycerides were reduced by approximately 10 μmol/g tissue in the ASA, PTX and ASA+PTX group compared to the HFD group; however, the reduction was not statistically significant (P = .2513). Liver total cholesterol (P = .6773), ALT (P = .8314), AST (P = .8314), ALP (P = .9450), bilirubin (P = .5554) and albumin (P = .1515) did not differ between HFD, ASA, PXT and ASA+PTX.

3.2 | Plasma lipids

Plasma levels of triglycerides (P = .2775), total cholesterol (P = .3160) or free fatty acids (P = .1332) were not different between HFD, ASA, PXT and ASA+PTX (Table 3).

3.3 | Hepatic gene expression

Overall, inflammatory markers were not different between groups, but a tendency towards decreased hepatic expression was noted for monocyte chemoattractant protein 1 (Mcp1, also known as Cc12) and interleukin 8 (Il8) in the intervention groups (ASA, PTX and ASA+PTX) (Figure 7A). Hepatic expression of Mcp1 was decreased (21%-33%) in intervention groups compared to HFD, but the groups were not overall different (P = .4397). Il8 expression was also reduced (21%-35%) in the ASA, PTX and ASA+PTX group compared to HFD, but the expression was not statistically different between groups (P = .3730). Tumour necrosis factor α (Tnf) expression did not differ between HFD and intervention groups (P = .9682).

Fibrogenic genes were not affected by interventions (Figure 7B) as collagen 1a1 (Col1a1) (P = .8697), platelet-derived growth factor β (Pdgfb) (P = .8259) and transforming growth factor β (Tgfb1) displayed similar expression in all groups (P = .6639).
FIGURE 4 Liver histopathology and disease activity. ASA and PTX, alone or in combination, did not affect hepatic steatosis (A), hepatocyte ballooning (B), lobular inflammation (C), portal inflammation (D) or disease activity measured as the NAFLD activity score (E) and SAF activity (F) when compared to HFD. Data (only HFD and intervention groups) were analysed using the Kruskal-Wallis test followed by Dunn’s multiple comparisons test comparing to the HFD group if applicable. Medians with individual values

4 | DISCUSSION

The current study investigated the effect of ASA and PTX alone and in combination on NASH end-points in a guinea pig model. ASA and PTX did not affect the primary histological outcome of steatosis, hepatocyte ballooning, lobular inflammation or fibrosis. Whole-liver expression of inflammatory and fibrogenic genes was not affected, although a nominal reduction in Mcp1, Il8 and Pdgfb as well as relative liver weight and liver triglyceride content was recorded. The guinea pig is a well-validated model of NASH that develops advanced (F3) fibrosis when fed a human-like “Western diet” enabling the study of this critical NASH end-point. Accordingly, high-fat-fed guinea pigs developed NASH with steatosis, inflammation, ballooning and fibrosis prior to intervention. ASA and PTX alone or in combination exerted no statistically significant effects on NASH histology, biochemistry or gene expression. However, all treatments did nominally decrease relative liver weight and liver triglyceride levels indicating an anti-steatotic effect, in line with previous preclinical and clinical reports of ASA and PTX. Although this was not corroborated by the CT scans in the current study. Furthermore, 86% of untreated HFD animals had grade 3 inflammation while 45%-60% of treated animals had grade 3 inflammation. Additionally, Mcp1 and Il8 mRNA levels—which regulate immune cell infiltrations—were decreased 21%-33% and 21%-35% compared to HFD, respectively, in ASA- and PTX-treated groups. While these decreases were not statistically significant, they could indicate that ASA and PTX have a potential anti-inflammatory effect in guinea pigs with NASH.

An isolated effect of PTX is supported by two small meta-analyses suggesting that PTX improves lobular inflammation. Furthermore, one of the meta-analyses also found a beneficial effect of PTX on liver fibrosis. In the present study, 40%-55% of animals treated with PTX, alone or in combination with ASA, had advanced (F3) fibrosis at the end of the study while 71% of untreated HFD animals had advanced fibrosis. In addition, hepatic Pdgfb expression was reduced by 11%-25% in treatment groups compared to HFD. Though these decreases were not statistically different, hepatic fibrosis constitutes an important prognostic marker in NASH, with increasing severity associated with decreased liver function and increased mortality.

A similar tendency to improve fibrosis was not observed with ASA alone, which is in contrast to previous studies. In Mdr2−/− mice with biliary fibrosis, platelets were identified as a source of hepatic PDGFB that could activate hepatic stellate cells and induced fibrosis. Accordingly, ASA (30 mg/kg/d) supplied in the diet for 1 year reduced PDGFB levels and liver fibrosis in mice. Furthermore, PTX reduced PDGF-induced collagen synthesis and proliferation
**FIGURE 5** Liver histology. Representative liver sections stained with haematoxylin and eosin (A, C, E, G and I) or picro sirius red (B, D, F, H and J) from healthy chow controls (A and B), HFD (C and D), ASA (E and F), PTX (G and H) and ASA+PTX (I and J) at euthanasia (week 25). Scale bar is 200 μm.

**FIGURE 6** Liver fibrosis. (A) Fibrosis grade did not differ between HFD and intervention groups. (B) Nominally, less animals had severe (F3-4) fibrosis in the PTX and ASA+PTX group compared to HFD. (C) The relative fibrosis area did not differ between groups, although a nominal reduction was observed for PTX and ASA+PTX. Medians with individual values (A) or means with SD (C). Analysed with the Kruskal-Wallis test (A) or 1-way ANOVA (C) followed by Dunn’s or Dunnett’s multiple comparisons test, respectively, comparing to the HFD group, if applicable. (B) Intervention groups were compared separately to HFD by the Chi-square test and subsequently Bonferroni-corrected for multiple comparisons.
Liver markers at study end

<table>
<thead>
<tr>
<th>Parallel intervention groups</th>
<th>Reference values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFD</td>
<td>ASA</td>
</tr>
<tr>
<td>Liver weight [g]</td>
<td>63.9 ± 12.8</td>
</tr>
<tr>
<td>Relative liver weight [%]</td>
<td>7.19 ± 1.07</td>
</tr>
<tr>
<td>Liver TG [μmol/g]</td>
<td>55.1 (36.5-83.3)</td>
</tr>
<tr>
<td>Liver TC [μmol/g]</td>
<td>30.1 ± 7.55</td>
</tr>
<tr>
<td>ALT [U/L]</td>
<td>108.7 ± 37.6</td>
</tr>
<tr>
<td>AST [U/L]</td>
<td>949.4 (706.2-1272)</td>
</tr>
<tr>
<td>ALP [U/L]</td>
<td>37.7 ± 7.02</td>
</tr>
<tr>
<td>Bilirubin [μmol/L]</td>
<td>3.53 ± 1.43</td>
</tr>
<tr>
<td>Albumin [g/L]</td>
<td>27.2 ± 1.18</td>
</tr>
</tbody>
</table>

Note: Means with SD or geometric means with 95% confidence intervals. Data (only HFD compared to intervention groups) were analysed using the one-way ANOVA followed by Dunnett’s multiple comparisons test comparing to the HFD group if applicable.

Abbreviations: ALP, Alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TC, total cholesterol; TG, triglycerides.

aAnalysed by Welch ANOVA test.
bValues below lower limit of quantification (1.4 μmol/L) were set to 1.4 μmol/L, and this was done for all values in the Chow group and four out of five values in the HFD Pre-intervention group.

dohepatic stellate cells in vitro, supporting an anti-fibrotic effect of both compounds. Notably, randomized clinical trials investigating PTX had a considerably longer (12 month) intervention period and PTX was administrated three times daily compared to our 8-week intervention period and continuous administration through the diet. The meta-analyses of retrospective cohort studies, showing an association between ASA and hepatic fibrosis, also had a considerably longer follow-up time (6-24 m) compared to the relatively shorter time span of the current study. Thus, it is possible that longer treatment periods are necessary/required to detect significant beneficial effects of ASA and PTX alone and in combination.

