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Investigating the Metabolic and Pathophysiologic Ramifications of Elevated Dietary Cholesterol and Macronutrient Manipulation in Guinea Pigs (Cavia porcellus) with Non-alcoholic Fatty Liver Disease (NAFLD)

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Non-alcoholic fatty liver disease (NAFLD) is a clinicopathological entity that includes a spectrum of histological and biochemical anomalies. NAFLD begins with excessive lipid accumulation within hepatocytes (hepatic steatosis) and can transition to non-alcoholic steatohepatitis (NASH), characterized by hepatic inflammation and injury that can render the liver vulnerable to bridging fibrosis, cirrhosis, and hepatocellular carcinoma. Dietary cholesterol has been proposed as a potential mediator of the transition from steatosis to NASH. Guinea pigs are similar to humans in terms of hepatic cholesterol metabolism and were implemented in three studies to explore its contribution to NAFLD.

In study one male guinea pigs ($n = 10$) were randomly assigned to either a $0.25\%$ high-cholesterol (H-Chol) or a $0.04\%$ low-cholesterol diet (L-Chol) for six weeks. H-Chol feeding promoted weight loss ($p = 0.0009$), hypercholesterolemia, elevated plasma alanine and aspartate aminotransferases ($p = 0.042$, $p = 0.049$ respectively), and accumulation of total hepatic cholesterol ($p < 0.0001$) and triglyceride ($p = 0.002$). H-Chol livers developed moderate steatosis along with evidence of mild inflammation and injury. Therefore, these results support the participation of increased dietary cholesterol
in promoting NAFLD. Macronutrient composition also influences NAFLD, therefore study two sought to determine the impact of feeding a diet low in carbohydrate and high in fat (LCD; n = 9) or a high carbohydrate diet (HCD; n = 10) to guinea pigs with cholesterol-induced NAFLD. Both groups failed to improve liver histology despite greater lipid accumulation in LCD livers. Biochemical liver injury was similar between groups, suggesting that both LCD and HCD are unable to improve cholesterol-mediated NAFLD. Study three demonstrated that phospholipids (PL) and triglycerides from H-Chol livers had an increased unsaturated and decreased saturated fatty acid profile. H-Chol also significantly lowered levels of PL arachidonic acid (p = 0.004). There were no differences between LCD or HCD, however both had significant reductions in overall PL content (p = 0.001) and similar increases in unsaturated and decreases in saturated fatty acid in PL and triglyceride when compared to H-Chol. In conclusion, elevated dietary cholesterol participates in NAFLD development and impacts the effectiveness of dietary intervention in guinea pigs.
Investigating the Metabolic and Pathophysiological Ramifications of Elevated Dietary Cholesterol and Macronutrient Manipulation in Guinea Pigs (Cavia porcellus) with Non-alcoholic Fatty Liver Disease (NAFLD)

Ryan C. deOgburn

B.S., University of Connecticut, [2007]
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Doctor of Philosophy Dissertation

Investigating the Metabolic and Pathophysiological Ramifications of Elevated Dietary Cholesterol and Macronutrient Manipulation in Guinea Pigs (Cavia porcellus) with Non-alcoholic Fatty Liver Disease (NAFLD)

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[2013]
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I want to begin by expressing my sincerest appreciation for all that you have done for me these past six years. It has been a true pleasure to have gotten to know you and it is a tremendous source of pride for me to call you my major advisor. I will always reflect fondly on our conversations, where, if we were not intensely discussing our own data, would inevitably involve one of us excitedly asking the other their thoughts about the latest paper to be published, or about the future direction of the field in general. For me, these were some of the most instructive experiences I had throughout graduate school. Your unwavering enthusiasm and dedication to your work is truly inspirational and something I hope to emulate in the years to come. I also want to thank you for all of the confidence you had in me, and the independence you afforded me throughout. Your constant support, availability, and genuine concern made the entire process an incredibly enjoyable and fulfilling experience, a description that one does not always associate with graduate school. Lastly, for you to say that you are proud of me is one of the most deeply satisfying encouragements I have ever had, and something that I will always remember. My quiet hope is to continue onward and do all that I can to keep that opinion alive. Thank you Dr. Volek.

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become a better scientist, and meet amazing people from all over the world, many of which are now very good friends. You always made me, as well as all of your students, feel welcome and a part of something special. I still marvel at how you can manage so many students at once and yet make time for each of us as if we were the only member of the lab. I know that no matter what research I do in the future, I will forever remain intrigued by cholesterol, the liver, lipoproteins, and will always have a soft spot for guinea pigs. Thank you for being such a great teacher and advisor; my time in your lab was a tremendous opportunity and something that I hope other future nutrition graduate students out there have the pleasure to experience. Thank you.

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<tr>
<td>15 deoxy-(\Delta)12,14-prostaglandin J2</td>
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GENERAL INTRODUCTION: NON-ALCOHOLIC FATTY LIVER DISEASE

Opening Remarks:

If one were to select at random a scientific publication concerning non-alcoholic fatty liver disease (NAFLD) it would almost invariably contain a rather bland introductory statement describing NAFLD as a ‘spectrum beginning with benign simple steatosis and progressing to non-alcoholic steatohepatitis, which ultimately can result in cirrhosis and liver failure’. Quite apart from the pervasiveness of these sentiments in academic literature, this type of statement is severely misleading in several respects. First, this description suggests a relative uniformity in NAFLD pathogenesis amongst all individuals regardless of some rather key differences like ethnicity, gender, and biological age. Second, and perhaps somewhat more seriously, this phraseology insinuates that fatty deposition and infiltration (herein uniformly referred to as hepatic steatosis) is a simplistic process and of minor pathological concern compared to the more progressive stages of the disease. Lastly this description leads one to believe that the NAFLD spectrum is unidirectional and culminates inevitably with cirrhosis, in essence implying a lack of potential regression or improvement of NAFLD.

The danger of the aforementioned introductory statement is not the emergence of these specific misconceptions themselves, for any sound discussion concerning the epidemiology and histopathology of NAFLD would undoubtedly clarify many of these issues. What is particularly dangerous is the ubiquity of these vague, uninformative phrases throughout the literature and how this reflects a serious lack of critical questioning on the part of researchers themselves. Certainly the field of NAFLD...
research has undoubtedly made enormous progress over the past few decades since its initial discovery, fueled by comprehensive experimental approaches in cellular, animal, and human studies. It is therefore of great importance to continue moving forward, which involves not only performing solid research, but effectively communicating the intricacies of such a disease to other scientists and the general public as well. Accordingly, the aims of this introduction are two-fold: First, to unequivocally correct and summarily dismiss the misconceptions that inevitably arise from such a damagingly trite description of a massively complex disease such as this, and second, to offer an alternative narrative of NAFLD that is more comprehensive in scope and truthful in detail.

The ensuing chapters of this dissertation place a heavy emphasis on dietary considerations—specifically cholesterol and macronutrient composition—in NAFLD pathogenesis. Building off of the introductory section, Chapter 1 of the literature review follows with an overarching description of cholesterol metabolism, that is its dietary absorption, entrance into the liver, intracellular trafficking, and regulatory mechanisms governing homeostasis. It then sifts through some key studies that highlight the pathophysiological ramifications of excess dietary cholesterol on the liver and how this spurs NAFLD progression in animals and humans. It concludes by making a case for the guinea pig as an attractive experimental approach for exploring the nuances of hepatic cholesterol metabolism and NAFLD. Chapter 2 shifts the attention to macronutrients and how dietary carbohydrate and fat elicit changes in hepatic metabolic pathways that are prominent in NAFLD. It then proceeds with a discussion of
carbohydrate-restricted diets and how, on a biochemical level, this dietary scheme has the potential to improve metabolic parameters associated with NAFLD. The dissertation then proceeds with three studies, each with a specific research aim designed to build upon preexisting evidence in the field. Finally, the project concludes with some closing remarks and suggestions for the direction of NAFLD research.

**Nomenclature: Acronyms galore**

More than thirty years have passed since Ludwig et al’s seminal encounter with a “hitherto unnamed disease” (1), which they pointedly termed nonalcoholic steatohepatitis (NASH). Unnamed no longer, the histopathological lesions observed by Ludwig’s group enjoy distinction today as the leading cause of chronic liver disease in adult and pediatric populations the world over, ranging from the United States, South America, and Europe in the West to the Middle East, Australia, and Asia in the East. Considering the enormity of societies affected by the emergence of this disease, it stands to reason that a uniform nomenclature system exist to appropriately refer to NASH and its associated histological spectrum. At present Ludwig’s original definition of NASH is recognized as an entity within the more comprehensive framework of nonalcoholic fatty liver disease or NAFLD.

To those not intimately involved in this research field (as well as to the more reflective researchers within it), the very term NAFLD appears to rely more on a lack of association with excessive alcohol intake than with any specific underlying pathological phenomena. And given that NAFLD is almost universally recognized as the hepatic manifestation of the metabolic syndrome (2), this has in turn prompted an outpouring of
alternate descriptions such as “metabolic syndrome steatohepatitis (MESH)”, “metabolic fatty liver disease”, “insulin-resistance-induced steatohepatitis (IRISH)”, and so on (3). While the debate is certain to continue as more studies are published, NAFLD seems descriptive enough to remain in use for the foreseeable future; this is fortunate given that there are also distinct differences in the pathological progression of NAFLD and alcoholic liver disease (ALD).

**A Global Continuum: NAFLD epidemiology in America and abroad**

One often hears or reads today about the global extent of noncommunicable diseases such as cardiovascular disease and type 2 diabetes and how these conditions reflect an increasingly sluggish lifestyle and poor dietary behaviors that result in rampant obesity. The statistics certainly are startling. For example it is currently estimated that more than 1.1 billion adults are overweight across the globe, and even more depressing is that roughly 300 million adults and at least 155 million children are actually classified as obese (4). Obesity itself is reflective of the many metabolic derangements such as dyslipidemia and poor glucose tolerance that define the metabolic syndrome, thus clinical measures such as plasma lipids, glucose, and body mass index are easily obtainable and are the most reliable markers for proper diagnosis. There is also a hepatic component to obesity and metabolic syndrome (2) that deserves consideration, for the last twenty years have witnessed an increase in the prevalence of NAFLD which closely parallels that for obesity (5). Unlike the standardized clinical tests that evaluate obesity and metabolic syndrome, there are no set standards for evaluating the presence of NAFLD with the exception of a liver biopsy.
Typical biochemical measures such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP)—the so-called liver enzymes—are notably unreliable at predicting hepatic steatosis or fibrosis (6). Despite being heralded as the ‘gold standard’ for NAFLD diagnosis, a liver biopsy is an intrusive procedure fraught with risks of complications, especially for children, and is also quite expensive financially. Moreover, biopsies are prone to sampling error as well as differences in histopathological interpretation (7). Ultimately these inherent difficulties pose significant obstacles to obtaining true estimates of NAFLD prevalence.

Despite these pitfalls of NAFLD diagnosis, there is a general consensus that NAFLD poses an emerging and formidable public health concern. In the United States, for example, typical estimates for adults range between 2.8%-46% (8) while approximately 10% of all children are estimated to display some features indicative of NAFLD (9). Ethnicity has been shown to be influential in terms of susceptibility to NAFLD and progression along the histological spectrum. A seminal study in the United States conducted by Browning et al (10) found that fatty infiltration or steatosis is greatest amongst older men when compared to women, and the frequency is highest amongst Hispanics, followed by white, and black populations respectively. This was a population-based study and did not speculate on potential mechanisms, but these findings, along with others (11,12) suggest that gender and ethnic differences likely play prominent roles in determining overall susceptibility to disease progression.

NAFLD is also rapidly emerging throughout various global populations, many of which live in developing nations that have increasingly adopted a so-called ‘Western’
diet. Data is hard to come by, however the most recent and comprehensive reports estimate approximately 20% of the entire global population has some degree of NAFLD (8). There are also several estimates that have been published for populations of South America (13), Japan (14), Australia (15), northern Europe (16), southern Europe (17), and the Middle East (18). Much of this data however is from the 1990’s, therefore present-day estimates and population-based studies are eagerly awaited to further characterize the epidemiology of NAFLD. Argo et al (19) and Chitturi et al (20) have published two very well researched and comprehensive reviews that discuss in detail all of the available data to date.

‘Hepatomania’: A brief appreciation of liver physiology

The sheer magnitude of physiological functions that the liver is responsible for makes it an organ like no other. It serves as the central hub for virtually all metabolic processes and is equipped with a staggering enzymatic arsenal—one can conceive of the liver as a kind of biochemical powerhouse, simultaneously coordinating a vast network of metabolic pathways that engage nutrients, macromolecules, and xenobiotics alike. It is of course no great surprise that the liver has been a subject of inquiry since the earliest civilizations, as Hesiod the eighth century poet attests with his description of Zeus’ vengeful punishment of the rebellious Prometheus. This now well known capability of the liver for regeneration is interesting from an evolutionary perspective in the fact that the liver is the only organ capable of regeneration. This attests to its crucial importance in ensuring metabolic homeostasis and general survival of the organism itself. We will not attempt here a thorough description of liver physiology as a whole.
Instead the aim is to emphasize how aspects peculiar to the liver, such as its anatomical location and diverse cell population ensure smooth and efficient coordination of the many basic tasks fundamental for metabolic balance and general survival.

