

## Guinea Pigs Present Hypercholesterolemia, Hepatic Steatosis and Liver Injury Congruent with Cholesterol-Induced Non-Alcoholic Fatty Liver Disease Following a Dietary Cholesterol Challenge

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### Abstract

Dietary cholesterol challenges could be lipotoxic mediators in the transition of hepatic steatosis to non-alcoholic steatohepatitis (NASH), a critical event in the progression of non-alcoholic fatty liver disease (NAFLD). Here, we sought to examine the impact of a dietary cholesterol challenge on NAFLD progression in phenotypically normal guinea pigs, animals that closely approximate humans in terms of hepatic cholesterol handling and metabolism. Male Hartley guinea pigs ( $n = 10/\text{group}$ ) were fed either a high-cholesterol (H-Chol) diet containing 0.25% dietary cholesterol or a control, 0.04% low-cholesterol (L-Chol) diet for six weeks and evaluated by metabolic and histological parameters. Interestingly, H-Chol fed guinea pigs had lower final body weights ( $p = 0.002$ ) and decreased daily food intake ( $p < 0.0001$ ) compared to the L-Chol group. Compared to L-Chol controls, H-Chol guinea pigs developed marked hypercholesterolemia (284 vs 48 mg/dL;  $p = 0.0001$ ) and elevated plasma activity of ALT and AST, which are biochemical indicators of hepatic injury ( $p < 0.05$ ) while displaying no differences in plasma glucose, non-esterified fatty acid, or triglycerides (TG). Plasma total cholesterol was shown to be correlated with plasma ALT activity ( $r = 0.78$ ,  $p = 0.02$ ) in the H-Chol group only. H-Chol feeding resulted in increased hepatic accumulation of TG ( $p = 0.002$ ) along with total, free, and esterified cholesterol ( $p < 0.0001$  for all), resulting in modest hepatic steatosis. Moreover, features of hepatic inflammation and injury such as single cell necrosis were evident in livers of H-Chol fed guinea pigs. These results demonstrate that a dietary cholesterol challenge is sufficient to elicit not only steatosis, but more advanced features of hepatic injury in guinea pigs over a six week period.

**Keywords:** Hepatic steatosis; Cholesterol challenge; Hypercholesterolemia; Liver enzymes; Guinea pigs

**Abbreviations:** CE: Cholesterol Esters; FC: Free Cholesterol; H-Chol: High Cholesterol; L-Chol: Low Cholesterol; LD: Lipid Droplets, MUFA: Monounsaturated Fatty Acids; NAFLD: Non-Alcoholic Fatty Liver Disease; NASH: non-alcoholic steatohepatitis, PPAR $\alpha$ : peroxisome proliferator activated receptor  $\alpha$ SCD-1: stearoyl-CoA desaturase 1 TG: triglycerides, NEFA: Non Esterified Fatty Acids; TC: Total Cholesterol; ALT: Alanine Transaminase; AST: Aspartate Transaminase

### Introduction

In situations of macronutrient abundance, such as immediately after a meal or during situations of prolonged caloric overload, cells such as adipocytes and hepatocytes store metabolic energy in the form of triglycerides (TG). These inert, highly hydrophobic lipid molecules reside within the core of lipid droplets (LD), specialized organelles designed to shelter neutral lipids such as TG as well as cholesterol esters (CE) away from the aqueous cytoplasm [1,2].

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When circumstances dictate, the stored lipid can be hydrolyzed and released as fatty acids where they can then serve as intermediates in the synthesis of various lipid species or as bioactive ligands by binding to key nuclear hormone receptors like peroxisome-proliferator activated receptor $\alpha$  (PPAR $\alpha$ ), which in turn elicits key changes in overall hepatic lipid metabolism [3]. Thus by regulating the flux of lipid in cells, LDs serve as crucial components of lipid metabolism suggesting a prominent role for these organelles during times of health and disease [4].