In addition, both compounds were administered via the diet, and while this circumvents the stress associated with injections or gavage, continuous administration via the diet may not provide a sufficiently high plasma concentration to provide a therapeutic effect. Preventive therapy with 9 mg/kg PTX provided in drinking water did not affect hepatic steatosis, inflammation or fibrosis nor ALT and AST in rats fed a choline-deficient diet. However, oral or intraperitoneal administration of PTX with doses identical to our study improved ALT and AST, steatosis and inflammation in rats fed a high-fat diet with or without cholesterol (2%) and cholate (0.35%). In contrast to the current study, ASA reduced liver fibrosis induced by CCl4 in rats, liver steatosis in mice fed a high-fat or methionine and choline-deficient diet and liver steatosis and fibrosis in mice fed a choline-deficient L-amino acid-defined diet. However, oral or intraperitoneal administration of PTX with doses identical to our study improved ALT and AST, steatosis and inflammation in rats fed a high-fat diet with or without cholesterol (2%) and cholate (0.35%).

The pathoaetiology of many of these studies (toxin and micronutrient deficient) differs from the diet-induced NASH in guinea pigs, which may explain some of the discrepancies. Furthermore, these studies initiated drug administration concurrent with disease induction, thereby differing fundamentally from the current study, which investigated the compounds as an intervention against established NASH, a situation, which arguably mimics the clinical setting more closely. Ranging from 100 to 150 mg/kg/d, doses were also higher, which may explain the absence of statistically significant effects of ASA in the present study. Thus, the route of administration, dose of the compounds and when the therapy is initiated are clearly important factors.

The decision of drug administration through diet formulation was based on the narrow anatomical confinements of the guinea pig oral and pharyngeal cavity, preventing dosing by gavage in this species without imposing unacceptably high stress levels and risking iatrogenic deaths. Dietary dosing ensured stress-free dosing and continuous exposure, however, prevented the control of the exact dose per animal. This may have increased variation, thereby decreasing statistical power. To improve the translational validity of the study, animals not displaying adequately decreased liver density prior
TABLE 3  Plasma lipids at study end

<table>
<thead>
<tr>
<th>Parallel intervention groups</th>
<th>Reference values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HFD Pre-intervention</td>
</tr>
<tr>
<td></td>
<td>Plasma TC</td>
</tr>
<tr>
<td>HFD</td>
<td>4.44 (2.72-7.22)</td>
</tr>
<tr>
<td>ASA</td>
<td>4.99 (4.16-6.00)</td>
</tr>
<tr>
<td>PTX</td>
<td>4.35 (3.15-5.99)</td>
</tr>
<tr>
<td>ASA+PTX</td>
<td>4.94 (3.81-6.40)</td>
</tr>
</tbody>
</table>

Note: Means with SD or geometric means with 95% confidence intervals. Data (only HFD compared to intervention groups) were analysed using the one-way ANOVA followed by Dunnett’s multiple comparisons test comparing to the HFD group, if applicable.

Abbreviations: FFA, free fatty acids; TC, total cholesterol; TG, triglycerides.

FIGURE 7  Liver expression of inflammatory and fibrogenic genes in intervention groups relative to HFD. (A) Expression of the inflammatory markers chemokine monocyte chemoattractant protein 1/c-c motif ligand 2 (Ccl2/Mcp1), interleukin 8 (Il8) and tumour necrosis factor α (Tnf) was not statistically significant affected by interventions, although all interventions led to reduced Mcp1/Ccl2 and Il8 expression. (B) Fibrogenic markers collagen 1α1 (Col1a1), platelet-derived growth factor β (Pdgfb) and transforming growth factor β1 (Tgfb1) did not change following the intervention period. A reduction in Pdgfb was observed, without being statistically significant. Means with ranges. Analysed with a one-way ANOVA followed by Dunnett’s multiple comparisons test comparing to the HFD group, if applicable.

to treatment start—reflecting liver steatosis—were excluded prior to data analyses and interpretation. This ensured that treatment effects were only investigated in animals with fatty livers, more closely mimicking a clinical setting, but also reducing group sizes, which in turn decreased statistical power. We have recently reported differences in sensitivity to diet and vitamin E intervention in NASH guinea pigs, suggesting the presence of high and low responders when investigating effects, for example on inflammatory markers in NASH. This could have contributed to increased variability within groups and potentially underestimated a statistically significant effect of treatment in the present study. However, the applied CT scan inclusion criterion and subsequent reductions in group sizes prevents any meaningful further subdivision, for example into low responders.

In conclusion, the current study suggests that 50 mg/d ASA and PTX alone or in combination for 8 weeks does not ameliorate NASH or hepatic fibrosis in guinea pigs. Though findings indicate improvement in some groups and on some markers, the absence of statistical significance suggests that treatment periods should be extended and/or doses increased in order to mediate a potential therapeutic effect.

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CONFLICT OF INTEREST
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ORCID
David Højland Ipsen  https://orcid.org/0000-0002-2065-8497
Pernille Tveden-Nyborg https://orcid.org/0000-0002-5574-5742

REFERENCES


Paper V

Article

Vitamin C Deficiency May Delay Diet-Induced NASH Regression in the Guinea Pig

Josephine Skat-Rørdam 1, Kamilla Pedersen 1, Gry Freja Skovsted 1, Ida Gregersen 1, Sara Vangsgaard 1, David H. Ipsen 2, Markus Latta 3, Jens Lykkesfeldt 1,2 and Pernille Tvend-Nyborg 1,3

1 Department of Veterinary and Animal Sciences, Section of Experimental Animal Models, University of Copenhagen, Grønegårdsvej 15, DK-1870 Frederiksberg, Denmark; jsr@sund.ku.dk (J.S.-R.); kamilla.pedersen@sund.ku.dk (K.P.); gryfreja@sund.ku.dk (G.F.S.); idagregersen@sund.ku.dk (I.G.); saravangsgaard@sund.ku.dk (S.V.); jolpi@sund.ku.dk (J.L.)
2 Global Obesity and Liver Disease Research In Vivo Pharmacology DK II, Novo Nordisk A/S, Novo Nordisk Park 1, 2760 Måløv, Denmark; DVI@novonordisk.com
3 Global Obesity & Liver Disease Research, Global Drug Discovery, Novo Nordisk A/S, Novo Park 1, 2670 Måløv, Denmark; mrlq@novonordisk.com
* Correspondence: ptn@sund.ku.dk

Abstract: Oxidative stress is directly linked to non-alcoholic fatty liver disease (NAFLD) and the progression to steatohepatitis (NASH). Thus, a beneficial role of antioxidants in delaying disease progression and/or accelerating recovery may be expected, as corroborated by recommendations of, e.g., vitamin E supplementation to patients. This study investigated the effect of vitamin C deficiency—often resulting from poor diets low in fruits and vegetables and high in fat—combined with/without a change to a low fat diet on NAFLD/NASH phenotype and hepatic transcriptome in the guinea pig NASH model. Vitamin C deficiency per se did not accelerate disease induction. However, the results showed an effect of the diet change on the resolution of hepatic histopathological hallmarks (steatosis, inflammation, and ballooning) (p < 0.05 or less) and indicated a positive effect of a high vitamin C intake when combined with a low fat diet. Our data show that a diet change is important in NASH regression and suggest that a poor vitamin C status delays the reversion towards a healthy hepatic transcriptome and phenotype. In conclusion, the findings support a beneficial role of adequate vitamin C intake in the resolution of NASH and may indicate that vitamin C supplementation in addition to lifestyle modifications could accelerate recovery in NASH patients with poor vitamin C status.