The liver finds itself constantly exposed to an array of substances, three-quarters of which travel through hepatic lobules via transport from the portal vein. Portal blood emanates from the intestine, spleen, pancreas, as well as gallbladder and consequently is enriched in nutrients and foreign xenobiotic compounds; the remaining one quarter of hepatic blood, supplied by the hepatic artery, is concentrated with oxygen and circulating hormones and exit via the terminal arterioles branching throughout the hepatic parenchyma. Arterioles, along with the portal venules and major biliary duct are extended throughout the liver parallel to one another, forming the conspicuous portal triad characteristic of the hepatic lobule. The average human liver consists of approximately 100,000 lobules, each about 1 millimeter in diameter. The lobules are hexagonally shaped structures with a total of six portal triads present at each corner. Situated at the center of the lobules are the central veins which collect the blood delivered by the portal vein and hepatic artery and eventually empty into the inferior vena cava.

Hepatocytes represent the overwhelming cell type of the liver, accounting for 78% of total liver volume (21). These polarized epithelial cells are arranged as cords one cell thick and because their surface faces fenestrated sinusoidal endothelial cells, they have direct access to both incoming dietary and exogenous substances. They are also capable of secreting endocrine substances such as lipoproteins directly into the
blood stream. Each hepatocyte is closely bordered by neighboring ones that form tight junctions, generating a canaliculus that is responsible for the collection of bile acids and salts which travel across the apical hepatocyte surface (22,23). In addition to bile secretion hepatocytes are the cell type responsible for a litany of functions that include the metabolism of cholesterol, lipids, carbohydrates, proteins, as well as regulation of the acute phase response and blood clotting (23).

Hepatocytes are complimented by a panoply of other distinct cell types. Sinusoidal endothelial cells (about 2.5% total volume) are unique cells with distinct fenestrations that allow uptake of circulating molecules, sometimes which may be quite large (a classic example is chylomicron remnants) and because they lack a basement membrane, substances are able to travel to hepatocytes for processing (24). Other cells such as cholangiocytes (about 3% of total cell population) control the rate of bile flow (23), while still others such as stellate cells (1.4%) are involved in the maintenance of the extracellular matrix and storage of vitamin A (25). Kupffer cells (2%) are the resident macrophage cell of the liver and represent the largest population of macrophages of any organ in the human body, making the liver a potent participator in immunological processes (26). The diversity of the liver cell population should be kept in mind when the discussion turns to the pathological changes that characterize NAFLD. For instance, it is the hepatocytes which are prone to excessive lipid accumulation and stellate cells which when activated by cytokine stimulation trigger the fibrogenic response to injury (see below). It is therefore critical that NAFLD be thought of as a
disease of multi-cellular collaboration, which is where the focus of the discussion now turns.

**Homogeneous No More: Pathological characteristics of the NAFLD spectrum**

The entire NAFLD pathological spectrum begins with an increased cytoplasmic accumulation of lipids (in the form of triglyceride) within various liver cells, predominantly hepatocytes, and is referred to as hepatic steatosis. Steatosis is the *sine qua non* of NAFLD and presents histologically as either large globules with displacement of the nucleus to the cellular periphery (macrovesicular) or as smaller indentations around the centrally located nucleus, creating a ‘foamy’ cytoplasmic appearance (microvesicular) (27). A mixture of both macrovesicular and microvesicular patterns tends to predominate in human NAFLD (27-29) and is typically localized within perivenular hepatocytes of zone 3 (30). In contrast, truly microvesicular steatosis never occurs in isolation in NAFLD and when present, usually occurs in a nonzonal distribution within contiguous patches of hepatocytes that often harbor megamitochondria (31). Lastly, steatosis that is present without accompanying inflammation or liver injury is often incorrectly referred to as ‘simple’ steatosis; there is however, nothing actually simple about steatotic development given that it involves a complex interplay of molecular, biochemical, and metabolic events both at the level of the liver as well as peripheral organs such as adipose and muscle.

While steatosis is the histological hallmark of NAFLD, this pathological abnormality alone is insufficient for a diagnosis of the more inflamed and dangerous NASH stage of the spectrum. In NASH, hepatocytes begin to transition from being
merely lipid-engorged to being inflamed and architecturally distorted, thus accurate diagnosis of NASH relies on the presence of steatosis, inflammation, and several features of hepatic injury (32). In NASH, inflammation can be either lobular or portal in nature, although evidence acquired from human liver biopsies suggests that lobular inflammatory infiltrates predominate (31). Typically much of the inflammation occurs in zone 3, which anatomically is exposed to lower circulating oxygen and consists of small foci of mixed inflammatory infiltrates composed of lymphocytes, eosinophils, occasionally polymorphonuclear leukocytes, and aggregates of Kupffer cells (27,30,31). Clusters of Kupffer cells are referred to as microgranulomas or pigmented Kupffer cells and form as a result of their migration from their normal periportal location in zone 1 to a perivenular zone 3 position where they engulf and phagocytose apoptotic and necrotic hepatocytes (33). As the resident macrophages of the liver the participation of Kupffer cells in hepatic inflammation is unsurprising, however it is interesting that very little attention has been paid to Kupffer cell aggregation as a pathological entity of NAFLD, although there are some exceptions (27). When hepatic macrophages contain noticeable lipid droplets they are referred to as lipogranulomas that may be observed in either perivenular, portal, or lobular areas. It should be emphasized that the inflammatory pattern of NASH is drastically different from that typically observed in other severe liver diseases such as chronic hepatitis or cholestasis, in that NASH inflammation is generally mild and tends to occur primarily within the lobules rather than portal areas (34).
Concomitant with inflammation are features of hepatic injury that can include ballooned hepatocytes, Mallory-Denk bodies (MDB), megamitochondria, glycogenated nuclei, acidophil bodies, and spotty necrosis (27,28,30). Ballooning degeneration of hepatocytes is one of the most crucial determinants of NASH diagnosis and refers to the enlargement or swelling of hepatocytes, most often in zone 3 in the midst of surrounding steatotic hepatocytes (27). Morphologically, ballooned hepatocytes have a loss of their normal hexagonal shape, are larger, and retain rarified cytoplasm that may be due to intracellular fluid accumulation (32). It is worth mentioning here that histological identification of ballooned hepatocytes can be quite challenging and it is not uncommon for pathologists to have to distinguish these lesions from swollen, glycogen-rich hepatocytes (29). Additionally, ballooned hepatocytes are often one of the most difficult lesions to consistently reproduce in animal studies of NAFLD. MDB, which are dense eosinophilic intracytoplasmic perinuclear inclusions, are often found within ballooned hepatocytes and result from impaired proteosomal degradation of cytoplasmic proteins, mainly those that bind to ubiquitin (28,30). Often this type of lesion is found within cells of zone 3 and can be detected through immunohistochemistry using monoclonal antibody staining specific to either cytoplasmic filaments or ubiquitin itself (35).

Distorted organelle morphology can also be present in NASH and often this involves the mitochondria. Megamitochondria are observed as intracellular eosinophilic inclusions often in hepatocytes with microvesicular steatosis (29) and ultrastructurally present as multi-lamellar membranes with paracrystalline inclusions, often with an
apparent loss of cristae (28). Whether these mitochondrial abnormalities reflect a progressive adaptation to cellular stress (30) or indeed implicate NASH as a disease of the mitochondria themselves as has recently been proposed (36), is still relatively unclear.

All cells retain the requisite molecular machinery to engage in a program of calculated self destruction referred to as apoptosis (37). Existence of such a dramatic pathway offers protection to the tissue and organism itself from the far more deleterious consequences of uncontrolled release of cytoplasmic contents and unwarranted activation of the immune system, which are features characteristic of a not so coordinated type of cell death, necrosis (33). A thorough description of the molecular details in both modes of cell death will not be attempted here, but thankfully there is a relative abundance of informative reviews available, much of which emanates from research conducted by Gregory Gores and colleagues (38-41). Germane to the current discussion is that in NASH, hepatocytes undergo both forms of death, creating unique histological features. Activation of the apoptotic signaling cascade, either through the extrinsic, death receptor mediated pathway or the intrinsic, intracellular organelle pathway results in the collapse and shrinking of individual hepatocytes along with distinctive chromatin condensation and nuclear fragmentation, culminating in the ‘blebbing’ of the plasma membrane into small vesicles termed apoptotic bodies (40,42). In NASH, the presence of these bodies tends to correlate with the severity of apoptosis itself (43). Whereas apoptosis represents a metabolically active form of cell death, oncostic necrosis occurs in situations when cellular energy in the form of adenosine
triphosphate (ATP) is limited and thus represents a form of uncontrolled cell death (38,39). Hepatocyte swelling, loss of membrane integrity, rupture, and release of cytoplasmic components are distinctive features of oncotic necrosis that typically trigger a robust hepatic inflammatory response. Histologically this can be observed in foci of Kupffer cell aggregates that surround single hepatocytes within the sinusoid (43), however often times both features of cell death coexist in liver pathology. Thus in the pathophysiological setting of NASH it is likely that both apoptosis and necrosis occur, especially considering they share several of the same initiating factors and signals (33).

There comes a point in the NAFLD spectrum, as in other chronic liver diseases, when the liver seeks to protect itself from these previously enumerated onslaughts by enacting a wound-healing response; this is done in an attempt to encapsulate the existing injury and is accomplished through accelerated deposition of extracellular matrix components which collectively give rise to the hepatic scar (44). Such components include a vast array of proteins that include fibril-forming collagens, proteoglycans, and fibronectin and their accumulation reflects both increased synthesis as well as decreased degradation (44,45); here it should be pointed out that the extent of extracellular matrix remodeling which occurs in fibrosis is a chronic process, typically over the course of several years, sometimes even decades, which is in stark contrast to organs such as kidney and lung which have a much more rapid fibrotic progression course (46). These differences are likely due to the unparalleled regenerative capacity of the liver. Accordingly, the liver is quite resilient to the early pathological changes that accompany fibrosis and if the source of injury is eliminated, fibrosis can potentially
reverse course (44). Cirrhosis on the other hand, represents the most advanced stage of fibrosis, is not reversible, and is characterized by even greater hepatic scarring along with the distortion of the hepatic parenchyma and the formation of nodules of regenerating hepatocytes. These alterations to hepatic architecture culminate in impeded blood flow and portal hypertension that substantially increase the risk of overall hepatic failure (46).

The evolution of fibrosis is not surprisingly, quite complex and involves a multicellular collaborative effort, which for brevity's sake will not be further delineated here, although the interested reader should consult the litany of insightful reviews composed by Scott Friedman that have greatly facilitated current molecular and clinical conceptions of this process (44,46-48). The work by Friedman and colleagues throughout the years has demonstrated that the principal mediator of fibrosis is the stellate cell (49), which normally resides in the Space of Disse as a quiescent, inactive cell. When stimulated by appropriate stimuli, be it reactive oxygen species (ROS) due to oxidative stress, apoptotic bodies that are engulfed by Kupffer cells, cytokines or other soluble mediators, stellate cells undergo activation which results in several phenotypic changes that include increased proliferation, contractility, altered matrix degradation, and inflammatory signaling (46).

In general, despite varying etiologies (viral hepatitis, alcohol abuse, iron or copper overload for example) fibrotic development tends to retain several of the same general cellular and molecular features. This does not mean however that the pattern or relative distribution of fibrotic tracts within the lobules themselves are similar in all
instances. A case in point is NASH. NASH has a uniquely distinctive fibrotic signature compared to other chronic liver diseases like hepatitis and cholestatic diseases in that the initial collagen deposition begins within the pericellular or along the perisinusoidal spaces, predominantly within zone 3 (27,29,31,32,34,43). This so-called ‘chicken wire’ type of fibrosis tends to extend and radiate outward from the central veins. As the collagen fibers begin to form bridges across the parenchyma, hepatocytes become trapped and form islands, resulting in seriously distorted hepatic architecture and not surprisingly, impaired functioning of the liver itself (34).

Having discussed, albeit somewhat briefly, the vast extent of pathological changes visited upon the liver during NAFLD, the focus now shifts inwards towards the cellular pathways that, on a molecular and biochemical level, strongly govern these hepatic alterations. The attentive researcher should nonetheless be aware of the necessity of both fields of study; that is, one cannot simply focus on the style or pattern of pathological lesions nor solely on mechanistic data and hope to produce a comprehensive picture of NAFLD pathogenesis. A deeper understanding instead requires an appreciation for both pathology and biochemistry alike.