Given the massive amount of TG and CE that can be stored within LDs it should be of no surprise that their accumulation leads to excessive lipid deposition, which in the liver manifests itself as hepatic steatosis. This condition is of clinical significance as it represents the initial stage and histological hallmark of non-alcoholic fatty liver disease (NAFLD), a clinic-pathological condition that represents a spectrum of histological abnormalities typically observed during excessive alcohol intake [5]. Steatosis that accompanies hepatic inflammation and features of injury like ballooned hepatocytes and apoptotic bodies is referred to as non-alcoholic steatohepatitis (NASH), the development of which represents a critical transition in the continuum of NAFLD [6]. NASH livers from animal models and humans are prone to further alterations to liver architecture that include bridging fibrosis, cirrhosis, and in some cases hepatocellular carcinoma, culminating eventually with liver failure [7]. Exactly what mechanisms mediate the transition from pure steatosis to NASH is a research question of fundamental importance, but has yet to be convincingly answered. At the forefront of proposed explanations has been the 'two-hit' hypothesis [8], which proposes that excess hepatic lipid accumulation brought about by metabolic derangements such as insulin resistance and obesity is an initial insult to the liver. Rendered vulnerable by the presence of steatotic hepatocytes, the liver then experiences an onslaught of secondary hits in the form of oxidative injury and inflammatory pathways that enable hepatic inflammation and injury to ensue [9]. According to this hypothesis the capability of certain by-products of lipid peroxidation could in turn initiate many of the cellular processes such as apoptosis, necrosis, and pro-inflammatory cytokine signaling characteristic of lesion formation, thus offering a unifying framework of steatohepatitis progression.

However, a noticeable shift has emerged recently that suggests disagreement with this hypothesis in NASH development [10-12]. Rather than focus solely on the magnitude of fatty deposition as a crucial effector of disease progression, many investigators are instead directing their attention to the nature of the lipid species themselves [13,14] or lipotoxic mediators of hepatic injury, of which excessive cholesterol might be a prime culprit [15,16].

Experiments conducted in cells have shown convincingly that cholesterol loading induces cytotoxicity of the ER [17], depletes mitochondrial glutathione content and sensitizes cells to cytokine-mediated NASH [18]. Additionally, a recent metabolomic assessment concluded that excessive cholesterol was a causal factor in both steatosis and hepatic inflammation [19]. Moreover, there exists now an abundance of evidence from animal studies that has shown that hepatic accumulation of cholesterol aggravates hepatic histology by dysregulating homeostatic mechanisms [20] through interactions with dietary fats [21,22]. Pharmacological doses of dietary cholesterol also further distort hepatic architecture and promote injury in hypercholesterolemic mouse models such as LDLR and ApoE knockout mice [23,24]. Most importantly human studies have also demonstrated a resounding connection between dysregulated hepatic cholesterol metabolism and the severity of NAFLD progression [25]. In fact, the very appearance of hepatic cholesterol crystals in livers of both mice and humans appears to distinguish between steatosis and NASH as shown by Ioannou, *et al.* [26].

Thus the available evidence to date confirms the importance of cholesterol as a lipid mediator of NAFLD progression in a variety of experimental settings. These data led to the current study which sought to investigate how challenging guinea pigs with excess dietary cholesterol influences NAFLD. The animal was chosen purposefully, given its close similarities with humans in terms of hepatic cholesterol handling [27].

## Materials and Methods

### Study design

Male Hartley guinea pigs ( $n = 20$ ) approximately 3 months of age (Charles River Laboratories, Wilmington, MA) were housed in cages in groups of two or three on a 12 hour light-dark cycle in a temperature and humidity controlled room. All guinea pigs were acclimated to the facility for one week prior to the experimental period and were fed standard rodent non-purified diet. Guinea pigs were then randomized to consume either a 0.04% low-cholesterol control diet (L-Chol) ( $n = 10$ ) or a 0.25% high-cholesterol diet (H-Chol) ( $n = 10$ ) for six weeks. Diets were prepared by Research Diets and were identical in terms of macronutrient and ingredient composition with the only exception being the cholesterol content. The composition of the diet has been previously reported [28]. Vitamin and micronutrient composition were formulated to meet the National Research Council requirements for guinea pigs and all experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Connecticut.