Keywords: non-alcoholic fatty liver disease (NAFLD)/steatohepatitis (NASH); vitamin C; guinea pig model

1. Introduction

Redox imbalance and consequent oxidative stress is an important driver of hepatocellular damage in non-alcoholic fatty liver disease (NAFLD) and the subsequent progression to steatohepatitis (NASH) and fibrosis; a primary indicator for liver transplantation [1,2]. A causal connection to a chronically high calorie intake (in the form of fat, cholesterol, and sugars) combined with a sedentary lifestyle ties NAFLD to the broadly termed “lifestyle associated diseases”, hereby representing the hepatic consequence of metabolic dysfuncion [3,4]. In this way, closely linked to dyslipidemia and metabolic stress, an imposing state of redox imbalance and resulting oxidative stress in NASH induces hepatocellular damage and release of inflammatory cytokines and proinflamatory factors, driving disease progression with serious consequences for liver function and patient health [2,5].

By quenching free radicals, antioxidants are pivotal in maintaining intracellular redox balance and are likely to play an important role in NAFLD/NASH progression and resolution. In agreement, vitamin E (vitE) supplementation has been shown to improve NASH...
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histology in experimental animal models [6–8] and in humans [9], including large clinical trials (PIVENS (placebo vs. 800IU vitE or 30 mg Pioglitazone) [10] and TONIC (placebo vs. 800IU vitE or 1000 mg Metformin) [11]). Though mechanisms are not completely disclosed, vitE has been included as part of the treatment guidelines for some NAFLD patients [12–16].

However, other antioxidants may also play a role in NAFLD progression and resolution. Of these, vitamin C (vitC) may be the most important. VitC is considered the most important antioxidant in the blood and the only antioxidant capable of preventing lipid oxidation [17]. Moreover, decreased vitC levels are reported in NAFLD patients and are associated with several NAFLD/NASH co-morbidities such as obesity, cardiovascular diseases, and type 2 diabetes, though a causal relationship between vitC deficiency and accelerated NAFLD/NASH progression has not yet been established [18–24]. Acting as an electron donor and situated low in the free radical ‘pecking order’, ascorbate (the reduced form of vitC) provides reducing equivalents to diminish oxidative stress, but also to recycle other antioxidants such as vitE [25,26]. In addition, vitC acts as a cofactor in multiple enzymatic reactions, including in the formation of mature collagen and in the regulation of gene expression through DNA methylation, supporting a role in NAFLD progression [27–29].

Like humans, guinea pigs depend on a dietary vitC intake, contrary to most other mammals that have an endogenous vitC synthesis [30]. Moreover, guinea pigs develop NASH with hepatic fibrosis when subjected to a high fat diet, and share a high degree of similarity with the human NAFLD/NASH transcriptome and accompanying NASH histopathology [31–35]. Together, this supports a uniquely high translational potential of the guinea pig NASH model including a high predictability of mechanistic responses related to disease phenotype as well as vitC homeostasis.

This study investigated the potential burden of vitamin C deficiency—often resulting from a poor diet low in fruits and vegetables and high in fat—on the guinea pig NAFLD/NASH phenotype and hepatic transcriptome. The consequences of high versus low vitC intake on diet-induced NASH progression were assessed, as well as effects of vitC supplementation on subsequent NASH resolution combined with or without a low fat/low cholesterol diet, as recommended by clinical guidelines to patients.

2. Materials and Methods

Animal experiments were approved by the Danish Experimental Animal Inspectorate (License No: 2018-15-0201-01591) and in accordance with European legislation on animal experimentation (Directive 2010/63/EU on the protection of animals used for scientific purposes).

Eighty female Hartley guinea pigs between 301 and 350 g (Charles River Laboratory, Lyon, France) were equipped with an 1.4 mm subcutaneous chip (E-vet, Haderslev, Denmark) and allocated by randomization into three weight stratified groups upon arrival, all receiving a low fat high vitC diet (LFH) (control) diet. Following one week of acclimatization, high fat diets were introduced gradually over a 5-day period, establishing the three experimental groups for the 16 weeks of NASH induction. Groups consisted of the following: low fat-high vitC (control) (LFH, n = 16) (3.8% fat, 0% cholesterol, 0% sucrose, 2000 mg vitC/kg feed); high fat-high vitC (HFF, n = 16) (20% fat, 15% sucrose, 0.35% cholesterol, 2000 mg vitC/kg feed); and high fat-low vitC (HFL, n = 48) (20% fat, 15% sucrose, 0.35% cholesterol, 50 mg vitC/kg feed). For a more detailed diet composition, see Table S1.

HFH and HFL groups received feed ad libitum, whereas the LFH group was pair fed to the HFH group, ensuring the LFH group as a lean and metabolic healthy control with comparable vitC intake [36]. During the 16-week induction period, two animals (one from the LFH group and one from the HFH group) were euthanized because of a lack of sufficient weight gain, consequently reaching the humane endpoint of a maximum of 20% lower body weight compared with the group average. Neither of the animals displayed any signs of underlying disease in vivo or in the post mortem necropsy.
After 16 weeks on diets, oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were performed on a subset of animals from each group (selected by randomization and stratified by bodyweight) (LFH n = 7; HFL n = 7 and HFH n = 8) prior to euthanization (see Figure 1 for study overview). The remaining animals in LFH and HFH groups (n = 8/group) continued on their diet until study termination. Animals in the HFL group (n = 40) were randomized into four weight-stratified intervention groups (n = 10/group) to receive one of the following diets in a 2 by 2 factorial design: high fat-low vitC (HFL/HFL); high fat-high vitC (HFL/HFH); low fat-high vitC (HFL/LFH); or a low fat-low vitC (HFL/LFL) (LFL: 3.8% fat, 0% cholesterol, 0% sucrose, 50 mg vitC/kg feed). The 50 mg vitC/kg feed was obtained by titrating feed with 100 mg vitC/kg with feed containing 0 mg vitC/kg feed. The new diets were introduced gradually over 5 days. The HFL/LFH group was pair-fed to the HFL/HFH group, and the HFL/LFL group was pair fed to the HFL/HFL group to ensure equal intake of vitC. After 16 weeks on diets, OGTT and ITT were performed on all animals (n = 56), after which all animals were euthanized.

Figure 1. Study overview. Eighty guinea pigs were weight stratified into HFH, HFL, or LFH groups. One animal from each of the HFL and LFH groups was euthanized owing to weight loss. Following 16 weeks on diets, a subset was selected for OGTT/ITT and subsequent euthanization (HFL and LFH n = 7; HFH n = 8). The remaining animals in LFH and HFH groups continued on diets, whereas those in the HFL group (n = 40) were weight stratified into HFL/HFL, HFL/HFH, HFL/LFL, or HFL/LFH. Animals continued on diets until week 32, where all were subjected to OGTT and ITT before termination. HFL: high fat low vitC; HFH: high fat high vitC; LFH: low fat high vitC; LFL: low fat low vitC; ITT: insulin tolerance test; OGTT: oral glucose tolerance test; vitC: vitamin C.