**A Not So ‘Simple’ Spectrum: Pathological and biochemical anomalies in hepatic steatosis**

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 surfeit metabolic energy in the form of triglycerides (TG). These inert, highly reduced lipid molecules reside within the hydrophobic core of lipid droplets (LD),
specialized organelles designed to, among other functions, shelter neutral lipids like TG as well as cholesterol esters (CE) away from the aqueous cytoplasm (50,51). 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 α (PPARα), which in turn elicits key changes in overall hepatic lipid metabolism (52). Thus by regulating the flux of lipid in cells, LDs serve as crucial components of lipid metabolism, with increasing evidence suggesting a prominent role for these organelles during times of health as well as disease itself (see reference 53 for a recent review).

There is also another side to LDs, which is their participation in the development of steatosis. Mechanistically this occurs when TG begins to be stored in amounts that exceed mechanisms of export and breakdown and clinically manifests when lipid content exceeds 5% of the liver by weight. Normally the liver contains only relatively small amounts of stored lipids, yet in the face of excessive fatty acids hepatocytes can enact several mechanisms that effectively funnel incoming fatty acids towards pathways responsible for TG production, and ultimately, LD incorporation.

At the level of the liver, TG content is reflective of fatty acid uptake (either from circulating fatty acids or dietary lipoproteins), endogenous synthesis, fatty acid oxidation, and secretion in the form of very-low density lipoprotein (VLDL). The latter two mechanisms are the major means hepatocytes use to reduce hepatic TG content (54). Donnelly et al (55), using stable isotope methodology found that the overwhelming
majority, or 60%, of hepatic TG were derived from the plasma fatty acid pool. Approximately 26% of liver TG were accounted for through endogenous production or *de novo* lipogenesis (DNL), while 15% was shown to come from the diet. Thus in this key study the major sources in humans with NAFLD seem to come principally from peripheral fatty acids and DNL. Among some of the offered explanations for steatotic development aside from increased fatty acid uptake and lipogenic production is possible impairments in the mitochondrial oxidation of fatty acids and defective hepatic VLDL assembly and release (56). Available evidence, at least in humans, largely seems to indicate that fatty acid oxidation is normal, or even increased in patients with steatosis as demonstrated by Sanyal et al (57). Using nondiabetic patients with steatosis or NASH, Sanyal et al found that serum beta-hydroxybutyrate levels were greater in both groups compared to non-obese control subjects, although the highest rates were observed in the NASH group. Impaired VLDL assembly or secretion on the other hand, does in fact seem to be implicated in generating fatty liver in humans (58,59). For instance, Charlton et al (58) evaluated the absolute synthesis rate of apoB-100 using stable isotope methodology and found a marked decrease in apoB-100 synthesis in patients with steatosis and NASH compared to controls. Thus while the study by Donnelly et al (55) indicates elevated DNL and uptake of circulating fatty acids as the prime culprits in promoting steatosis it is important to keep in mind the potential participation of impaired mobilization of VLDLs, which is now starting to garner more intensive interest in the field.
Assaults from All Angles: The folly of the two-hit hypothesis in the steatosis to NASH transition

Metabolism in its barest sense represents the totality of all biochemical reactions that enable survival. Of the over 8,700 reactions and 16,000 metabolites documented in the Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg/pathway.html) three core processes predominate: 1) the construction of simple or more complex macromolecules (anabolism); 2) the degradation of molecules for generation of energy (catabolism); and 3) the elimination of reactive and harmful biochemical intermediates (disposal). As the field of biology itself continues to splinter into more refined sub-disciplines with increasing precision and refinement of experimental techniques, there has been a call for a more comprehensive understanding of the nuances of metabolism as it relates to disease. DeBerardinis et al are quite correct in their perspective of the impossibility to view metabolism “merely as a self-regulating network operating independently of other biological systems” (60). Certainly any perturbation within a particular metabolic pathway will affect the performance of other metabolic routes in a particular physiological context. Pathological conditions such as diabetes, insulin resistance, cardiovascular dysfunction, and NAFLD, among others, are poignant illustrations of distinct disturbances within the metabolic web that wreak havoc both locally within cells and globally throughout the organism. Thus, just as ripples in a pond extend beyond the pebble, interruptions in metabolic nodes create ripples throughout the entire biological network leaving no system unscathed.
This comprehensive approach to metabolism and how it factors into the genesis of disease seems an appropriate philosophy to adopt towards NAFLD, whose pathophysiology involves multiple derangements within several distinct, but interconnected hepatic pathways. Accelerated flux of metabolites (glucose, fructose, certain amino acids) through the lipogenic pathway, dysregulated synthesis of VLDL particles, inefficient decarboxylation and oxidation of fatty acids (both mitochondrial and peroxisomal), and inappropriate handling and storage of fatty acid pools are the primary mechanisms at the level of the liver that are responsible for steatosis, the *sine qua non* of NAFLD. External to the liver are adipocytes that through alterations in their own signaling pathways are unable to respond to insulin’s suppressive effect on lipolysis, producing high levels of unesterified fatty acids that mobilize to the liver. The situation becomes even more daunting during the inflammatory transition of steatosis to NASH. Here, metabolically stressed organelles like the mitochondria and endoplasmic reticulum (ER) react to a variety of cellular insults by engaging pro-inflammatory mechanisms such as apoptosis, ER stress, and the unfolded protein response (UPR) that promote organelle destruction and cellular death, excessive release of dangerous pro-inflammatory mediators, alterations in nutrient utilization and energy production, and most seriously, distort liver architecture to produce the conspicuous inflammatory infiltrates, ballooned hepatocytes and cross-linked fibrotic tracts that define NASH.

So, not only does one have to be cognizant of metabolic interruptions within the liver in understanding the emergence of NAFLD—we would also do well here to remember the diversity of the liver cell population and that these metabolic ripples
extend throughout hepatocytes as well as stellate and Kupffer cells alike- but also within the adipose and to some extent, skeletal muscle as well. To gain some insight as to why a broader, more encompassing metabolic perspective is better suited towards the study of NAFLD we need to first revisit those earlier conceptions articulated by Christopher Day and Oliver James in what has come to be known as the “two-hit” hypothesis (61).

In reflecting on the years following Ludwig et al’s publication of NASH as a distinct, pathological phenomena it seems fair to say that by the late 1990’s, research had come to somewhat of a crossroads. For instance it was understood that many patients with severely fatty liver often never progressed to steatohepatitis (62). Somewhat counter intuitively, it was also known that despite varying etiologies, the progression of steatohepatitis could be characterized by several of the same histological features. This prompted researchers to ask whether there was the existence of some unifying mechanism that could sufficiently explain both the tendency of certain individuals to progress beyond steatosis as well as histological similarities that present during different clinical situations.

In 1998, Day and James in an editorial published in the journal Gastroenterology (61), posited that one such unifying mechanism linking steatosis to hepatic inflammation and fibrosis was lipid peroxidation. Citing data produced by Dominique Pessayre’s lab in which rat liver mitochondria, hepatocytes, or rats themselves were exposed to drugs capable of producing so called ‘classical’ steatohepatitis (cationic amphiphilic amines) (63), Day and James hypothesized that
lipid peroxidation potentially “explains most, if not all, of the diverse histological features observed in this condition”. In Berson et al’s study, the administration of these drugs inhibited mitochondrial β-oxidation and respiratory capacity and increased the production of various reactive oxygen species (ROS), triggering a 5-10 fold increase in lipid peroxidation. The appeal of these data for Day and James was that they convincingly demonstrated that a cellular insult capable of initiating lipid accumulation, in this case the inhibition of fatty acid oxidation, could serve as a first “hit”. The impairment of mitochondrial respiration and function that followed could then theoretically serve as a second “hit” in which free radicals emanating from the mitochondria were free to attack susceptible fatty acid chains of membrane polyunsaturated fatty acids (PUFA). According to this hypothesis the capability of certain bi-products of 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.

It may help to consider for a moment the ‘lipocentric’ nature of the “two-hit” hypothesis. Lipids, as fatty acids are here needed first to secure the development of steatosis which emerges from an overload of the oxidizing systems responsible for fatty acid deconstruction. Such an overabundance of fatty acids in turn increases the availability for them to serve as substrates for the principal microsomal cytochrome P450 enzymes, CYP2E1 and CYP4A. Normally these enzymes function to hydroxylate and convert non-polar substrates, including fatty acids, to more polar compounds for eventual conjugation or excretion. As their catalytic activity is dependent on oxygen
however, there are a great many harmful metabolites produced from this system and it is these superoxide anions (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (·OH), that when produced in amounts exceeding antioxidant species lead to a state of cellular oxidative stress (64). ROS can also be produced from complexes of the mitochondrial respiratory chain and because of their fairly short half-life, many of the cellular effects occur in a close proximity to mitochondria themselves; these local effects include the depletion of adenosine triphosphate (ATP) and nicotinamide dinucleotide (NAD), damage to cellular macromolecules such as DNA, protein denaturation, and not surprisingly the reduction of antioxidant defenses, in particular glutathione (65). Here again is where lipids come into play. With limited capacity for cellular defense free radicals (in particular the hydroxyl radical) are able to migrate unopposed into membrane lipid bilayers and attack vulnerable unsaturated side chains of membrane PUFAs, owing to their decreased carbon to hydrogen bond strength. This in turn creates lipid hydroperoxides that release aldehyde bi-products, of which 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA) are of considerable importance in biological systems (66). Longer lived than free radicals, 4-HNE and MDA pose a much greater cytotoxic risk to cells as they can readily diffuse from membranes and attack distant intracellular or extracellular targets (66); these attacks can include the covalent modification of cellular proteins into adducts and the disruption of organelle membrane integrity. Lipid peroxidation itself also acts as an amplifier of free radical and aldehyde generation, further depleting antioxidant capacity and thereby exacerbating the state of oxidative stress (67). Lastly, aside from impairing organelle function, aldehydes such as
4-HNE and MDA can directly induce the production of cytokines, in particular tumor necrosis factor-α (TNF-α), which in turn acts as a chemoattractant for neutrophils leading to chemotaxis, inflammation, and eventually apoptotic-mediated cell death. Hepatic stellate cells, the principal mediators of liver fibrosis can also be directly activated following membrane lipid peroxidation through the production of transforming growth factor β (TGF-β) (68,69).

Though the ‘two-hit’ hypothesis has commandeered the attention of researchers of NAFLD there are several emerging issues that have caused this framework to be questionned. As discussed in detail in chapter 1 of the upcoming literature review, the concept that liver TG accumulation is a necessary condition for the transition from steatosis to NASH is in need of a serious re-evaluation in light of powerful studies which demonstrate quite the opposite (70-72). In fact, TG accumulation likely serves a hepatoprotective function by sheltering bioactive fatty acids in the TG molecule and it is only when the fatty acids are mobilized from TG that they have the opportunity to serve as signaling molecules in lipotoxic processes. Examples of such species include non-esterified fatty acids (primarily saturated) which trigger apoptotic activation (71), diacylglycerols which interfere with insulin signaling (73-75), and cholesterol, specifically in its free form (FC) (see following chapter).

**Of Mice, Men, and Everything in Between: Animal models in NAFLD research**

Because of the inherent difficulty and ethical considerations involved in obtaining a liver biopsy, research in animals provides an advantageous approach to studying different facets of NAFLD. For example, dietary (76) and genetic manipulations (77-86)
can be combined or studied separately in order to tease apart the many manifold events in NAFLD propagation. Much of the work to date is centered around the mouse or rat, which provide the overwhelming source for much of the genetic manipulations currently available, though studies using non-genetically modified rabbits are starting to emerge as viable alternatives, especially with regard to liver disease and excessive dietary cholesterol (87,88). Even less utilized and appreciated is the guinea pig, an animal that more closely resembles humans with regards to lipoprotein metabolism and which also handles hepatic cholesterol similarly (see next section). Currently there are very few publications utilizing guinea pigs to study NAFLD, though it appears the tide may be turning (89,90). It has even been proposed that guinea pigs, rather than being appropriate models for atherosclerosis, are in fact much more appealing for liver diseases, NAFLD in particular (91).