Throughout the six week study period guinea pigs consumed diets and water *ad libitum*. Animal body weight was measured at the beginning of every week on a digital scale and recorded in grams. Food intake, measured every two days, was calculated as the difference between the original amount offered and the amount remaining in the hopper. At the conclusion of the six week study period guinea pigs were fasted overnight for 12 hours and then anesthetized under isoflurane vapours followed by cardiac puncture. Whole blood and livers were then subsequently collected for analysis.

### Plasma analysis

Whole blood was obtained through cardiac puncture and collected into EDTA-containing 30 ml screw tubes and immediately centrifuged at  $2000 \times g$  for 20 minutes (Beckman Model TJ-6) to separate plasma from red blood cells. To prevent proteolytic degradation a preservation cocktail composed of sodium azide (0.1 ml/100 ml) and aprotinin (0.5 ml/100 ml) was added to plasma following centrifugation. Plasma was aliquoted and stored at  $-80^{\circ}\text{C}$  for subsequent analysis. Plasma cholesterol, LDL and HDL cholesterol, glucose, liver enzymes and triglycerides were analyzed using the Cobas c111 analyzer (Roche Diagnostics, Indianapolis, IN).

Plasma free cholesterol (FC) and neutral free fatty acids were quantitatively determined according to enzymatic methods (Wako Diagnostics, Richmond, VA). The concentration of NEFA was determined at 550 nm and the results are expressed as mEq/L.

### Hepatic lipid analysis

Whole livers were excised and their weights recorded. Livers were photographed with a Canon PowerShot A720 IS digital camera and then approximately 1 g of tissue from the left sub lobe of the quadrate lobe was sliced with a razor, placed in a 1 ml snap tube and immediately stored at  $-80^{\circ}\text{C}$  for hepatic lipid extraction. Once all tissue had been removed for lipid and histological analysis the remnant liver mass was tightly wrapped in aluminium foil and stored at  $-80^{\circ}\text{C}$  for future analysis. The concentration of hepatic lipids is expressed as mg/g liver with the exception of phospholipid, which is expressed as  $\mu\text{g/g}$  liver.

For hepatic TG, approximately 1 g of liver was finely sliced with a razor blade and added to 50 ml screw-top glass test tubes containing 10 ml of 2:1 chloroform: methanol. Tubes were then capped, mixed and left to incubate for 24 hours at RT. The homogenate was then filtered gravimetrically with Whatman grade #1 filter paper, added to a separatory funnel, mixed with 3 ml 0.05% sulphuric acid, and left to incubate for 1 hour or until adequate phase separation took place. Hepatic TG were measured enzymatically using a commercially available assay (Pointe Scientific Inc, Canton, MI) which sequentially hydrolyzes, oxidizes, and condenses the TG molecules into a red coloured quinoneimine that is measured spectrophotometrically at 500 nm.

The isolation of total cholesterol (TC) from the liver followed the same steps as that detailed for liver TG until the phase separation step. Once the final volume of the lipid extract was adjusted to 10 ml with Folch solution, a 200  $\mu\text{L}$  aliquot was transferred in triplicate to 13 x 100 mm glass test tubes and allowed to evaporate overnight uncapped. Samples were resuspended in 200  $\mu\text{L}$  ethanol, vortexed,

and analyzed using a commercially available assay (Pointe Scientific Inc, Canton, MI). Free cholesterol was measured utilizing a kit from Point Scientific (Pointe Scientific Inc, Canton, MI). The amount of cholesterol stored as cholesteryl ester within the liver was determined indirectly by calculating the difference between liver TC and FC.

### Liver histology

To evaluate whether H-Chol impacts liver pathology, approximately 1-2 g of tissue from the left lobe or the medial sub lobe of the right lobe were sliced with a razor blade and submerged in 10% neutral buffered formalin solution for approximately 1 week, and were then processed to paraffin using standard methods.