All diets were chow based with vitC provided in the form of phosphorylated ascorbate (Stay-C) and content confirmed by postproduction analysis (Sniff Spezialdiäten, Soest, Germany). Feed intake was measured biweekly in each group by subtracting the amount of feed remaining with the amount given the previous day. All groups had ad libitum access to water and access to fixed amounts of hay throughout the study period. Body weights were measured once weekly and animal welfare was monitored daily by caretakers. No changes in behavior or clinical indications of disease or severe vitC deficiency (scurvy) were recorded.

2.1. Oral Glucose Tolerance Testing (OGTT)

All animals were semi-fasted (allowed access to hay and water) 12 h prior to testing. Testing at week 16 (n = 22) was performed over the course of two days. Testing at
week 32 was on n = 56 animals and performed over the course of 3 days. Animals were weight stratified and randomized within groups ensuring equal distribution across testing days. On the day of testing, animals were weighed and 2 g/kg of a 150% glucose solution was administered orally through a syringe and voluntary swallowing (not gavage). Micro (50 μL) blood samples for glucose monitoring were obtained by puncturing the ear vein with a 27 G needle at time-points 0 (baseline), 30, 60, 90, and 120 min after glucose administration, as previously described [36,37]. Blood glucose was measured in duplicates by an Aviva Accu-check glucometer (Roche A/S Diagnostics, Hvidovre, Denmark).

2.2. Insulin Tolerance Testing (ITT)

Animals were fasted and randomized as described for OGTT. On the day of testing, animals were injected subcutaneously with 0.5 U/kg insulin (Actrapid® Novo Nordisk A/S, Bagsvaerd, Denmark) with a 27 G needle. Blood samples were obtained as described for OGTT at 0 (baseline), 30, 60, 90, and 120 min after insulin administration. At week 32, one animal in the HFL/LFH group was excluded owing to inaccurate dosing.

2.3. Euthanasia and Sampling

As euthanasia proceeded across five days, animals were block randomized according to the day and time of euthanasia within groups. All animals were semi-fasted overnight and pre-anesthetized with 1.25 mL/kg Zoletil-mix (125 mg Tileamin, 125 mg Zoizapam (Zoletil 50 Virbac Laboratories, Carros, France) + 200 mg xylazin (Narcovet vet 20 mg/mL; Intervet International, Boxmeer, Holland) + 7.5 mg butorphanol (Turbogesic vet 10 mg/mL; Scanvet, Fredensborg, Denmark)). The anesthetized animal was then placed on isofluorane (3–5%) and, upon disappearance of inter-digital reflexes, intracardial blood was collected for vitC, free fatty acids (FFAs), triglycerides (TGs), total cholesterol (TG), alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST), as previously described [35,36,38].

HbA1c was collected from intra-cardial blood in 10 μL Na-Heparinized Endo-To-End Vitrex® Pipettes (Vitrex medical A/S, Herlev Denmark) and mixed with 1 mL Hemolyzing Reagent nr. 11488457 122 (Cobas, Roche diagnostics, Rotkreuz, Switzerland). The mixture was left to incubate on ice for 10 min, after which a maximum of 200 ul was transferred to a cobas cup (Sample cup micro 13/16, Roche Diagnostics, Mannheim, Germany) and stored at 20 °C until analysis on a Cobas 6000 (Roche Diagnostics, Berne, Switzerland), according to the manufacturer’s instructions.

Liver samples were collected as previously described [35,36]. The three liver sections collected for TG, TC, and vitC were immediately frozen on dry ice, and stored at 80 °C. The liver section collected for RNA-sequencing was immediately frozen in liquid nitrogen and stored at 80 °C.

2.4. Histology

Liver sections for histology were fixed in 10% formalin and paraffin embedded prior to slicing in 2–4 μm thick sections and staining with hematoxylin and eosin (H&E) or picrosirius red (PSR) with Weigert’s hematoxylin solution. All histopathological scorings were performed in a randomized and blinded manner, as previously described for the guinea pig model and according to Kleiner et al. [33,35,39,40]. The reliability of scoring was assessed by Cohen’s Kappa index [41,42]. The index was calculated by scoring 10 randomly selected sections in a blinded manner, then re-blinding and re-scoring and comparing the results to confirm scoring integrity (Kappa index). The observer was only allowed to continue with the scoring of all sections if Cohen’s Kappa values were 0.8 for the following categories: steatosis, ballooning, and fibrosis and >0.7 for inflammation. Steatosis, inflammation, and ballooning were evaluated on H&E-stained sections. Steatosis and ballooning were evaluated across the entire liver section, and scored as 0 (≤5%), 1 (5%–33%), 2 (33%–66%), 3 (>66%), and 0 (none), 1 (few), and 2 (many) respectively. For lobular inflammation, a lobule was defined as two portal areas and one central vein, and
an inflammatory focus was defined as three or more inflammatory cells in close proximity. Inflammatory foci were counted in five separate lobules dispersed across the liver section and scored as 0 (no foci), 1 (<2), 2 (2–4), and 3 (>4). Fibrosis scoring and quantification was performed across the entire section on PSR-stained sections. Fibrosis was scored as 0 (none), 1 (perisinusoidal or periportal), 2 (perisinusoidal and periportal), 3 (bridging), and 4 (cirrhosis). For fibrosis quantification, the relative fibrosis area was obtained by quantifying the amount of collagen stained tissue in relation to the total amount of liver tissue, using the Visiopharm software (version 2020.08.4.9377, Visiopharm, Horsholm, Denmark), and in accordance with quantification of fibrosis from PSR-stained sections in preclinical and human studies [43,44]. NAFLD activity score (NAS) was derived from the cumulative sum of steatosis, inflammation, and ballooning ranging from 0 to 8 [40].

2.5. Transcriptome Analysis

RNA was extracted from 10 mg liver tissue from randomly selected animals from each experimental group at the 32-week time point (LFH: \( n = 8 \), HFH: \( n = 8 \), HFL/HFH: \( n = 10 \), HFL/HFH: \( n = 6 \), HFL/LFH: \( n = 6 \), HFL/LFL: \( n = 6 \)), using the RNaseasy Lipid Tissue Mini Kit in accordance with the manufacturer’s instructions (Qiagen, Valencia, CA, USA). Twenty-five microliters of the purified RNA (200–500 ng/mL measured by nanodrop 2000 (Thermo Fisher, Waltham, MA, USA) was shipped to Novogene (Novogene, Cambridge, UK) for paired-end 175-nucleotide read length, 30 million reads per sample, sequencing on an Illumina NovaSeq 6000 platform (Illumina Inc., San Diego, CA, USA). The resulting FASTQ files were trimmed using the Trimmomatic software (version 0.38.0) [45]. Any reads shorter than 50 nucleotides were removed as well as any Illumina specific adaptors. Furthermore, the initial nine bases were removed from each read to avoid biased sequence composition. The trimmed reads were mapped to the Ensembl Cavia Porcellus genome Cav.Por.3.0 (Ensembl release 98, September 2019) with Hisat2 (version 2.1.0) [46]. Transcript assembly and quantification was performed using Stringtie and Strangtie merge (version 2.1.1) [47] with the Cav.Por.3 annotation as a guide. To improve annotation, BioMart (version 2.42.0) [48] was used to obtain human orthologues, as previously described [34]. Genes without annotation and total read counts <200 for all samples were excluded from further analysis. The final list of genes was then used as input for differential expression analysis with DESeq2 (version 1.26.0) [49]. Gene set enrichment analysis (GSEA) was performed on log2 fold change pre-ranked values, using the gsea package in R [50]. Hallmark pathways used as input were obtained from the Molecular Signature Database (MSigDB) [51–53]. Human top 150 NASH-associated advanced fibrosis genes were identified from gene expression data (GSE49541) consisting of 40 patients with mild (F0-F1) and 32 patients with advanced NAFLD/NASH and F3–F4 fibrosis [54].