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**LITERATURE REVIEW: CHAPTER 1**

*Key aspects of cholesterol homeostasis, the interaction of cholesterol, phospholipids, and triglycerides, and emerging role in NAFLD progression*

Cholesterol. What does one think of when confronted with this seemingly loaded word? For some, particularly biophysicists and biochemists, thoughts presumably turn
to the crucial biophysical properties of this molecule in cellular membrane physiology and eukaryotic cell survival. Nutritional scientists on the other hand, likely direct their attention to the essentiality of cholesterol in dietary lipid absorption and capacity to serve as precursors for the production of key vitamins and reproductive hormones, while still others like clinicians, focus on the pathophysiological ramifications of excess cholesterol in human disease. Indeed, cholesterol is responsible for a vast realm of biological functions, so it is no wonder that it spurs such intense scientific inquiry in so many diverse research fields. It is puzzling though, how such a molecule so crucial to survival itself can also simultaneously pose such severe physiological and pathophysiological risks, and how this nefarious aspect of cholesterol’s identity tends to predominate in the minds of many scientists as well as the lay masses themselves. While certainly dysregulated cholesterol metabolism is of major importance to the emergence of many devastating disease states, atherosclerotic vascular disease in particular, it should be kept in mind that these pathophysiological ramifications take years, sometimes decades, to develop and only after a myriad of regulatory mechanisms governing uptake, synthesis, and transport are eventually overwhelmed. Evolution it seems, has equipped cells with quite the armamentarium to handle this essential, yet potentially dangerous lipid.

The sheer magnitude of biological functions that cholesterol is responsible for is staggering. As a conspicuous component of cellular membranes, cholesterol’s presence influences membrane fluidity, modulates the functions of integral membrane proteins, and enables the formation of lipid raft regions critical for membrane signaling cascades
It is involved in the conductance of neurotransmissions along neuronal axons as a rich lipid component of myelin. Along with these membrane effects, the fused, rigid four-ringed steroid backbone can undergo a series of oxidative cyclizations and demethylation reactions to yield valuable bioactive molecules, notably bile acids, cholecalciferol (vitamin D), and steroid hormones, which in turn can participate in such critical processes as dietary lipid absorption, calcium metabolism, and hormonal regulation of reproduction. Given the breadth of detail involved, these processes will not be further expanded upon, however the interested reader is directed to several erudite reviews that discuss thoroughly the physiology of cholesterol. In particular, Ira Tabas’ perceptive perspective offers one of the most insightful reads into the uniqueness of the cholesterol molecule itself.

Because cholesterol is now increasingly being recognized as a major culprit in diseases other than atherosclerosis and cardiovascular disease (CVD), particularly those of hepatic and neurological origin, it seems as urgent a time as ever to understand the regulatory schemes fundamental for maintenance of cholesterol homeostasis. Knowledge of the intricacies surrounding this unique and often underappreciated molecule will likely begin to help clarify as to how it participates in the progression of these other diseases, of which NAFLD is of particular salience. Therefore it is the aim of this chapter to briefly describe the major aspects of cholesterol metabolism; that is, the uptake of exogenous (dietary) cholesterol by the intestine and liver, the hepatic uptake of endogenous cholesterol from plasma-derived lipoproteins, and finally, the molecular regulation of cellular cholesterol homeostasis. A brief
discussion then follows of studies that illustrate the role of elevated cholesterol in NAFLD and NASH pathogenesis. Lastly, the chapter will conclude by emphasizing the guinea pig as an experimental approach to understanding the nexus between disturbances in hepatic cholesterol metabolism and NAFLD progression.

**Deliverance: Intestinal and hepatic cholesterol acquisition**

Higher eukaryotes such as mammals are endowed with the ability to absorb dietary sterols, including cholesterol, from the luminal environment of the small intestine. There is however, no actual dietary requirement for cholesterol itself as virtually all cells can construct the molecule *de novo* from a single precursor, acetate. The routine of dietary cholesterol absorption can be conceived of as three distinct phases (8). The first, or intraluminal phase, involves a coordinated release of pancreatic lipases and esterases which attack incoming lipid emulsions containing dietary cholesterol, triglycerides, and phospholipids. Much of the cholesterol acquired from the diet exists in its free, unesterified form (FC), with less than 15% existing in its esterified form (CE) (9). Pancreatic cholesterol esterase acts upon any CE molecules, cleaving the fatty acid from the A ring creating FC. Because of the near absolute insolubility of FC in pure aqueous environments, the molecule requires rapid solubilization in order for efficient absorption and this is provided through the detergency of bile acids. These amphipathic compounds aggregate spontaneously, forming simple micelles when their concentration exceeds the critical micellar concentration. While thermodynamically stable these aggregates can only solubilize a small amount of cholesterol, however when they combine with various fatty acids, monoglycerides, and lysophospholipids, mixed
micelles are formed. These are larger aggregates whose formation greatly enhances the solubility of cholesterol (within the hydrophobic core) and which also enable dietary lipid transport through a formidable diffusion barrier, the unstirred water layer, which lies at the interface between the water phase of the intestinal lumen and the apical membrane of the enterocyte (9). The obligatory nature of bile acids in promoting efficient intestinal cholesterol absorption should not be understated (10). Following transport the mixed micelles then disaggregate, dispersing lipid contents that include significant amounts of FC which is then ready for uptake by the intestine. It should also be pointed out that intestinal cholesterol uptake refers to the actual entry of FC and CE into enterocytes from the lumen. This is distinct from intestinal cholesterol absorption, which as previously defined (9), refers to the transfer of luminal cholesterol to the lymphatic system throughout the entirety of the small intestine although the majority occurs in the duodenum and proximal jejunum.

The second phase of intestinal lipid absorption involves the uptake of cholesterol, the vast majority of which is unesterified, across the enterocyte brush border. Until 2004, intestinal sterol influx was largely perceived of as a passive diffusion process with the incoming hydrophobic cholesterol passing down a concentration gradient through the enterocyte plasma membrane (8,10), although evidence did exist that collectively suggested the existence of an elusive sterol transport protein (11). Moreover, heterogeneous responses and interstrain differences in humans and rodents, respectively, pointed to the likelihood of genetic regulation in this process. Using a genomic-bioinformatics approach with intestinal scrapings from rat, Altmann et al...
successfully identified Niemann-Pick C1 Like 1 (NPC1L1) as the putative intestinal cholesterol transporter (12). Not only was NPC1L1 enriched and localized to the enterocyte epithelial layer bordering the luminal space, but NPC1L1 null mice also had a 69% reduction in the absorption of radiolabeled cholesterol and did not respond when treated with a known pharmacological inhibitor of intestinal and biliary cholesterol, ezetimibe. Following the identification of NPC1L1, it was shown subsequently that this transporter not only was responsible for the uptake of cholesterol, but also the structurally related plant sterols, campesterol and sitosterol, as their plasma concentration in NPC1L1 null mice were reduced by more than 90% as compared to wild type mice (13). Genetic inactivation of NPC1L1 in mice also was found to dramatically reduce circulating plasma cholesterol on a high cholesterol, high-fat Paigen diet and prevented NPC1L1 null mouse livers and gallbladders from cholesterol-induced hepatic steatosis and cholestasis respectively (14). Interestingly this same study also demonstrated through messenger ribonucleic acid (mRNA) profiling experiments that humans and mice differ strikingly in the tissue expression of NPC1L1, with humans exhibiting the greatest expression in both the small intestine, as well as liver. In contrast, murine NPC1L1 is expressed primarily in the small intestine only (14). Direct biochemical (15) and molecular (16) evidence later emerged which unequivocally established NPC1L1 as the target of ezetimibe and hence as the bona fide intestinal cholesterol transporter.

The cellular basis for NPC1L1-mediated cholesterol transport has recently begun to be unraveled and provides an interesting insight as to how intestinally derived
cholesterol travels into enterocytes (see references 11 and 17 for detailed reviews that cover this process). Although not completely understood it appears that NPC1L1 functions in a cholesterol-sensitive endocytotic pathway (18); under conditions of cellular cholesterol depletion, NPC1L1 travels from the endocytotic recycling compartment to the apical plasma membrane where it binds and facilitates cholesterol uptake. The polytopic NPC1L1 then disappears from the plasma membrane and localizes to intracellular compartments where it releases its cholesterol load, therefore able to return to the cell surface when cholesterol is again limited. Ge et al (19) further demonstrated that FC promotes the internalization of NPC1L1 and that this requires microfilaments and the clathrin complex, which is important for endocytosis itself. Importantly, NPC1L1 transports only FC, not CE, which makes this pathway distinct from other membrane cholesterol receptors, particularly the low-density lipoprotein receptor (LDLR) and the scavenger class B type 1 receptor (SR-B1) which transport newly uptaken CE (11).

The third and final phase of cholesterol absorption involves the trafficking and incorporation of absorbed FC into chylomicrons, massive macromolecular spherical complexes composed of amphipathic phospholipids, apolipoproteins, triglycerides, and cholesterol. As mentioned previously, the overwhelming majority of cholesterol entering enterocytes is FC which if not handled properly, can elicit severe cytotoxic effects in cells (20). Fortunately cells have an efficient buffering system in place to prevent such occurrences, and this is mediated by acyl-CoA cholesterol acyltransferase (ACAT), which enzymatically transfers monounsaturated fatty acids (MUFA) such as
palmitoleate or oleate to the hydroxyl group on the third carbon of FC forming an esterified, and ultimately more hydrophobic molecule. In the major lipoprotein producing cells, that is, enterocytes and hepatocytes, ACAT-2 is the major isoform and is present as an ER-bound membrane protein (21). ACAT-mediated esterification enables the newly formed CE to package with newly synthesized triglycerides, phospholipids, and the substantially complex apolipoprotein B (apoB), lipidating and inflating the prechylomicron particle until it is ready for eventual egress into the lymphatic system. Exactly how the incoming FC is directed to the ER is still not defined, though it is likely that some form of vesicular or non-vesicular transport mechanisms exist. Dietary-derived cholesterol represents only a small fraction of chylomicrons, with FC and CE constituting 1% and 3% by weight respectively and this is due to the rich concentration of triglycerides which make up about 85% of the total weight of the particle. Because CE cannot intercalate into lipid membranes it is found stored within the huge hydrophobic core whereas the FC is scattered about the surface, helping to stabilize the spherical structure. The itinerary that enterocytes use to synthesize these gigantic particles is complex (see references 22-24 for informed discussions), yet at least for cholesterol it seems that ACAT-mediated esterification is the pivotal step in determining how much dietary-derived cholesterol gets packaged and ultimately, secreted from the intestine. For instance, mice that are genetically manipulated to have an intestinal-specific knockout of ACAT-2 have blunted cholesterol absorption and reduced plasma cholesterol, yet increased concentrations of total and free cholesterol along with a pronounced reduction of CE in intestinal segments (25,26). These findings would seem
to indicate that inhibition of cholesterol esterification at the level of the intestine is sufficient to prevent cholesterol accretion in other compartments such as plasma and liver and has prompted some (25) to speculate that ACAT-2 inhibition may be atheroprotective. One could contend however that limiting CE formation at the expense of increasing cellular FC concentration is a dangerous trade-off given the ramifications of excess FC accumulation (20).

To get to the liver, dietary cholesterol must first travel through the lymphatic vessels and venous circulation, sheltered within the core of chylomicrons. The myriad events that occur during chylomicron transport have been well studied and defined and for brevity’s sake will not be further discussed. (Two indispensible reviews (27,28) that explain beautifully the lipoprotein transport system and its intricacies are provided by Michael Brown and Joseph Goldstein, the proverbial ‘pioneers’ of cholesterol metabolism and discoverers of the LDLR). The focus here instead is the uptake of chylomicron delivered cholesterol by the liver.

Within the capillaries of adipose and muscle tissues lipoprotein lipase (LPL) depletes chylomicrons of their triglyceride cargo by liberating free fatty acids and monoglycerides, thereby forming a chylomicron remnant. This remnant, though smaller than the original particle (300-800 angstroms as compared to 800-5000 angstroms) is still quite large and must somehow traverse the fenestrated endothelial cells that line the hepatic sinusoids. This is provided through direct binding of the apolipoprotein E component to various heparan sulfate proteoglycans lining the Space of Disse (28), or alternatively, the remnants can be uptaken directly by the LDLR (via recognition of the
apolipoprotein E component). Hepatic LDLRs are the primary conduit for lipoprotein entrance into the liver and they function by plucking circulating lipoprotein particles from plasma (27), and are integral to the regulation of plasma cholesterol concentrations. To enter into hepatocytes, chylomicron remnants and other lipoprotein species including VLDL remnants, and LDL particles must first be internalized; by binding to the LDLR, a series of events is enacted in which the particle and receptor become internalized via a process known as receptor-mediated endocytosis (2). In terms of the particles’ cholesterol cargo, both the free and esterified molecules are liberated and travel through a coordinated organelle system known as the endosomal system, eventually entering into acidic lysosomes, and finally, traverse the lysosomal membrane and exit towards the ER (2). It is at this critical organelle that cholesterol exerts its homeostatic mechanisms through a remarkable sequence of molecular and cellular events, and where the spotlight is now directed.

**Keeping the Peace: Mechanisms of hepatic cholesterol homeostasis**

Cellular cholesterol concentrations are meticulously controlled by an array of membrane bound sterol sensing proteins that coordinate cholesterol biosynthesis and uptake through a highly regulated feedback system. This system functions at transcriptional and post-transcriptional levels, revealing an exceedingly complex cellular program devoted to maintain cholesterol and lipid homeostasis. Pivotal to the maintenance of cellular cholesterol concentrations are the ER-membrane embedded SREBP transcription factors which upon proteolytic cleavage travel to the Golgi complex
and then the nucleus to initiate transcription of genes involved in cholesterol biosynthesis and uptake.