Sections were cut at 4-5  $\mu$ M, and stained with hematoxylin and eosin (H&E). Histological evaluation of liver sections from L-Chol ( $n = 10$ ) and H-Chol ( $n = 10$ ) was performed by a board-certified pathologist (JAS), who was blinded to the treatment groups, using the scoring system for steatosis that was originally proposed by Brunt, *et al.* [29] and validated by Kleiner, *et al.* [30]. Grade 0: Minimal fat accumulation in < 5% of hepatocytes; Grade 1: Mild fat accumulation in 5-33% of hepatocytes; Grade 2: Moderate fat accumulation in 34-66% of hepatocytes and Grade 3: Severe fat accumulation in > 66% of all hepatocytes. Additionally, the occurrence of other features such as inflammatory cell infiltration, fibrosis, biliary hyperplasia, ballooning degeneration, necrosis, and glycogenated nuclei were specifically observed for.

### Statistical analysis

All data were analyzed using the Prism statistical program from Graph Pad Software version 5.0c (San Diego, CA). Two-tailed independent student's t-tests were performed when comparing the differences between L-Chol and H-Chol groups. An unpaired t-test with Welch's correction was used in cases where variances were significantly different as determined by the F-test. All data were expressed as the mean + standard error of the mean (SEM). An  $\alpha$ -level of  $p < 0.05$  was chosen to denote statistical significance.

## Results and Discussion

### Body weight and adiposity

Initial body weights at week one did not differ between groups ( $369 \pm 4.5$  g vs  $372 \pm 15$  g,  $p = 0.86$ ). Both groups gained weight over the course of six weeks yet surprisingly the L-Chol guinea pigs weighed more at week 6 compared to the H-Chol group ( $639 \pm 17$  vs  $555 \pm 17$ ,  $p = 0.002$ ) (Figure 1A). The increased weight gain of L-Chol guinea pigs can be explained by an increased daily consumption of the diet (grams/day) relative to the H-Chol group ( $57 \pm 1.8$  vs  $45 \pm 1.9$ ,  $p = < 0.0001$ ) (Figure 1B). Despite the differences in body weight, there were no differences in the degree of adiposity between L-Chol and H-Chol animals according to adipose weight (g) ( $10 \pm 1.2$  vs  $7 \pm 1.2$ ,  $p = .085$ ).

### Plasma Lipids, Glucose and NEFA

H-Chol guinea pigs displayed higher total cholesterol than the L-Chol controls ( $p = 0.0001$ ). Consonant with these data, plasma free cholesterol was also significantly elevated in H-Chol guinea pigs relative to the L-Chol group ( $p < 0.0001$ ). LDL-C ( $p = 0.0003$ ) as well as HDL-C ( $p = 0.0003$ ) were also greatest in H-Chol group compared to L-Chol group (Figure 2). The cholesterol profile of L-Chol and H-Chol guinea pigs demonstrated that the majority of the cholesterol in plasma is transported in the LDL fraction, which as discussed previously is one of the features of guinea pigs, which mimic human lipoprotein metabolism.

There were no differences on plasma triglycerides ( $58 \pm 10$  vs  $61 \pm 6$  mg/dL), non-esterified fatty acids ( $0.5 \pm 0.3$  vs  $0.5 \pm 0.4$  mEq/L), or glucose ( $186 \pm 12$  vs  $184 \pm 10$  mg/dL) for the L-Chol and the H-Chol groups, respectively.