2.6. Statistics

All statistical analysis was performed in the GraphPad Prism version 9.0.1 (GraphPad Prism software, La Jolla, CA, USA), or R version 4.1.1 (R Core Team, 2021.08.10). All normally distributed data with equal variances among groups were analyzed by one-, two-, or three-way ANOVA, with repeated measures if applicable, and Dunnett’s or Tukey’s correction for multiple comparisons presented as means with standard deviation (SD). Data deviating from normality were log transformed, re-analyzed, and subsequently presented as medians with 25th and 75th quartiles. In cases of continued deviation, or for categorical data, analysis was performed using a non-parametric Kruskal–Wallis test with a Dunn’s test for multiple corrections, and presented as medians with 25th and 75th quartiles. For differential expression analysis, differentially expressed genes (DEGs) were defined as genes with a Benjamini–Hochberg corrected p-value (q-value) <0.05. For GSEA, a q-value <0.1 was considered significant. Principle component analysis was performed on normalized and transformed values for each gene, unless otherwise stated.
3. Results

3.1. Body Weights and Energy Intake

Body weights and energy intake were monitored pre- and post-intervention for all groups. While there were no differences in body weights in the pre-intervention period, Figure 3A shows a clear effect of diet (p < 0.01) in the post-intervention period and no effect of vitC. The HFH group had an increased energy intake compared with HFL in the pre-intervention period (Figure 3B), and displayed the highest body weight of all groups in the post-intervention period (week 16 and on). Interestingly, all intervention groups switching to a high vitC diet (HFL/HFH and HFL/LFH) show a weight pattern similar to the reference groups (HFH and LFH), rather than their low vitC counterparts, and despite similar energy intake between HFL/HFH and HFL/HFL, and between HFL/LFL and HFL/LFH (Figure 3C). Average energy intake per group was calculated as the mean of the bi-weekly measurements of feed intake once animals were completely adapted to their respective diets, hereby yielding n = 13 weeks pre- and post-intervention.

3.2. Glucose Homeostasis

The effect of diet and vitC on glucose homeostasis was assessed by OGTT, ITT, and determination of HbA1c concentration in plasma. At week 16 (pre-intervention), the OGTT response showed an overall effect of diet and increased AUC in HFH compared with LFH (p < 0.01) (Figure 2A,B). There was no difference in ITT responses or HbA1c (data not shown). At week 32 (post- intervention), HFH and HFL/HFH displayed the highest glucose peak levels and AUC of all groups (Figure 2E,F). The altered glucose homeostasis of these two high fat fed groups was supported by increased HbA1c levels compared with LFH (p < 0.01) and HFH/LFH (p < 0.001) (Table 1). Glucose measurements in the ITT showed a higher AUC in LFH and HFL/HFH compared with HFL/HFL animals (Figure 2H). ANOVA showed a significant effect of diet and of vitC on ITT glucose measurements (p < 0.001 and p < 0.01, respectively) with no diet/vitC interaction.

3.3. Plasma Markers

The lipid profile was assessed by measuring TG, TC, and FFA levels in plasma (pre-intervention values are presented in Supplementary Table S2). In line with the dietary cholesterol content (0.35%) in the high fat diets (HF groups), TC levels were clearly affected by diet (p < 0.001), and increased in all HF groups compared with animals on the low fat (no cholesterol) diets (LF groups) (p < 0.001) at week 32 (Table 2). TG and TC levels indicated an overall effect of vitC with increased levels in low vitC groups compared with their high vitC counterparts (p < 0.01) (Table 2). As expected, liver damage marker AST was increased in all HF groups compared with LF groups (p < 0.001), and ALT increased in HF compared with LF intervention groups (p < 0.01). Both ALT and AST levels were decreased in HFL/HFH compared with HFH (p < 0.05) (Table 2). Finally, vitC levels were significantly decreased in all low vitC groups (p < 0.001) (Table 2).

Table 1. HbA1c plasma levels in all groups at week 32.

<table>
<thead>
<tr>
<th>Diet</th>
<th>HbA1c</th>
<th>Diet/vitC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFH</td>
<td>3.90 ±0.13</td>
<td>ns</td>
</tr>
<tr>
<td>HFH</td>
<td>4.25 ± 0.22 **</td>
<td>ns</td>
</tr>
<tr>
<td>HFL/HFH</td>
<td>4.20 (3.80–4.23) ***</td>
<td>ns</td>
</tr>
<tr>
<td>HFL/HFL</td>
<td>3.90 (3.80–4.00)</td>
<td>ns</td>
</tr>
<tr>
<td>Diet/vitC</td>
<td>3.90 (3.80–4.00)</td>
<td>ns</td>
</tr>
</tbody>
</table>

Data are presented as medians with Q25–Q75 (in brackets) or means ± SD and analyzed by two-way ANOVA with a Tukey’s test for multiple comparisons, or a one-way ANOVA (all groups compared to LFH) (n = 8–10/group). Difference from LFH: ** p < 0.01, *** p < 0.001; difference from HFL/HFH: ††† p < 0.001. For overall effect of factor (diet, vitC, and diet/vitC) ns p > 0.001. ns: not significant. HbA1c: hemoglobin A1C, HFH: high fat high vitC, HFL: high fat low vitC, LFH: low fat high vitC, LFL: low fat low vitC, vitC: vitamin C.
Figure 2. Glucose homeostasis. (A) OGTT at week 16. Log transformed data were analyzed by a two-way ANOVA (only results from the overall ANOVAs are shown). (B) AUC for OGTT at week 16.
Data were analyzed by a one-way ANOVA with a Tukey’s test for multiple comparisons. (C) ITT at week 16. Log transformed data were analyzed by a two-way ANOVA (the overall ANOVA was not significant). (D) AUC for ITT at week 16. Normalized data were analyzed by a one-way ANOVA. (E) OGTT at week 32. Data were analyzed by a three-way ANOVA (only results from the overall ANOVA are shown). (F) AUC for OGTT at week 32. All groups were compared to LFH by a one-way ANOVA with a Dunnett’s test for multiple comparisons. In a separate analysis, intervention groups were analyzed by a two-way ANOVA with a Tukey’s multiple comparisons test. (G) ITT at week 32. Data were analyzed by a three-way ANOVA (only results from the overall ANOVA are shown). (H) AUC for ITT at week 32. Normalized data for all groups were compared to LFH by a one-way ANOVA with a Dunnett’s test for multiple comparisons. In a separate analysis, intervention groups were analyzed by a two-way ANOVA with a Tukey’s multiple comparisons test. All data are presented as means ± SD n = 7–10. **p < 0.01 compared to LFH; ##p < 0.01 compared to HFL/HFL. ns: not significant. AUC: area under the curve, HFH: high fat high vitC, HFL: high fat low vitC, ITT: insulin tolerance test, LFL: low fat low vitC, LFH: low fat high vitC, OGTT: oral Glucose tolerance test, vitC: vitamin C.