In the mammalian genome three SREBP isoforms are present as SREBP-1a, SREBP-1c, and SREBP-2. Both SREBP-1a and 1c are produced through alternative transcription start sites on exon 1 whereas SREBP-2 is encoded by a separate gene located on human chromosome 22 (29). The physiological roles of the SREBP isoforms differ, with SREBP-1a being a strong activator of all SREBP target genes and SREBPs-1c and 2 confined to more restricted roles. SREBP-1c preferentially activates transcription of genes involved for fatty acid synthesis whereas SREBP-2 enhances genes required for cholesterol synthesis (29). Tissue and organ analysis have revealed that SREBPs-1c and 2 are primarily localized to the liver and several other cholesterol dependent tissues whereas in cultured cell lines SREBPs-1a and 2 are the predominant isoforms (30).

Immediately after their synthesis, SREBPs are anchored to ER membranes as inactive precursors through two membrane spanning helices and adopt an orientation in which both the amino-terminal and carboxy-terminal domains face the cytoplasm. All SREBP precursors consist of 1,150 amino acids and are organized into an amino-terminal domain which contain a bHLH-Zip crucial for binding to DNA and a carboxy-terminal regulatory domain of roughly 590 amino acids. The two transmembrane domains are separated by a small loop of 30 amino acids which projects into the ER lumen. SREBPs remain anchored to the ER membrane through an interaction between the regulatory carboxy-terminal domain of the SREBP and the cytosolically-oriented
carboxy terminal domain of the polytopic membrane protein SREBP-cleavage-activating protein (Scap). The carboxy terminal of Scap consists of multiple tryptophan and aspartate repeats (WD repeats) which foster protein-protein interactions (31).

To serve as a transcription factor, SREBPs must first exit the ER membrane bound to Scap and then travel to the Golgi complex where two sequential proteolytic cleavages occurs. Once the Scap/SREBP complex enters the Golgi, the complex dissociates and the SREBP becomes embedded in the Golgi membrane where it encounters two membrane bound proteases designated as Site-1 (S1P) and Site-2 (S2P). S1P is a serine protease that cleaves the SREBP in the luminal membrane spanning loop, thereby cleaving the SREBP in half (32). The amino terminal half of SREBP then serves as the substrate for the S2P, a zinc metalloproteinase that cleaves at a site within the membrane spanning helix thereby releasing the active amino-terminal domain located within the cytoplasm (33). The amino-terminal domain is designated nSREBP which then enters the nucleus and binds to sterol response elements (SREs) composed of the E-box inverted DNA repeat (5’-CANNTG-3’) as well as the direct DNA repeat (5’-TCACNCCAC-3’) (29).

Transport of the Scap/SREBP complex from the ER is a process highly regulated by cholesterol and oxysterols, potent cholesterol derivates with additional hydroxyl or keto groups at the 7-position on the B ring or at several positions on the side chain. When cellular concentrations of sterols decline, Scap escorts the SREBP from the ER and travels to the Golgi for proteolytic processing, thereby enabling nSREBP to bind to SREs located within promoter/enhancer regions of specific genes dedicated to
cholesterol synthesis and uptake including HMG-CoA reductase, the rate-limiting enzyme of cholesterol biosynthesis which converts HMG-CoA to mevalonate, and LDLRs. Conversely, if cellular sterol concentrations are elevated, Scap serves as a sterol sensor and retains the SREBP within the ER membrane thereby inhibiting Scap/SREBP transport to the Golgi, formation of the transcriptionally active amino terminal SREBP fragment, and transcription of SREBP target genes. Scap exhibits dual roles in maintaining cellular cholesterol balance, both as an escort protein and as a sterol sensor and therefore represents a crucial feature of this highly regulatory system.

Scap is a polytopic ER membrane protein composed of approximately 1,277 amino acids which contain two domains of between 600-700 amino acids each. The amino-terminal domain embeds the protein in ER membranes through a series of eight transmembrane helices, each of which are separated by hydrophilic loops. The carboxy terminal domain is composed of multiple copies of a tryptophan and aspartate (WD) repeat sequence, which serves to bind the carboxy terminal domain of SREBPs, thereby retaining SREBPs within the ER membrane (31). Within the amino terminal domain, transmembrane helices 2-6 represent the intramembrane sterol-sensing domain (SSD), a sequence of five transmembrane helices consisting of roughly 180 amino acids which play essential roles in controlling lipid homeostasis and facilitating lateral membrane protein movement (34, 35). Originally identified in Scap (34), SSDs have been identified in several other polytopic membrane proteins including HMG-CoA reductase, the Niemann Pick C1 protein (NPC1), a lipid transport protein involved in the
intracellular trafficking of cholesterol, and 7-dehydrocholesterol reductase, one of the final enzymes catalyzing the formation of cholesterol (36).

The dual roles of Scap as both a sterol sensor and escort protein were originally established in studies of mutant Chinese hamster ovary cells (CHO) that displayed defects in the regulation of cholesterol synthesis (37). In cells engineered to lack Scap, SREBPs are retained in the ER membranes and the cells require exogenous cholesterol administration for growth (38). Cells that contain point mutations in Scap, specifically at amino acids D443N or Y298C display sterol resistance in which the Scap sterol sensing function is diminished and the mutant Scap escorts SREBPs to the Golgi complex even in the presence of elevated cellular cholesterol (39). This ultimately leads to a situation of endogenous cholesterol overproduction and cellular overloading. These point mutation experiments, along with others (39) occur within the SSD of Scap and solidified this region of Scap as a 

bona fide

SSD. Thus, in the presence of sterols, Scap remains bound to SREBP in the ER membrane, access to proteolytic cleavage is denied, and transcription of cholesterol synthesizing enzymes declines.

In situations when cellular cholesterol levels are elevated, Scap remains embedded in the ER membrane bound to SREBPs and cholesterol biosynthesis is abolished; this inhibition is contingent on the sterol-sensing function of Scap as well as binding of the Scap/SREBP complex to Insigs, newly characterized ER retention proteins (40). Two Insig isoforms, Insig-1 and Insig-2 have been successfully identified as Scap binding proteins (41) and are central to the suppression of SREBP Golgi transport and nuclear import; remarkably these proteins also serve a crucial role in the
sterol-mediated ubiquitination and degradation of HMG-CoA reductase, also embedded
in the ER membrane (42). Cholesterol binds to the SSD of Scap and triggers a
conformational change thereby enabling its binding to Insigs (41) however cholesterol
does not participate in HMG-CoA degradation; it appears that lanosterol, a cholesterol
precursor, is a much more potent stimulus for this degradation. Elevated lanosterol
concentrations are presumed to interact with the SSD of HMG-CoA reductase which in
turn triggers the binding of the enzyme to Insig proteins, which ultimately facilitate the
degradation process (43). Why lanosterol is the primary regulator of this process is
relatively unknown, however it may be due to the cytotoxic nature of lanosterol itself
(44). By stimulating the degradation of the reductase, lanosterol inhibits its own
production farther along the cholesterol synthetic pathway and thus prevents excessive
accumulation. Cells can then efficiently metabolize lanosterol, thereby avoiding the
detrimental effects of this molecule (43,44).

The understanding that eukaryotic cells regulate cholesterol and lipid
homeostasis by the coordinated movement of ER membrane bound transcription factors
to the Golgi and eventually to the nucleus has fostered intense research efforts
dedicated to understanding the molecular events by which this process occurs.
Elevated concentrations of cholesterol and oxysterols in cells selectively inhibit this
process by facilitating Scap/SREBP binding to Insig-1, thus retaining this complex
entirely within ER membranes. When cells are depleted of cholesterol, the Scap/SREBP
complex exits the ER by clustering laterally along with other proteins into COPII-coated
vesicles, which then bud from the ER (45, 46). COPII binding begins with the
attachment of Sar1, a small GTP binding protein which attaches to ER membranes by exchanging GTP for GDP. The now membrane bound Sar1 subsequently attracts the Sec23/24 complex. The Sec23 component of this heterodimer can recruit additional proteins, notably the Sec13/31 complex, which forms the coat of the COPII vesicle (47). Binding of Scap to these vesicles is accomplished through an interaction between Sec24 and a MELADL (methionine, glutamate, leucine, alanine, aspartate, leucine) hexapeptide sequence of Scap, which is located in the cytoplasmic loop between the transmembrane helices 6 and 7, beginning at amino acid residue 447 and ending at residue 452 (48). This sequence was identified through several mutagenesis studies in which it was determined that mutations within this sequence abrogate Sec23/24 binding to Scap and thus inhibit Scap/SREBP incorporation into COPII vesicles for export to the Golgi (49). As cells begin to build up cholesterol, Scap undergoes a conformational change causing it to bind to Insig-1. The binding of Scap to Insig-1 precludes the binding of the Sar1/Sec23/24 complex to Scap and the Scap/SREBP complex ultimately remains in the ER membrane bound to Insig-1. While many aspects of this regulatory scheme remain to be answered, it is clear that that cells employ a remarkable spectrum of regulation and feedback control in monitoring cellular cholesterol levels. The elucidation of these molecular mechanisms provides a stunningly comprehensive picture that helps explain the long recognized ability of cells to inhibit cholesterol uptake and synthesis when concentrations are elevated.

*Agents Provocateurs: Hepatic free cholesterol, ER stress, and organelle dysfunction in NASH progression*
Experiments conducted in cells have shown convincingly that cholesterol loading induces cytotoxicity of the ER (50), depletes mitochondrial glutathione content and sensitizes cells to cytokine-mediated NASH (51). Additionally, a recent metabolomic assessment concluded that dietary cholesterol was a causal factor in both steatosis and hepatic inflammation (52). 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 (53) and through synergistically interacting with dietary fats (54,55). Dietary cholesterol also further distorts hepatic architecture and promotes injury in hypercholesterolemic mouse models such as \emph{LDLR} and \emph{ApoE} knockout mice (56,57). Most importantly human studies have also demonstrated a resounding connection between dysregulated hepatic cholesterol metabolism and the severity of NAFLD progression (58). In fact, the very appearance of hepatic cholesterol crystals in both mice and humans appears to distinguish between steatosis and NASH as shown by Ioannou et al (59). In a very recent study the group of Geoffrey Farrell showed quite convincingly that in an insulin-resistant, obese genetic mouse model (\emph{foz/foz}), NASH was successfully induced by feeding a cholesterol-rich diet, and perhaps more significantly, was completely abolished when pharmacological cholesterol-lowering agents (ezetimibe/atorvastatin) were administered (60). Musso et al (61) have compiled the most comprehensive review to date regarding the molecular and biochemical events initiated by excess cholesterol in NAFLD pathogenesis. It is anticipated that more publications will emerge in the near future, considering the mounting evidence that now exists against excessive dietary cholesterol. Now is the
time for serious investigations into other ramifications of excess cholesterol, beyond the arterial wall.

*The ‘Common’ Guinea Pig: Opportunities for investigating hepatic cholesterol and NAFLD*

The gamut of animal models for NAFLD is extensive and involves an array of both genetic and dietary models, the majority of which are mice and rats (62,63). Genetically altered models of hepatic steatosis often display increased hepatic lipogenesis (*PEPCK-n-SREBP-1α* mice) or decreased hepatic fatty acid oxidation (*PPARα* −/− mice). Models like the *ob/ob* mouse and *fa/fa* rat have naturally occurring mutations in the synthesis of leptin, a satiety hormone produced predominantly by white adipocytes, and in the gene encoding the leptin receptor respectively. As a consequence these animals exhibit severe disturbances in energy metabolism and develop hyperphagia, hyperinsulinemia, hyperglycemia, dyslipidemia, and spontaneously fatty livers (62) thereby making them a worthy model for NAFLD etiology. Phenotypically normal rodents have also been utilized to investigate the effects of dietary composition and individual nutrients on hepatic steatosis. Rodent diets high in fat, sucrose, or fructose have been developed to enhance hepatic lipogenesis while methionine- and/or choline deficient diets are often implemented to impair fat oxidation. Taken together, these genetic and dietary rodent models constitute the conceptual framework for our current understanding of the molecular and metabolic processes germane to steatosis and NAFLD.

The reality however, is that mice and rats differ substantially from humans in the genetic expression and activity level of several genes involved in hepatic lipid and
cholesterol metabolism, thus the pathogenesis of this disease in these animals does not appropriately emulate that of the human situation. Rather than relying solely on the tools of molecular biology to procure transgenic or genetic knock-out animals researchers must also direct their efforts towards identifying species that naturally replicate human metabolism. An ideal model should exhibit similar genetic and enzymatic programs related to the pathway(s) of interest and respond to dietary, exercise, or pharmacological treatments in a manner analogous to that of human interventions. Indeed, the guinea pig is perhaps the most excellent representation of such a model with regards to hepatic and whole body trafficking and processing of lipids and cholesterol and may represent a novel template for elucidating the true mechanisms implicated in human steatosis and NAFLD.