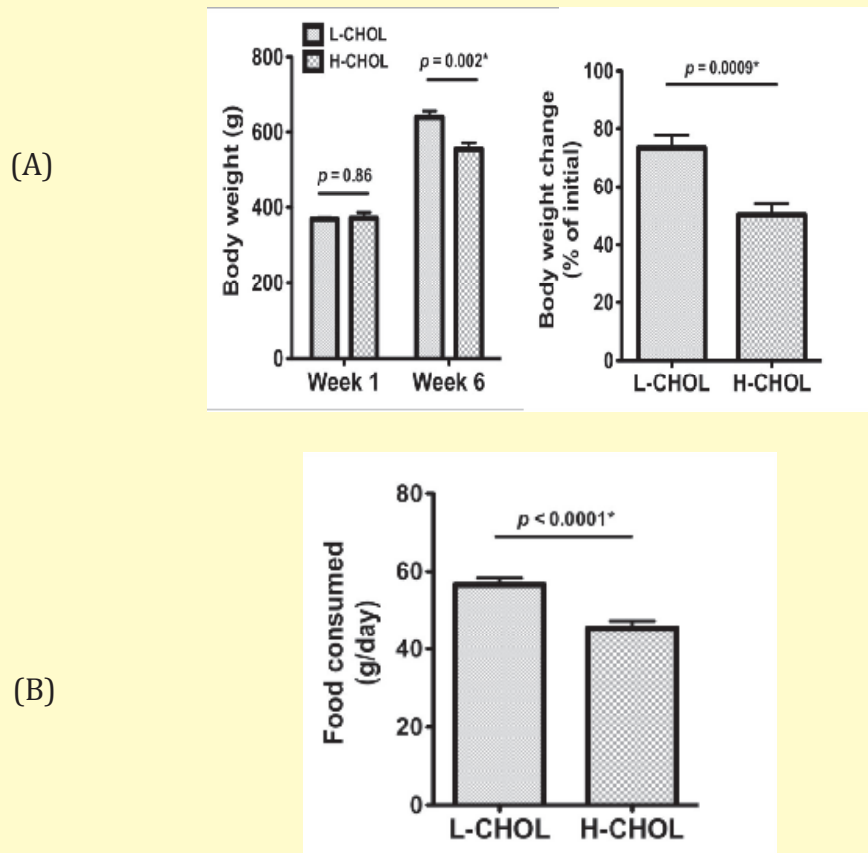
### Hepatic parameters: Lipids and liver enzymes

Guinea pigs in the H-Chol group displayed significantly elevated plasma concentrations of ALT ( $p = 0.042$ ) and AST ( $p = 0.049$ ) compared to the L-Chol controls (Figure 3A) indicating that excessive cholesterol feeding induces some form of hepatic injury. In support of

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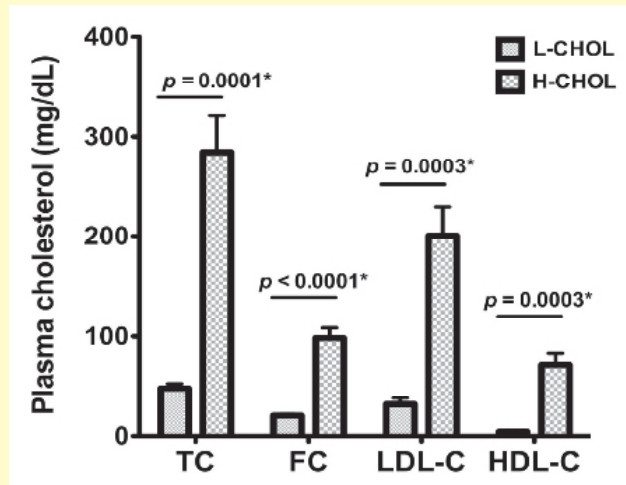
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this we noted a strong positive correlation between ALT and plasma total cholesterol in the H-Chol group only ( $r = 0.78, p = 0.02$ ) (Figure 3B). The livers from H-Chol guinea pigs were similar to L-Chol livers in wet weight, yet there were striking differences morphologically.



**Figure 1:** Initial and final body weights of guinea pigs fed low cholesterol (L-Chol) and high cholesterol (H-Chol) diets (Panel A) and average food consumption in g/day (Panel B).

Despite no differences in total liver weight, the liver/body weight ratio was greater in the H-Chol group ( $p = 0.001$ ), constituting almost 4% compared to 3% in L-Chol (Table 1). There was also an increase in liver TG content of H-Chol livers compared to L-Chol controls and this occurred with a concomitant accumulation of hepatic cholesterol, both total ( $p < 0.0001$ ) and free ( $p = 0.0004$ ). As TGs constitute the major stored neutral lipid in cells, it was particularly surprising to see hepatic cholesterol concentration exceed that of TG. For instance in the H-Chol group total cholesterol equalled 14 mg/g whereas liver TG was around 12 mg/g while L-Chol livers contained similar quantities of these two lipids (Table 1).