Figure 3. Body weights and energy intake. (A) Body weights presented as means ±SD; error bars are shown as one-sided on the graph. There were no differences in body weight in the pre-intervention overall effects of factors (diet, VitC, and diet/vitC) depicted on the graph (n = 10). (B) Average energy intake/animal/day is presented as means ± SD, and was analyzed by one-way ANOVA with a Tukey’s
multiple comparisons test, n = 13 weeks. (C) Average energy intake per animal is presented as means ± SD. LFH (control) was compared to all other groups by one-way ANOVA with a Tukey’s multiple comparisons test. In a separate analysis, all intervention groups were compared by one-way ANOVA with a Tukey’s multiple comparisons test, n = 13 weeks. Compared to LFH: * p < 0.05, *** p < 0.001; compared to HFL/HFH: # p < 0.05, ### p < 0.001; and compared to HFL/HFH: ††† p < 0.01. ns: not significant. HFH: high fat high vitC, HFL: high fat low VitC, LFH: low fat high vitC, LFL: low fat low vitC, vitC: vitamin C.

3.4. Liver Status

At the 16 weeks pre-intervention time point, NASH was successfully induced in both high fat diet groups (HFH and HFL), measured by increased steatosis, inflammation, ballooning, and consequently cumulated NAFLD activity scores compared with LFH animals (p < 0.001 for HFH and p < 0.05 for HFL) (Figure 4A–E). Fibrosis was also increased in both high fat groups (p < 0.01 and p < 0.001 in HFH and HFL respectively), with a median of F2 (range: F1–F4) stage in HFH and F3 stage (range F2–F3) in HFL animals. Significant differences in the histological distribution of fibrosis were only partially reflected by fibrosis quantification (p < 0.05 for HFH animals) (Figure 4F). Biochemical markers in the liver are presented in Supplementary Table S3.

Figure 4. Hepatic histopathological scoring and fibrosis quantification at week 16. Data are represented as individual values (scores) with medians (A–E) or means ± SD (F) (n = 7–8/group). Semi-quantitative scoring results are depicted in (A) steatosis, (B) inflammation, (C) ballooning, and (D) fibrosis. (E) Cumulative NAFLD activity score for each animal. (F) Fibrosis fraction quantified by image analyses of picrosirius red stained sections and displayed as % of total tissue (section) area. Scoring was analyzed by a non-parametric Kruskal–Wallis with a Dunn’s multiple comparisons test, comparing all groups to LFH. For fibrosis quantification, LFH vs. all groups was analyzed by one-way ANOVA with Dunnett’s multiple comparisons test on log transformed data. Difference from LFH: * p < 0.05, ** p < 0.01, *** p < 0.001. HFH: high fat high vitC, HFL: high fat low vitC, LFH: low fat high vitC, VitC: vitamin C.
Table 2. Plasma levels for all groups following 22 weeks on diet.

<table>
<thead>
<tr>
<th>Group</th>
<th>TG (mg/dL)</th>
<th>ALP (U/L)</th>
<th>AST (U/L)</th>
<th>GGT (U/L)</th>
<th>ALT (U/L)</th>
<th>ALTM (U/L)</th>
<th>ASTM (U/L)</th>
<th>ALTM (U/L)</th>
<th>2HSL (U/L)</th>
<th>2HSM (U/L)</th>
<th>2HIM (U/L)</th>
<th>2HLM (U/L)</th>
<th>HFL/LFH</th>
<th>HFL/LFH</th>
<th>LFH/LFH</th>
<th>HFL/HFH</th>
<th>LFH/HFH</th>
<th>LFH/LFH</th>
<th>LFH/LFH</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFL</td>
<td>33.92 ± 8.02</td>
<td>205.75 ± 67.76</td>
<td>22.85 ± 6.76</td>
<td>6.04 ± 0.57</td>
<td>4.92 ± 0.53</td>
<td>0.62 ± 0.54</td>
<td>0.64 ± 0.53</td>
<td>0.18 ± 0.57</td>
<td>0.54 ± 0.57</td>
<td>0.14 ± 0.57</td>
<td>0.54 ± 0.57</td>
<td>0.14 ± 0.57</td>
<td>0.63 ± 0.57</td>
<td>0.64 ± 0.57</td>
<td>0.18 ± 0.57</td>
<td>0.54 ± 0.57</td>
<td>0.14 ± 0.57</td>
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</tr>
<tr>
<td>LFH</td>
<td>33.92 ± 8.02</td>
<td>205.75 ± 67.76</td>
<td>22.85 ± 6.76</td>
<td>6.04 ± 0.57</td>
<td>4.92 ± 0.53</td>
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<td>0.54 ± 0.57</td>
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<td>0.54 ± 0.57</td>
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<td>0.54 ± 0.57</td>
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<td></td>
<td></td>
</tr>
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<td>0.62 ± 0.54</td>
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<td>0.64 ± 0.57</td>
<td>0.18 ± 0.57</td>
<td>0.54 ± 0.57</td>
<td>0.14 ± 0.57</td>
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</table>
Post-intervention hepatic TG and TC levels were increased in all HF groups compared with LF, hereby showing a clear effect of diet \( (p < 0.01) \), while there was no effect of vitC (Table 3). Post-intervention (32-week time-point) hepatic vitC levels were lower in the low vitC groups compared with high vitC groups, reflective of plasma values \( (p < 0.001) \) (Table 3). In line with previous findings from our group, HF groups demonstrated lower vitC levels compared with LF \( (p < 0.05) \) [35,36].

**Table 3.** Liver levels of measured markers following 32 weeks on diets.

<table>
<thead>
<tr>
<th>Marker</th>
<th>LFH</th>
<th>HFH</th>
<th>HFL/HFL a</th>
<th>HFL/HFL b</th>
<th>HFL/LFH c</th>
<th>HFL/LFL d</th>
<th>Diet</th>
<th>VitC</th>
<th>Diet/VitC</th>
</tr>
</thead>
<tbody>
<tr>
<td>VitC 1</td>
<td>1682.00 (1497.00–1913.00)</td>
<td>1181.00 (1073.00–1426.00)</td>
<td>128.20 (103.60–147.60)</td>
<td>1198.40 (1015.30–1316.80)</td>
<td>1344.80 (1031.60–1691.20)</td>
<td>119.50 (95.40–139.10)</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>TG 2</td>
<td>4.10 ± 0.5</td>
<td>37.76 ± 6.81 **</td>
<td>42.29 (39.86–47.86) **</td>
<td>46.94 (43.86–56.33) ***</td>
<td>6.58 (5.09–10.72) **</td>
<td>8.00 (6.89–14.29) **</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>TC 3</td>
<td>4.10 (3.77–4.46)</td>
<td>27.28 (24.70–32.53)</td>
<td>31.58 (29.92–33.19) **</td>
<td>31.79 (28.87–33.15) **</td>
<td>4.57 (4.17–5.14) **</td>
<td>4.69 (4.46–5.10) **</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

1. Log transformed data were analyzed by two-way ANOVA with a Tukey’s test for multiple comparisons.
2. Comparison to LFH was analyzed by a Kruskal–Wallis test with a Dunn’s test for multiple comparisons, and comparison between intervention groups was analyzed by two-way ANOVA on log transformed data. Data are presented as means ± SD or medians with Q25–Q75 values in brackets \( (n = 8–10/group) \). LFH was compared to all groups by one-way ANOVA with a Dunnett’s test for multiple comparisons. Post-intervention groups (HFL/HFL; HFL/HFH; HFL/LFH; and HFL/LFL) were compared by two-way ANOVA with a Tukey’s test for multiple comparisons. Difference from LFH: * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \). Differences between intervention groups are illustrated by different superscript letters a, b, c, and d: \( p < 0.05 \) or less. For overall effect of factors (two-way ANOVA): ns \( p > 0.01 \); ns: not significant.