The most drastic difference between guinea pigs and rodents is that the majority of cholesterol is transported within LDL particles (64), an observation which has made guinea pigs the ultimate animal model for human lipoprotein metabolism (65); this does not hold true for rats or mice which predominantly carry cholesterol in HDL particles (66). Furthermore, similar to humans, guinea pigs display a high LDL-to-HDL ratio (67) and possess several key enzymes involved in the intravascular processing of lipoproteins, notably CETP, lecithin cholesterol acyltransferase (LCAT), and LPL (65). These inherent similarities between guinea pigs and humans implicate this animal as a bona fide model for lipoprotein metabolism yet may also expand current knowledge pertaining to NAFLD and cardiovascular disease. NAFLD has been proposed as the hepatic manifestation of the metabolic syndrome (68) and is also closely linked to
cardiovascular dysfunction (69,70). Given the striking similarities between humans and guinea pig lipoprotein metabolism, in conjunction with recent research that has suggested guinea pigs as a suitable model to evaluate inflammation and atherosclerosis (71), it appears feasible that guinea pigs may represent an innovative approach to discerning the mechanisms by which NAFLD contributes to the pathophysiology of cardiovascular disease.

Guinea pigs also resemble humans in the synthesis, storage, and catabolism of cholesterol in the liver. This observation is particularly relevant for hepatic steatosis and NAFLD, as emerging research has suggested cholesterol as a central molecule in the transition from hepatic steatosis to NASH. Although excessive cholesterol storage may not solely cause hepatic steatosis per se, the accumulation of free cholesterol may exacerbate the degree of steatosis and hepatocellular dysfunction in humans. Both humans and guinea pigs exhibit higher concentrations of hepatic free cholesterol versus esterified cholesterol (72) as well as similar enzyme activities of ACAT, the ER-localized enzyme that esterifies cholesterol, and HMG-CoA reductase (73). Moreover, guinea pigs demonstrate moderate rates of hepatic cholesterol synthesis (67) and catabolism (74) comparable to humans. While it remains unclear as to whether the expression and activity of enzymes involved in hepatic fatty acid synthesis and combustion correlate between guinea pigs and humans, the striking similarities in hepatic cholesterol metabolism warrant the use of this animal as a novel model for elucidating the role of cholesterol in the progression of hepatic steatosis and the transition to more advanced stages of NAFLD. Lastly, guinea pigs have been repeatedly demonstrated to respond to
dietary interventions, particularly carbohydrate restriction, in a manner analogous to humans (75,76) and given the potential therapeutic role of carbohydrate restriction in attenuating NAFLD (see following chapter), guinea pigs may represent the most appropriate means to evaluate the role of dietary composition on NAFLD, thus standing at the crossroads between laboratory research and clinical application.

LITERATURE REVIEW: CHAPTER 2

Carbohydrates and fats in hepatic lipid accumulation and dietary modulation of NAFLD

Glucose Fattens Up: De novo lipogenesis and hepatic steatosis
The biochemical transformations of carbohydrates, notably the aldo- and ketohexoses glucose and fructose to varying fatty acid species for cellular storage as TG-a tightly choreographed process referred to as de novo lipogenesis (DNL)-offers a unique insight into metabolic regulation as a whole. Engaging glycolysis, the tricarboxylic acid (TCA) cycle and fatty acid biosynthesis, the lipogenic program responds to numerous regulatory signals emanating from dietary macronutrients and hormones whose effects are mediated by a selective group of nuclear receptors and transcription factors-all of which are intimately involved in the preservation of carbohydrate and lipid homeostasis. Certain nutritional scenarios particularly excessive carbohydrate consumption markedly enhance DNL within the liver and as the primary lipogenic organ in humans accelerated hepatic DNL represents a significant contributory mechanism to cytoplasmic TG accumulation and the development of hepatic steatosis. Appreciation of the nutritional and metabolic cues responsible for DNL requires an understanding of the critical enzymes involved and the molecular factors governing their expression and activity.

Under situations in which hepatic glycogen stores are replete, monosaccharides such as glucose and fructose enter hepatocytes and are directed towards the glycolytic pathway to be utilized in a series of sequential energy-yielding catabolic reactions. Phosphorylation of glucose and fructose via glucokinase (GK) and fructokinase respectively effectively traps the sugars within hepatocytes and represents the initial glycolytic step forming glucose-6 (G-6P) and fructose-1 phosphate (F-1P). Fructose metabolism occurs much more rapidly than glucose presumably due to a higher affinity
of fructokinase for fructose (77) as well as its circumvention of the first committed glycolytic enzyme phosphofructokinase-1 (PFK-1). Subsequent conversion of F-1P to dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3 phosphate effectively avoids the complex allosteric regulation aimed at PFK-1; these differences enable fructose to be considerably more lipogenic than glucose. Glycolysis proceeds with the formation of several intermediates, culminating in the substrate-level phosphorylation of phosphoenolpyruvate (PEP) to pyruvate via liver-type pyruvate kinase (L-PK). Newly formed pyruvate then enters into the mitochondrial matrix where it undergoes an oxidative decarboxylation through the multi-enzyme pyruvate dehydrogenase (PDH) complex yielding acetyl-CoA. Rather than enter into the TCA cycle for complete oxidation acetyl-CoA condenses with the four-carbon oxaloacetate to form the six-carbon citrate which then exits the mitochondria and into the cytosol where it immediately undergoes an ATP-dependent cleavage to oxaloacetate and acetyl-CoA through the enzyme citrate lyase (ACL). This reaction is of considerable importance as it links carbohydrate catabolism to the energetically expensive biosynthesis of fatty acids. The newly liberated acetyl-CoA is then able to serve as a substrate for the rate-limiting enzyme in lipogenesis, acetyl-CoA carboxylase (ACC) which catalyzes the formation of malonyl-CoA, an important molecule that serves as both a carbon donor in fatty acid synthesis and a potent inhibitor of the carnitine-palmitoyl transferase (CPT) system and hence mitochondrial fatty acid oxidation (78). Two isoforms of ACC (ACC1 and ACC2) and distinct cellular pools of malonyl-CoA are responsible for these contrasting phenomena; ACC1 located in the cytosol generates the malonyl-CoA
necessary for lipid synthesis while the close proximity of ACC2 to the mitochondrial membrane generates the malonyl-CoA required for CPT inhibition (79). Following a series of condensation and reduction reactions catalyzed by fatty acid synthase (FAS), the malonyl-CoA molecule is extended to ultimately form the sixteen carbon saturated palmitate, the end product of FAS and the first fatty acid formed through DNL.

As a precursor to other various long-chain fatty acid species palmitate is a valuable substrate for the elongation and desaturation systems designed to produce longer chain saturated fatty acids (SFA) as well as mono- (MUFA) and polyunsaturated fatty acids (PUFA). Numerous endoplasmic reticulum (ER) membrane bound elongases, desaturases and acyltransferases participate in these coordinated reactions, the majority of which will not be discussed here (see (80) for a recent comprehensive review). Two critical enzymes, the elongase ELOVL-6 (elongation of very-long-chain fatty acid) and stearoyl-CoA desaturase 1 (SCD-1) deserve particular attention as both are known to be transcriptionally regulated in a manner similar to other enzymes involved in DNL (see next section). ELOVL-6, a member of the microsomal elongase family catalyzes the elongation of long chain (typically C12-C16) SFA and MUFA and relevant to TG synthesis ELOVL-6 elongates palmitate to stearate (81). SCD-1 is a delta-9 desaturase which introduces a single double bond into both palmitate and the eighteen-carbon stearate forming the MUFAs palmitoleate (16:1) and oleate (18:1) respectively and therefore serves as the rate-limiting reaction in endogenous MUFA synthesis. As a result of these two enzymes palmitate is successfully elongated to stearate as well as desaturated to palmitoleate and oleate. Finally, a series of
sequential acyltransferase reactions culminates in the production of diacylglycerol which in the final reaction catalyzed by diacylglycerol acyltransferase (DGAT) is covalently joined to a fatty acyl-CoA thereby forming TG (for an insightful and historical account of the major TG biosynthetic pathways and biochemical reactions see a recent review, (82)).

The extraction and storage of nutrient-derived energy represents a distinct survival mechanism exploited by nearly all organisms, the vast majority of which tend to store cellular energy as TG due to its highly reduced and concentrated nature; at more than 38 kJ/g TG yield the greatest energy content of all nutrients. In humans nutrient storage becomes crucial during times of nutrient scarcity such as fasting and starvation yet troublesome during caloric overindulgence and physical inexertion, behaviors that afflict a staggering proportion of the national and global populations. As just described, excessive intake of nutrients especially carbohydrate signals the liver to increase hepatic DNL to appropriately convert and store this incoming energy as TGs through the participation of several lipogenic enzymes. Understanding the manner in which these reactions are coordinated however, requires an investigation of the molecular factors responsible for the induction of hepatic DNL.

The liver X receptors α (LXRα) and β (LXRβ) are members of a subgroup of nuclear receptors termed the “adopted orphan” receptors (83) that serve as cellular sterol sensors through their binding of cholesterol derivatives or oxysterols. Accordingly LXRs regulate the expression and transcription of genes pivotal to cellular cholesterol efflux, reverse cholesterol transport, and hepatic cholesterol catabolism via bile acid
synthesis and biliary excretion (see 84,85 for reviews). Despite promising anti-
atherogenic effects upon LXR pharmacological activation, valid concerns exist with
regard to the lipogenic effect mediated by LXRs. An earlier study by Grefhorst et al (86)
was one of the first to demonstrate that oral administration of an LXR agonist to wild-
type C57BL/6J mice resulted in massive hepatic steatosis, a phenomenon associated
with a sharp induction in the mRNA of Fas, Acc1 and the lipogenic transcription factor
sterol regulatory element binding protein 1c (SREBP-1c). These observations were in
agreement with previous promoter studies that identified two functional LXR response
elements (LXREs) within the mouse SREBP-1c promoter region, both of which are
essential for LXR regulation (87,88). Moreover, selective deficiency of Srebp-1c in mice
was shown to result in a blunted response to a fasting/refeeding treatment and an
approximately 50% reduction in the rate of hepatic fatty acid synthesis (89).
Mechanistically it is still not entirely clear as to how LXR activation induces SREBP-1c
transcription and subsequent lipogenesis although in vitro experiments with isolated rat
hepatocytes have suggested a collaboration with insulin (90). Alternatively, Hegarty et al
(91) demonstrated that while LXR activation resulted in increased SREBP-1c induction,
insulin proved a much more potent stimulus in the induction and cleavage of the mature
SREBP-1c protein, thereby proposing that the full activation of the transcriptionally
active SREBP-1c is absolutely dependent on insulin. Lastly it is important to note that
LXR can directly activate lipogenic gene expression, particularly Fas independent of the
SREBP-1c (92). Even more striking was the fairly recent finding by Ji-Young Cha and
Joyce Repa (93) that the carbohydrate-sensitive transcription factor, carbohydrate-
response element-binding protein (ChREBP) (see below) is an apparent target gene of LXR. LXR agonists administered to wild-type mice significantly increased hepatic ChREBP mRNA compared to LXR\(\alpha/\beta\) double knock-out mice. Particularly exciting was that the mRNA of L-PK failed to increase in the double knock-out mice, a noteworthy finding given that L-PK expression is absolutely dependent on ChREBP transcriptional activity. These latest findings appear to further solidify LXR as a \textit{bona fide} master regulator of lipogenesis.