**Figure 2:** Plasma total cholesterol (TC) free cholesterol (FC), LDL cholesterol (LDL-C) and HDL cholesterol (HDL-C) of guinea pigs fed low cholesterol (L-Chol) or high cholesterol (H-Chol) diets for 6 weeks.

Liver Parameter	L-Chol (n=10)	H-Chol (n=10)	p-value
Liver weight (g)	19.3 ± 0.8	21.9 ± 1.3	0.103
Liver/body weight (%)	3.0 ± 0.1	3.9 ± 0.2	0.001
Triglycerides (mg/g)	6.0 ± 0.4	11.6 ± 1.3	0.002
Total cholesterol (mg/g)	5.1 ± 0.4	14 ± 1.3	< 0.0001
Free cholesterol (mg/g)	4.5 ± 0.3	11 ± 1.2	< 0.0004
Cholesteryl ester (mg/g)	0.6 ± 0.2	3.0 ± 1.0	< 0.0001
Steatosis score	0.2 ± 0.2	1.8 ± 0.2	< 0.0001

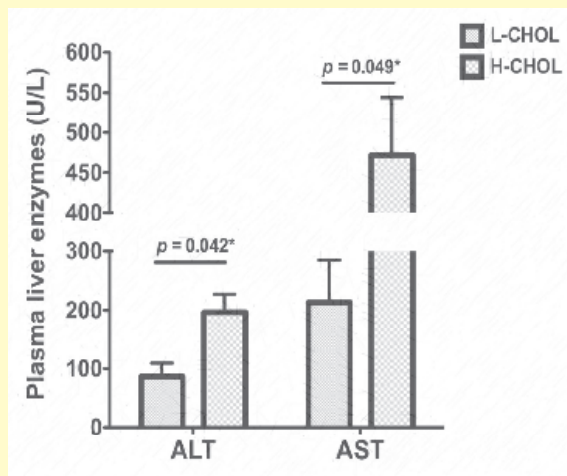
**Table 1:** Hepatic characteristics of guinea pigs fed .04% and .25% cholesterol for six weeks. All data presented as mean±standard error of the mean (SEM).

### Hepatic histology

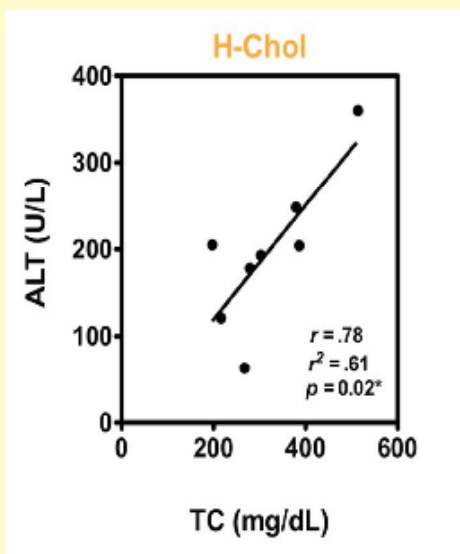
L-Chol livers were smooth and dark red-brown. H-Chol livers were paler, with an almost cloudy white appearance and a rough surface. Liver sections from each guinea pig were evaluated to assess the degree of fatty infiltration, inflammatory features, and fibrosis. All sections were examined twice and at different time points, thereby ensuring an unbiased evaluation. With regard to the L-Chol group, nine out of ten livers had very minimal fatty deposition and were classified as grade 0 while one liver had fine micro vesicular steatosis and was classified as grade 2. In contrast, all H-Chol livers presented some degree of steatosis with the majority (60%) being assigned a grade of 2 resulting in a significantly greater steatosis score compared to L-Chol ( $p < 0.0001$ ) (Figure 4).