The effect of a high fat diet corresponds with hepatic histopathology of severe steatosis in HF groups compared with LF \( (p < 0.001) \) (Figures 5A and 6C,E,G). The two HF intervention groups (HFL/HFL and HFL/HFH) did not exhibit significant differences in hepatic histopathology, and the median values were comparable to the HFF reference group (Figures 5 and 6E–H). Both LF intervention groups showed decreased NAFLD activity score compared with HF intervention groups (HFL/HFH and HFF/HFL, \( p < 0.01 \) or less). Fibrosis quantification demonstrated decreased fibrosis in both LF intervention groups (HFL/LFH and HFL/LFL) compared with the HF groups, though this was not reflected in the scoring of the overall morphological distribution pattern of fibrosis across the liver section (Figure 5D,F and Figure 6I–L).

An effect of vitC status on hepatic histopathology was not recorded in the HF groups. LF intervention groups (HFL/LFH and HFL/LFL) were not statistically different; however, an effect of vitC was indicated as HFL/LFH animals decreased fibrosis, inflammation, and ballooning cells compared with both HF intervention groups (HFL/HFH and HFL/HFF; \( p < 0.05 \) or less), whereas this was not as clear in the HFL/LFF group, where animals appeared to exhibit increased scoring on several parameters (Figure 5B,C).

### 3.5 Transcriptome Analysis

RNA-sequencing was performed on all (LF \( n = 8 \), HF \( n = 8 \), and HFL/HFL \( n = 10 \)) or a randomly selected subset of animals (HFL/LFH, HFL/LFL, and HFL/HFH, \( n = 6 \)). Principle component analysis (PCA) showed clear separation of all groups based on diet (Figure 7A), and showed a total of 1956 differentially expressed genes in common between groups receiving a control diet and their respective HF counterparts (Figure 7B).
Figure 5. Hepatic histopathological scoring and fibrosis quantification at week 32. Data are represented as individual values (scores) with medians (A–E) or means ± SD (F) (n = 8–10/group). Semi-quantitative scoring results are depicted in (A) steatosis, (B) inflammation, (C) ballooning, and (D) fibrosis. (E). Cumulative NAFLD activity score for each animal. (F). Fibrosis fraction quantified by image analyses of picrosirius red stained sections and displayed as % of total tissue (section) area. Scoring was analyzed by a non-parametric Kruskal–Wallis with a Dunn’s multiple comparisons test, comparing all groups to LFH or to post-intervention groups in a separate analysis. For fibrosis quantification, LFH vs. all groups was analyzed by one-way ANOVA with Dunnett’s multiple comparisons test on log transformed data and, for intervention groups, data were analyzed by a two-way ANOVA with Tukey’s test for multiple comparisons. Difference from LFH. ** p < 0.01, *** p < 0.001. Difference from HFL/HFH: † p < 0.05; †† p < 0.01; ††† p < 0.001. Difference from HFL/HFL: # p < 0.05; ## p < 0.01; ### p < 0.001. HFH: high fat high vitC, HFL: high fat low vitC, LFL: low fat low vitC, LFH: low fat high vitC, VitC: vitamin C.
Figure 6. Representative histological images for each group at the 32-week time point. (A,C,E,G,I,K): Hematoxylin and eosin stain. Scale bar shows 250 µm. (B,D,F,H,J,L): Picrosirius red stain. Scale bar shows 250 µm. Solid arrows indicate lipid vacuoles (macro- and microvesicular steatosis), and open arrows indicate fibrosis (in red). CV: central vein, PA: portal area, HFL: high fat low vitC, HFH: high fat high vitC, LFL: low fat low vitC, LFH: low fat high vitC.
Figure 7. Transcriptomic differences between groups. (A) Principal component analysis (PCA) plot based on normalized and transformed values for all genes in all investigated samples. (B) Venn diagram of differentially expressed genes between HFH and LFH; HFL/HFH and HFL/LFH; and HFL and HFL/LFL, showing overlapping and unique genes for each dataset. (C) PCA plot using the 83 differentially expressed genes between HFL/LFL and HFL/LFH. (D) Top 10 regulated pathways in HFL/LFL vs. HFL/LFH. The color bar depicts normalized enrichment score. Only significantly upregulated pathways are included (p < 0.1). (E) PCA plot using the top 150 differentially expressed genes from the human NAFLD/NASH dataset [54]. Encircled areas represent the normal data distribution within a 68% confidence interval. HF: all high fat fed groups; HFH: high fat high vitC; HFL: high fat low vitC; LF: all low fat fed groups; LFL: low fat low vitC; LFH: low fat high vitC, VitC: vitamin C.

Differential gene expression analysis was performed to assess if the slightly improved histopathology in the HFL/LFH animals compared with the HFL/LFL group was reflected
by changes in the hepatic transcriptome. This resulted in the identification of 83 DEGs between HFL/LFH and HFL/LFL (see Supplementary Table S4 for a complete list of identified genes). PCA of these genes in the remaining groups revealed a grouping of animals from the HFL/LFL group, indicating a similar expression pattern in all HF groups and, importantly, in the LFH (control) and HFL/LFH group compared with HFL/LFL animals (Figure 7C). Gene set enrichment analysis highlighted an upregulation of inflammatory pathways in HFL/LFL compared with HFL/LFH (Figure 7D). Finally, the expression pattern of the top 150 fibrotic DEGs derived from a human dataset of NAFLD/NASH patients [34] resulted in a distinct separation of all HF and LF diet groups. As indicated in Figure 7E, samples from the three LF groups appear to cluster in three separate bands, with the LFH (control) group furthest away from the HF groups, closely followed by the HFL/LFH group, and finally the HFL/LFL group closest to the HF cluster. The complete RNA sequence data set can be found through GSE 192497.

4. Discussion

This study investigated the effects of vitC on the progression and potential resolution of NASH following dietary induction and subsequent intervention in the guinea pig disease model. NASH with fibrosis was evident in induced groups regardless of vitC supplementation, confirming the model and the high fat/high cholesterol diet as the main driver of the disease. Importantly, following a change to a low fat/low cholesterol diet, a positive effect of vitC status on the NASH transcriptome during regression was recorded, suggesting a beneficial role of this antioxidant in supporting NASH treatment strategies.

4.1. Low Fat Diet Drives Resolution of NASH

Hepatic histopathology—steatosis, ballooning, and NAFLD activity score—improved in both LF intervention groups (HFL/LFH and HFL/LFL) to levels comparable to LF animals and below week 16 (pre-intervention) status of HFL counterparts, supporting NASH regression rather than a delayed progression during intervention. This was not as clear for the distribution of fibrosis, likely owing to the chronic nature of fibrotic scarring compared with the more dynamic response, e.g., of inflammatory cells, but limiting conclusions of fibrosis resolution as opposed to delayed progression. Post-intervention, both HFL/LFH and HFL/LFL groups displayed a lower fibrosis fraction compared with the HFL/LFH group, and approaching pre-intervention levels. Fibrosis fraction in HFL/LFH animals was also decreased compared with HFL/HFH. The semi-quantitative scoring of liver histopathology did not unequivocally support these differences; however, the pathological assessment is based on the morphological distribution of fibrosis within the hepatic parenchyma—such as the presence or absence of fibrotic bridging—aimed for diagnostic hallmarks, and is a descriptive methodology as opposed to quantifiable measures. Hence, the recorded reductions in overall liver fibrosis may not yet have reached a sufficient degree to be reflected in significant alterations of general histopathology. An improvement in hepatic health is supported by circulating enzymes ALT and AST with levels comparable to LFH controls in both LF intervention groups, whereas the high fat fed groups show a significant increase. The recorded increase in AST and ALT in HFL/HFH animals compared with the HFL/HFL group is encompassed with a very large variation for unknown reasons, and may not be directly reflective of the hepatic status in these animals.