As just discussed, the SREBP-1c transcription factor maintains a central role in mediating the lipogenic response to LXR and insulin action due to its ability to activate nearly all the genes pertinent to fatty acid synthesis notably \(\text{GK, ACL, ACC, FAS, ELOVL6, and SCD1}\) (94). SREBP-1c is one isoform of the well-characterized SREBP family of ER membrane-located transcription factors governing fatty acid and cholesterol biosynthesis (see 94,95 for comprehensive reviews on each respectively). \textit{SREBP-1c} transcription is dramatically enhanced by insulin in cultured hepatocytes (96), an \textit{in vitro} observation that nonetheless appears to correspond to insulin’s potent anabolic effects. Thus in response to carbohydrate-mediated insulin secretion \textit{SREBP-1c} expression and activity increase leading to an induction of lipogenic enzymes and the eventual storage of carbohydrate as TG. The overexpression of \textit{SREBP-1c} in the livers of \textit{ob/ob} mice results in both an increased mRNA expression of lipogenic genes as well as a grossly fatty liver, illustrating the significance of this transcription factor in DNL and liver physiology (97).
The discovery of ChREBP as a carbohydrate-responsive transcription factor (98) provided a crucial insight as to how simple carbohydrates such as glucose impact and coordinate gene expression. It also confirmed the suspicions of several laboratories that SREBP-1c was not the sole transcription factor responsible for the induction of lipogenesis. Subsequent \textit{in vivo} experiments in mice utilizing a global \textit{ChREBP} knockout (99) demonstrated an impairment in glycolysis and a 60% reduction in the rate of hepatic fatty acid synthesis after administration of a carbohydrate load while the generation of liver-specific ChREBP knockout mice (100) showed a marked improvement in hepatic steatosis, primarily through a decreased rate in hepatic DNL and expression of \textit{ACC}, \textit{FAS} and \textit{SCD-1}. Perhaps one of the more intriguing aspects of ChREBP-mediated transcription is the identity of the carbohydrate molecule which activates ChREBP: xyulose-5 phosphate (Xu-5P) (101), an intermediate in the pentose phosphate pathway whose concentration increases in response to an enhanced influx of glucose. Phosphorylation of glucose by GK, a transcriptional target of SREBP-1c enables glucose to engage the glycolytic pathway while some of the glucose is directed towards the pentose phosphate pathway leading to Xu-5P, ChREBP activation and increased ChREBP-mediated lipogenesis. Thus SREBP-1c and ChREBP appear to coordinate DNL in response to hormonal and nutritional cues, the former via insulin and the latter through dietary carbohydrate in the form of Xu-5P.

\textit{Food for Thought: Consideration of nutrient composition in the promotion and regression of NAFLD}
From a nutritional perspective, a critical component of any dietary intervention is the macronutrient composition. Physiologically, carbohydrate in the form of glucose serves as the most potent stimulator of pancreatic insulin release, thus it seems logical that in the context of impaired insulin action, this dietary stimulus should be lessened to minimize insulin release and thereby improve sensitivity. Furthermore, in humans, the liver is the organ most actively involved in lipid synthesis, a process predominately stimulated via insulin through transcriptional activation of SREBP-1c and through glucose activation of ChREBP. The catabolic actions of glucagon oppose these effects, therefore dietary measures which increase the glucagon to insulin ratio would abrogate such anabolic processes as lipid and glycogen synthesis and promote energy expenditure, effectively decreasing hepatic lipid content.

Several investigators have observed a strong association between excessive carbohydrate intake and an increased severity of hepatic steatosis and the inflammatory NASH (102,103) in humans. In a recent prospective study, Kang et al (103) evaluated 91 patients with clinically suspected NAFLD and characterized 31 patients (34%) as having metabolic syndrome. Those patients with metabolic syndrome were shown to consume significantly more carbohydrate ($p = 0.03$) and less fat ($p = 0.01$) and displayed higher histological severity scores for steatosis ($p = 0.004$) and NASH ($p = 0.0006$) compared to patients without metabolic syndrome. This particular study is noteworthy as it recapitulates current evidence which demonstrates a strong link between excessive carbohydrate consumption and the development of physiological factors associated with metabolic syndrome as elegantly described by Volek et al (104). A
carbohydrate restricted dietary intervention was recently conducted in an obese female patient diagnosed with NAFLD (105) that presented with abnormal plasma ALT levels and a hepatic triglyceride content of 44.6% as assessed by $^1$H MRS. The patient was instructed to follow for 2 weeks a ketogenic diet composed of 5: 41: 54 % carbohydrate: fat: protein respectively. At 5 weeks after the initiation of the diet, there was a complete correction of plasma ALT values and remarkably, a dramatic four-fold reduction in hepatic triglyceride content to 11.9%. While long-term controlled human studies designed to evaluate the role of carbohydrate restriction on improvements in NAFLD are sparse, the available evidence does indeed support a beneficial role for this dietary intervention in ameliorating the associated clinical and histological abnormalities inherent in this disease. In an interesting animal study (106) two groups of mice were fed diets high in carbohydrate (68%) that differed in starch composition in order to examine the effects of rapidly absorbed carbohydrate on hepatic steatosis. One group consumed a diet composed of 100% amylopectin, a highly branched chain glucose polymer which is rapidly metabolized within the small intestine, whereas the other group consumed 60% amylose and 40% amylopectin; amylose due to its linear structure, is hydrolyzed at a slower rate and therefore predicted to contribute to less severe hepatic steatosis. Indeed, histological examination indicated the presence of mixed hepatic steatosis in mice fed the rapidly absorbed carbohydrate and a two-fold greater hepatic triglyceride content (20.7 ± 9.4 mg/g vs 9.6 ± 4.9 mg/g; $p = 0.01$) when compared to the mice that consumed the slower absorbed carbohydrate. Again, long term clinical studies which examine the relationship between carbohydrate and NAFLD in humans
are warranted, however this study clearly points to the potential benefit(s) of reduced carbohydrate or low glycemic index diets as a novel dietary treatment for reversing the progression of hepatic steatosis.

To date, there have been relatively few long-term controlled studies investigating the role of dietary composition, in particular, carbohydrate and carbohydrate restriction on NAFLD although several lines of evidence suggest that limited carbohydrate consumption may be beneficial towards improved liver function. In a one year prospective study, Solga et al (102) found that a higher carbohydrate intake was significantly associated with higher odds of hepatic inflammation whereas a higher fat intake was associated with significantly lower odds of inflammation in morbidly obese patients with NAFLD. In addition, the authors observed a trend toward lower odds of steatosis and fibrosis in the highest tertile of fat intake suggesting that the presence of fat may improve NAFLD histopathology. The underlying mechanism(s) pertaining to carbohydrate-induced hepatic inflammation have yet to be clarified. A more recent study by Tendler et al (107) found that a low carbohydrate, ketogenic diet (< 20 g/day carbohydrate) significantly improved steatosis, inflammation, and fibrosis in NAFLD patients within a 6 month period concomitant with weight loss and lowered plasma insulin and glucose levels. These results support the prevailing notion that insulin resistance and hyperglycemia play salient roles in enhancing hepatic DNL and triglyceride synthesis thus diets aimed to reduce insulin release may mitigate carbohydrate-stimulated lipogenesis.
Dietary fat, in particular PUFAs are known to exert hypotriglyceridemic effects and are a potentially important dietary treatment in reducing cardiovascular disease risk. Evidence is also accumulating that suggests they may also exert various hepatoprotective effects. These fatty acids are well known regulators of hepatic gene expression with regard to carbohydrate and lipid metabolism, suppressing L-PK in the glycolytic pathway as well as several key lipogenic enzymes (see (108) for a brief review). Impressively, SREBP-1c is potently inhibited by PUFAs and although the mechanistic details require further studies, it has been demonstrated that PUFAs reduce $SREBP-1c$ transcription, enhance $SREBP-1c$ mRNA turnover and directly interfere with the proteolytic cleavage of the transcriptionally mature $SREBP-1c$ protein (108). It has also been recently demonstrated that PUFAs, specifically linoleate (18:2), eicosapentanoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) effectively inhibit ChREBP activity in mice, thereby decreasing expression of both L-PK and FAS (109). A recent study investigating the potential therapeutic effects of omega-3 FAs in patients clinically diagnosed as having hepatic steatosis (110) found that those receiving 2.7 grams daily of concentrated EPA for twelve months had significantly reduced plasma values of ALT and AST and a regression of steatosis as assessed by histological analysis. An eight week trial in patients with elevated hepatic TG content (as assessed by proton magnetic resonance spectroscopy) who received 9 grams/day of fish oil showed a 46% reduction in plasma TG, a 21% reduction in plasma VLDL and IDL cholesterol and a 15% decrease in plasma apolipoprotein B, yet no improvement in hepatic TG content (111). It must be mentioned that the sample population for this study
was fairly small (n=17) and included mainly African-Americans, who tend to accumulate less intraperitoneal fat and differ from other ethnic groups such as Hispanics and Caucasians in terms of the prevalence of steatosis (112).

Nutritional modulation of hepatic anomalies such as steatosis and the more severe NASH through carbohydrate restriction as well as increased unsaturated fatty acids are beginning to show promise as front-line dietary defenses. Although the evidence is still fairly limited for these approaches the metabolic basis for them seem fundamentally sound. Reduced dietary intake of carbohydrate, particularly rapidly absorbed sources such as refined grains and starches restricts insulin release and shifts metabolism towards a state of energy-deficit, prompting the oxidation of stored carbohydrate, as glycogen and fat, as adipose TG. Dietary unsaturated fatty acids appear to function at the molecular level to antagonize the transcriptional regulators responsible for inducing the lipogenic program. While drugs such as insulin sensitizers and nuclear receptor agonists are valuable in their own right, it is time for nutritional strategies to emerge as viable therapeutic modalities for diseases of metabolic origin. Given the strategic role of the liver in managing incoming dietary components, this seems a logical place to start.
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STUDY 1: Hypercholesterolemia, hepatic steatosis, and liver injury in guinea pigs with cholesterol-induced non-alcoholic fatty liver disease

Abstract:

Evidence from numerous animal and human studies have implicated dietary cholesterol as a lipotoxic mediator in the transition of hepatic steatosis to non-alcoholic steatohepatitis (NASH), a critical event in the progression of nonalcoholic fatty liver disease (NAFLD). Here, we sought to examine the impact of a dietary cholesterol challenge on NAFLD progression in phenotypically normal guinea pigs, an animal that more closely approximates humans in terms of hepatic cholesterol handling and metabolism than other commonly utilized animal models. Young (3-month) male Hartley guinea pigs ($n=10$/group) were subjected to 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 histopathological 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 ($p<0.05$ for both) while displaying no differences in plasma glucose, non-esterified fatty acid, or triglyceride (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. As these are the first studies to document histological changes in guinea pigs fed H-Chol, they add to the growing list of studies which implicate dietary cholesterol as a causative factor in NAFLD development and progression.
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 surfeit metabolic energy in the form of triglycerides (TG). These inert, highly reduced lipid molecules reside within the hydrophobic core of lipid droplets (LD), specialized organelles designed to, among other functions, shelter neutral lipids like TG as well as cholesterol esters (CE) away from the aqueous cytoplasm (1,2). 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, with increasing evidence suggesting a prominent role for these organelles during times of health as well as disease itself (see reference 4 for a recent review).

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 clinicopathological entity that represents a spectrum of histological abnormalities typically observed during conditions of 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 posits 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 bi-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.

A noticeable shift has emerged recently however 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). The search is on now for the so called lipotoxic mediators of hepatic injury, of which excessive dietary cholesterol is 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 dietary 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) and through synergistically interacting with dietary fats (21,22). Dietary cholesterol also further distorts hepatic architecture and promotes 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 both mice and humans appears to distinguish between steatosis and NASH as shown by Ioannou et al (26).

Thus the available evidence to date not only hints at, but affirms 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).
**STUDY 1: Materials and experimental methodology**

**Study design:**

Male Hartley guinea pigs ($n=39$) approximately 3 months of age (Charles River Laboratories, Wilmington, MA) were housed in the biology vivarium in the School of Pharmacy at UConn. Guinea pigs 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 chow. 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) ($n=29$) for six weeks. Both diets were prepared by Research Diets (New Brunswick, NJ: L-Chol, D01013102; H-Chol, D01013101) and were identical in terms of macronutrient and ingredient composition with the only exception being the cholesterol content (Table 1.1). The level of dietary cholesterol in the L-Chol group is equivalent to 300 mg/day for humans whereas the H-Chol diet is equivalent to the daily consumption of 1800 mg for humans. Therefore the H-Chol diet represents a true dietary cholesterol challenge. 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 (IACUC) at UConn.

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 per cage was calculated as the difference between the original amount offered and the amount remaining in the hopper and was
measured every two days. At the conclusion of the six week study period guinea pigs were fasted overnight for 12 hours and then anesthetized under isoflurane vapors followed by cardiac puncture. Whole blood and livers were then subsequently collected for analysis. For the current study, a total of 10 guinea pigs from the H-Chol group were randomly selected for sacrifice, while the remaining 19 guinea pigs were transitioned to a separate dietary intervention (see study 2 of this dissertation). Therefore all experimental data obtained in the current study represents a sample of \( n = 10 \) for L-Chol and \( n = 10 \) for H-Chol. It must be noted here that with regards to plasma measurements, data could only be analyzed in five of the L-Chol guinea pigs due to unintended hemolysis of blood following cardiac puncture; while the hemolyzed samples were still assessed for plasma parameters, the results differed dramatically from the non-hemolyzed samples and thus were excluded from final analyses due to their unreliability.