(A)



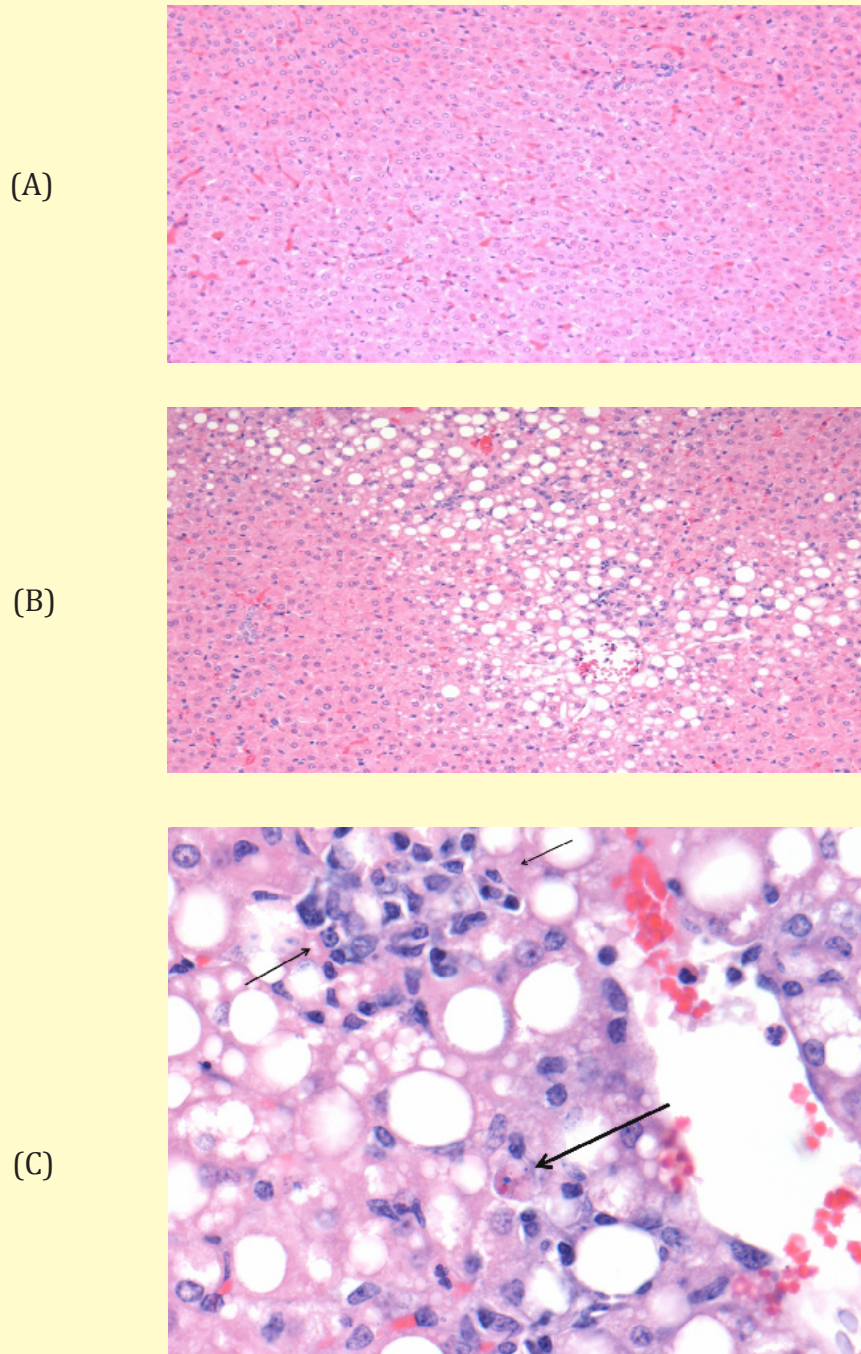
(B)



**Figure 3:** Plasma activity levels of liver enzymes in L-Chol and H-Chol fed guinea pigs (Panel A). Correlations between total cholesterol and ALT for the H-Chol group (Panel B).







**Figure 5:** Representative areas of H&E stained sections of liver in the low cholesterol group (L-Chol) (Panel A) and in the high cholesterol group (H-Chol) (Panel B). The latter guinea pig was assigned Steatosis Grade 2. Single-cell necrosis (long arrow) and occasional inflammatory foci (short arrows) was evident in liver of H-Chol guinea pigs (Panel C).