4.2. Vitamin C Deficiency Delays Recovery of the NASH Transcriptome

However, fibrosis quantification and RNA-sequencing results indicated a slightly superior effect of receiving the low fat/low cholesterol diet in combination with a high vitC level in diminishing NASH. The PCA of all genes demonstrated a separation of groups based on dietary fat and cholesterol and showed that the reversion to a normalized and healthy hepatic profile was dynamic and occurred after a relatively short intervention period. The second PCA plot showed similar regulation of the 83 identified DEGs in LFH and HFL/LFH groups, compared with the HFL/LFL group, indicating that the transcriptomic
profile of LFH animals more closely resemble that of HFL/LFH rather than HFL/LFL. This is perhaps not surprising as the two diets are alike. However, the HF groups do not separate clearly and display no difference due to vitC levels between intervention groups (HFL/HFH vs. HFL/HFL). Furthermore, the span between the transcriptomic profile of fibrotic genes in HFL/LFH and HFL/LFL indicates that NASH fibrosis is increased in vitC deficient animals. Compared with the parallel high fat intervention groups (HFL/HFH and HFL/HFL), this indicates that these changes in the hepatic transcriptome are linked to disease resolution (driven by a low fat diet) rather than delayed disease progression, supporting a beneficial effect of an LFH diet compared with an LFL diet on the recovering NASH transcriptome.

Interestingly, C-reactive protein is among the 83 identified DEGs between HFL/LFH and HFL/LFL groups, and is upregulated in the HFL/LFL group by a log 2 fold change of 1.78. In humans, C-reactive protein is an inflammatory marker linked to obesity and atherosclerosis [55–57], and more recently also to NAFLD [58–60]. Guinea pig C-reactive protein shares sequence and subunit homology with humans, confirming conservation of the protein, but is not characterized as an acute phase inflammatory reactant in vivo in this species [61,62]. However, gene set enrichment analysis of hallmark pathways revealed an overrepresentation of pathways involved in inflammatory signaling, which could indicate that the hepatic inflammatory signaling profile is improved in response to vitC supplementation. The gene encoding collagen type IV alpha 2 chain (COL4A2) was among the 83 DEGs identified and represented in the top 150 human fibrotic genes in NAFLD [54]. The Collagen IV subunit is a major constituent of a fibrous extracellular matrix and basement membranes, and is increased in mild and severe hepatic fibrosis [63]. We have recently identified a list of genes highly associated with fibrosis in the guinea pig NASH model with severe hepatic fibrosis and with similar expression pattern in transcriptomic data from NAFLD patients [34]. This list included carbohydrate sulfotransferase 11 (CHST11), also significantly upregulated in HFL/LFL compared with HFL/LFH in the present study, supporting an increased fibrotic gene expression in the HFL/LFL group. Corroborating these observations, principle component analysis of the top 150 human fibrotic genes revealed a marginally closer relationship between fibrotic gene regulation in LFH and HFL/LFH compared with the HFL/LFL group, indicating that the fibrotic gene expression pattern in the HFL/LFH group was closer to complete reversal than the HFL/LFL group.

Previous studies in guinea pigs on high fat or cholesterol diets combined with vitC deficiency have reported changes in hepatic lipid-metabolism, e.g., increased hepatic TG and cholesterol and hepatocellular necrosis and fibrotic tissue expansion (in severely deficient animals) compared with non-deficient counterparts [64,65]. A recent study in senescence marker protein 30 (SMP30) knockout mice devoid endogenous vitC synthesis reported that vitC deficient (vitC serum concentration below 2.5 μg/mL) failed to progress from simple steatosis to more advanced disease compared with wild type counterpart mice on a high fat diet during 11 weeks of study [66]. The authors propose that a chronic state of vitC deficiency may mediate cholesterol build-up and subsequent inhibition of de novo lipogenesis, in turn delaying NAFLD progression. Although this supports a role of vitC in NAFLD, e.g., through a role as a cofactor for CYP7A1 and bile acid metabolism [67,68], no differences in hepatic TC levels were detected between HFH and HFL/HFL groups, hence no apparent effect of vitC status on hepatic cholesterol content in the present study.

4.3. Study Limitations

It may be speculated that the effect of high fat diet used in our study overpowered any effects of vitC deficiency. This is supported by RNA-sequencing that revealed grouping of all HF fed animals in the principle component analysis regardless of vitC status. It should be noted that the caloric intake in the HFL group was smaller during the 16 weeks pre-intervention period compared with counterparts. Although energy intake was equal between groups at post-intervention, the body weight curve of HFL/HFL animals appeared closer to LF groups (LFH, HFL/LFH, and HFL/LFL) compared with HFL/HFH
and HFH animals. An initially reduced energy intake may have delayed induction of metabolic dysfunction and NASH progression in this group, leading to a reduction of the expected effects. This is likely also reflected in the OGTT responses, in which HFH—but not HFL groups—displayed a compromised glucose homeostasis compared with LF groups, supported by the overall effect of diet as a factor. Post-intervention, LF groups (HFL/LFH and HFL/LFL) show a return to a normalized metabolic competence. A high fat diet has previously been reported to suppress insulin tolerance in guinea pigs [69]; however, the ITT responses in the current study were not significantly different between groups, apart from a reduction in the HFL/HFL group at week 32. This may be due to the reduced weight gain in this group, but could also be caused by a difference in insulin production. Unfortunately, there are currently no commercially available detection methods for measuring insulin in guinea pigs, preventing further elaboration on this aspect. The increase of hepatic markers in HFL/HFH animals at week 32 is likely to be driven primarily by outliers and not directly linked to NASH. However, further exploration is required to determine if this effect is reproducible and reflective of a biologically relevant effect. Unfortunately, this could not be pursued in the current study owing to a lack of appropriate samples.

4.4. Final Remarks

While controlled clinical studies are yet to assess a role of vitC on the progression and regression of NAFLD/NASH, epidemiological data suggest a lower dietary vitC intake in NAFLD patients compared with healthy controls, linking vitC to disease propagation [23,24]. A beneficial effect of supportive antioxidant therapy in NASH is underlined by current guidelines that dictate lifestyle modifications as the primary treatment for NAFLD, while patients diagnosed with NASH may also be considered for vitamin E supplementation (800 IU/day) [12–14]. In line with this, the results presented in this study suggest that adequate vitC intake in addition to a diet change toward a low fat/low cholesterol regime has a beneficial effect in the treatment of NASH compared with dietary intervention alone. Thus, future clinical studies should explore if vitC supplementation in addition to lifestyle modifications may accelerate disease regression in recovering NASH patients with poor vitC status.

5. Conclusions

In conclusion, we show that vitC deficiency delayed hepatic improvements compared with animals with a sufficient vitC intake when switching from HF to LF diet, indicating a beneficial role of vitC in the regression of NASH. In contrast, vitC deficiency had a limited effect on NASH progression, though beneficial effects may be masked by the severity of lipotoxic lipids (cholesterol) driving the disease.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antiox11010069/s1; Table S1: Detailed diet composition; Table S2: Plasma markers following 16 weeks on diets (pre-intervention); Table S3: Liver markers following 16 weeks on diets (pre-intervention); Table S4: Complete list of identified genes (DEGs).


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Data Availability Statement: The DNA sequencing data are publicly available via GSE192497. Additional data and material supporting the conclusions of this article can be requested from the authors.

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