Hepatic cholesterol homeostasis is governed by a highly coordinated feedback system. When cholesterol availability is increased, such as during a high-cholesterol diet, cells enact a sequence of events designed to reduce their cholesterol content; this involves a down-regulation of the low-density lipoprotein receptors (LDLR) which are the major entrance points of extracellular cholesterol, as well as degradation and reduced transcription of HMGCR, a major regulatory point in cholesterol synthesis [31]. Though these experiments were not conducted in the current study, we think it likely that similar changes are also at play. Guinea pigs from the H-Chol group displayed hypercholesterolemia, indicating that there was a reduction in hepatic clearance of plasma cholesterol. It was interesting that only plasma cholesterol and not circulating triglyceride, non-esterified fatty acids, or glucose was impacted by the H-Chol feeding although mice and rabbits that have been fed just a cholesterol-enriched diet also have no changes in plasma lipids compared to controls [22,32]. We should also point out that the hepatic pathological changes that occurred in the H-Chol group did so in the absence of weight gain or hyperglycemia. This suggests that different metabolic factors are at play depending on the species, or alternatively that glucose metabolism and perhaps insulin resistance are not major players in the context of cholesterol-induced liver injury. The evidence supporting this notion is still relatively conflicted, with one study showing a reduction in plasma glucose and insulin in Japanese white rabbits with advanced fibrosis that were fed a 0.75% cholesterol diet for 9 months [33]. Another study in Syrian golden hamsters showed that the addition of 0.05-0.25% cholesterol to a 40% high sucrose diet aggravated insulin resistance and that the severity of changes were cholesterol concentration dependent [34].

An interesting observation from the present study was that H-Chol guinea pigs accumulated more hepatic cholesterol than triglyceride (TG). Though TG is the major storage form of lipid within cells, they are known to be influenced by the level of cellular cholesterol. The connection between cholesterol and TG likely centers around the endoplasmic reticulum embedded enzyme, stearoyl-CoA desaturase 1 (SCD-1), which is the enzyme responsible for the conversion of saturated fatty acids to monounsaturated fatty acids (MUFA) [35]. MUFA are the preferred substrates for incorporation into both TG as well as cholesterol esters, thus conditions that influence SCD-1 activity such as cholesterol excess likely impact the metabolism of both lipids [36].

One of the features making guinea pigs attractive for studying cholesterol's involvement in NAFLD is that like humans, guinea pigs contain most of the cholesterol within the liver in the free, unesterified form [27]. We also observed an overwhelming proportion of the liver cholesterol to be free in H-Chol guinea pigs. As free cholesterol has been shown to accumulate extensively in obese diabetic mice leading to NASH [20], it was anticipated that the H-Chol group would experience similar pathological changes. Indeed, hepatic free cholesterol was significantly greater in the H-Chol livers, although it should be mentioned that guinea pigs with the highest concentrations of free cholesterol did not always have the fattiest livers or greatest extent of hepatic injury. That said, the guinea pig that was classified as having the greatest histological evidence of inflammation did have the highest free cholesterol content, but also the highest average plasma glucose and interestingly, the greatest concentration of HDL-C. Therefore it seems that the hepatic response to increased accumulation of free cholesterol varies individually in guinea pigs.

### Conclusion

To summarize, we observed that feeding non-genetically modified male guinea pigs a diet with the cholesterol equivalent of 1800 mg per day for humans resulted in hypercholesterolemia, moderate mixed micro- and macrovesicular liver steatosis and mild inflammation, with some guinea pigs displaying single cell necrosis and glycogenated nuclei. Importantly, these changes occurred despite guinea pigs losing weight and having similar glucose metabolism to controls. These effects were diet-specific as the low-cholesterol fed guinea pigs displayed none of these changes and had overall normal liver histology. The extent of steatosis in H-Chol guinea pigs seems to depend more on hepatic cholesterol content than triglyceride and this could be useful in future studies that investigate whether there exist any potential differences between hepatic lipids in steatotic development.

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