**Plasma metabolite analysis:**

Whole blood was obtained through cardiac puncture and collected into EDTA-containing 50 mL screw tubes and immediately centrifuged at 2000 x 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°C for subsequent analysis. All plasma metabolites, with the exceptions of free cholesterol and non-esterified fatty acids (see below) were analyzed using the Cobas c111 analyzer (Roche Diagnostics, Indianapolis, IN).
**Total cholesterol (TC)**

Plasma TC was analyzed using an enzymatic and colorimetric method where cholesterol esters (CE) are first cleaved to free cholesterol and fatty acids via cholesterol esterase. The cholesterol molecule is then acted upon by cholesterol oxidase and undergoes oxidation to cholest-4-en-3-one and hydrogen peroxide. The reaction proceeds with hydrogen peroxide oxidatively coupling 4-aminoantipyrine and phenol in the presence of peroxidase, yielding a quinone-imine chromophore. The resulting red color intensity is directly proportional to the concentration of TC in the sample. Results are expressed as mg/dL.

**High-density lipoprotein cholesterol (HDL-C)**

Plasma HDL-C was measured directly with a homogeneous enzymatic colorimetric automated procedure. This method is based on the formation of water-soluble complexes of dextran sulfate and LDL, VLDL, and chylomicrons in the presence of divalent cations, particularly magnesium ions (28). Briefly, a HEPES buffer containing 1.5g/L dextran sulfate was added to plasma aliquots followed by the addition of a working solution of HEPES buffer containing polyethylene glycol (PEG)-modified cholesterol esterase and cholesterol oxidase. The modification of these enzymes by PEG (attached to the amino groups) causes them to show selective catalytic activities towards the various lipoprotein particles with the greatest reactivity towards HDL. Furthermore, LDL, VLDL, and chylomicrons are resistant to the actions of PEG-modified enzymes, thus the measured cholesterol corresponds to that within the HDL particle only. CE are then converted to FC and fatty acid following the action of PEG-modified
cholesterol esterase with subsequent oxidation of the FC to \( \Delta^4 \)-cholestenone and hydrogen peroxide through the action of cholesterol oxidase. Hydrogen peroxide then reacts with 4-aminoantipyrine and a sodium complex (sodium N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline), yielding water and a purple-blue chromophore whose intensity is directly proportional to the concentration of cholesterol within the HDL particles. Results are expressed as mg/dL.

**Low-density lipoprotein cholesterol (LDL-C)**

The concentration of LDL-C was calculated indirectly according to the Friedwald equation as shown here; LDL-C = TC – [HDL-C + (TG/5)]. As the plasma triglyceride levels were not markedly elevated (table 1.2) the use of this formula to estimate LDL-C was considered appropriate. LDL-C concentration is expressed as mg/dL.

**Free cholesterol (FC)**

Plasma FC was quantitatively determined according to an enzymatic method as per the instructions of the manufacturer (Wako Diagnostics, Richmond, VA). The principle of this test is the enzymatic oxidation of FC by cholesterol oxidase to \( \Delta^4 \)-cholestenone with the simultaneous production of hydrogen peroxide. The hydrogen peroxide produced along with the presence of peroxidase enables a oxidative condensation reaction between 3,5-dimethoxy-N-ethyl-N-(2-hydroxy-3-sulfopropyl) aniline sodium (DAOS) and 4-aminoantipyrine producing a blue pigment whose absorbance is then measured at 600 nm and reflects the concentration of FC present in the sample, expressed as mg/dL. The reaction scheme is shown here:
Triglyceride (TG)

Plasma TG was measured using an enzymatic colorimetric method in which lipoprotein lipase hydrolyzes the TG molecule generating three fatty acids and the glycerol moiety. Glycerol kinase then phosphorylates glycerol to produce adenosine diphosphate and glycerol-3-phosphate, which then undergoes oxidation to dihydroxyacetone phosphate via glycerophosphate oxidase. This reaction step also yields hydrogen peroxide that reacts with 4-aminophenazone and 4-chlorophenol and, in a peroxidase-catalyzed reaction, results in the production of a red dyestuff, 4-(p-benzoquinone-monoimino)-phenazone. The resultant intensity of the red dyestuff is directly proportional to the TG concentration and is measured photometrically in the Cobas c111 analyzer. Plasma TG is expressed as mg/dL.

Non esterified fatty acid (NEFA)
To quantitatively determine plasma NEFA concentration an enzymatic colorimetric method was employed (Wako Diagnostics, Richmond, VA) and conducted in accordance with the manufacturer’s instructions. This method relies on the acylation of coenzyme A by fatty acids in the presence of acyl-CoA synthetase and adenosine triphosphate (ATP). This acylation results in the formation of thiol esters in the form of acyl-CoA along with adenosine monophosphate (AMP) and pyro-phosphate as byproducts. The procedure proceeds with the oxidation of the acyl-CoA in the presence of added acyl-CoA oxidase, producing hydrogen peroxide, which, in the presence of peroxidase, then enables the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple-colored chromophore. The concentration of NEFA is then determined from the optical density of the chromophore when measured at 550 nm. The results are expressed as mEq/L. The overall reaction scheme is represented here:
Glucose

Plasma glucose was quantitatively determined photometrically by monitoring the rate of NADPH formation. Glucose was first phosphorylated to glucose-6-phosphate (G6P) by hexokinase in the presence of ATP and then oxidized to gluconate-6-phosphate following G6P-dehydrogenase activity. In this second reaction, the oxidation of G6P proceeds with the reduction of NADP+ to NADPH, the rate of which is directly proportional to the plasma glucose concentration. It is worth noting that glucose is the only carbohydrate oxidized in this assay. Results are expressed as mg/dL.

Alanine aminotransferase (ALT) activity

Hepatic injury was assessed biochemically by measuring the enzymatic activity level of ALT in plasma. The assay relies on the enzymatic transfer of the amino group from L-alanine to 2-oxoglutarate, catalyzed by ALT, with subsequent formation of pyruvate and L-glutamate. Nicotinamide adenine dinucleotide (NADH) then reduces pyruvate to L-lactate in a reaction catalyzed by lactate dehydrogenase. The rate of NADH oxidation to NAD+ is directly proportional to the catalytic activity of ALT and is determined by measuring the decrease in absorbance. Importantly, this assay includes pyridoxal phosphate (PLP) which functions as a coenzyme in the transfer of amino groups and therefore ensures full enzymatic activation of ALT. Results are expressed as U/L.

Aspartate aminotransferase (AST) activity

Plasma AST activity is also utilized as a biochemical assessment of hepatic injury and is measured by the Cobas c111 analyzer in a manner similar to ALT. In the
first transfer reaction, AST catalyzes the transfer of the amino group from L-aspartate to 2-oxoglutarate, forming oxaloacetate and L-glutamate. The assay proceeds with the reduction of oxaloacetate by NADH and in the presence of malate dehydrogenase forms L-malate and NAD+. AST activity reflects the rate of NADH oxidation and is determined by measuring the decrease in absorbance. Full AST activation was ensured by the presence of PLP in the assay reagents. Results are expressed as U/L.

**Hepatic lipid analysis:**

Whole livers were excised and immediately weighed on a digital scale and their weights recorded in grams. Livers were photographed with a Canon PowerShot A720 IS digital camera and then approximately 1 g of tissue from the left sublobe of the quadrate lobe was sliced with a razor, placed in a 1 mL snap tube and immediately stored at -80°C for hepatic lipid extraction. Once all tissue had been removed for lipid and histological analysis (see below) the remnant liver mass was tightly wrapped in aluminum foil and stored at -80°C for future analysis. The concentration of hepatic lipids are expressed as mg/g liver with the exception of phospholipid, which is expressed as µg/g liver.

**Hepatic TG**

The procedure for lipid extraction from liver is based on the original protocol published by Folch et al (29) with some minor variations. 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 Folch solution (2:1 chloroform:methanol). Tubes were then capped, vortexed, and left to incubate for 24 hours in a dark room at room temperature.
The homogenate was then filtered gravimetrically with Whatman grade #1 filter paper, added to a separatory funnel, mixed with 3 mL 0.05% sulfuric acid, and left to incubate for 1 hour or until adequate phase separation took place. The lower phase, which contains the pure lipid extract, was collected into 30 mL glass screw-top tubes and the total volume was adjusted to 10 mL by adding Folch solution followed by vortexing. Then a 2 mL aliquot was collected into 10 mL glass screw top test tubes, combined with 500 µL of sulfuric acid, vortexed, and left uncapped overnight at room temperature. The following day the upper layer was removed and the total volume adjusted to 2 mL with chloroform. Next, a 200 µL aliquot was added to 13 x 100 mm glass tubes and evaporated under a steady stream of nitrogen gas. Samples were rehydrated with approximately 1 mL of 1% triton X-100 (triton/water) and then stored in the dark at 4 °C prior to analysis. 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 colored quinoneimine that is measured spectrophotometrically at 500 nm. The TG concentration in the liver sample is directly proportional to the intensity of the red chromophore.

**Hepatic TC**

The isolation of TC from the liver followed the same steps as that detailed for liver TG until the phase separation step (see above). Once the final volume of the lipid extract was adjusted to 10 mL with Folch solution, a 200 µL aliquot was transferred in triplicate to 13 x 100 mm glass test tubes and allowed to evaporate overnight uncapped. Samples were resuspended in 200 µL ethanol, vortexed, and analyzed using a
commercially available assay (Pointe Scientific Inc, Canton, MI). The assay utilizes the peroxidase, phenol, and 4-aminoantipyrine color system in which the intensity of the red colored quinoneimine is measured at 500 nm and directly proportional to the concentration of liver TC.

**Hepatic FC**

Steps for the extraction and isolation of hepatic FC are identical to that for hepatic TC. Once samples were rehydrated with 200 µL ethanol they were then analyzed according to the manufacturer’s instructions (Pointe Scientific Inc, Canton, MI). This assay is the same used to measure plasma FC (see above for reaction scheme).

**Hepatic cholesterol ester (CE)**

The amount of cholesterol stored as CE within the liver was determined indirectly by calculating the difference between liver TC and FC; CE = TC – FC.

**Hepatic phospholipid (PL)**

Total PL concentration was quantified in the liver through capillary gas chromatography (see study 3 materials and experimental methodology).

**Liver pathology:**

To evaluate whether H-Chol impacts liver pathology, approximately 1-2 g of tissue from the left lobe or the medial sublobe of the right lobe were sliced with a razor blade and submerged in 10% neutral buffered formalin solution. Formalin is an example of an aqueous aldehyde solution and contains approximately 4% weight/volume (w/v) formaldehyde in water at a neutral buffered pH of 7.2-7.4. All samples remained
immersed for approximately 1 week in order for full stabilization of histological structure to occur and were then delivered to the Histology section of the Connecticut Veterinary Medical Diagnostic Laboratory in the Department of Pathobiology and Veterinary Science for processing, sectioning, and staining. It must be mentioned here that the selection of the left lobe or medial sublobe for representative histology was done in an attempt to avoid any interpretive difficulties resulting from a phenomenon known as tensional lipidosis. This can occur when liver tissue that is in close proximity to the gall bladder is removed and this can often exaggerate the degree of hepatosteatosis present.

A note on histological methods

The ultimate aim of histological staining is to examine the structural framework of tissue with as minimal alteration as possible to the original structural and chemical composition. This requires considerable vigilance when it comes to preparation and handling of microscopic samples as well as an appreciation for certain technical difficulties that inevitably arise during the course of tissue procurement, fixation, embedding, sectioning, and staining.

All freshly isolated tissues are chemically and physically unstable and need to be stabilized in order to arrest post-mortem decay. Moreover, many histological staining methods will only succeed when this process, referred to as fixation, is implemented. The most obvious result of fixation is the hardening of tissue which enables the eventual slicing of the samples into sections. However, often unbeknownst to the researcher, common fixation methods that rely on liquid fixatives also introduce chemical and
structural artifacts due to swelling and shrinking of tissue. Complicating matters further is that these physical changes often do not affect all regions of the tissue equally, resulting in visibly noticeable ‘empty spaces’ that one must take into account when selecting the most appropriate section(s) for analysis.

In order for embedding to successfully occur, specimens must be equilibrated with a solvent that is miscible with wax (typically, but not always, this is paraffin). This is accomplished by replacing the natural water present in the specimen first with alcohol and then with a paraffin solvent (also referred to as a clearing agent), often xylene. Molten paraffin wax is then allowed to infiltrate the tissue, which is eventually embedded (blocked out) in solidified paraffin blocks that are used for sectioning. Physical changes are continually visited on tissues till the time of embedding, such that the volume of paraffin-embedded specimen is typically 60-70% that of what it was at the time of excision. One should also be cognizant of the potential loss of some lipid constituents, which invariably dissolve out during the dehydration and embedding process. If lipids are of significant interest experimentally then quick freezing of tissue via immersion in liquid nitrogen followed by sectioning on a cryostat can significantly minimize loss of tissue lipid constituents. Germane to histology, this type of freezing technique limits ice crystal formation which is known to distort tissue architecture by appearing as series of meaningless holes scattered throughout the section.

Paraffin blocks are then sliced into thin sections (typically 5-10 µM), floated on warm water to remove wrinkles and mounted on glass slides. The slides are dried in warm air and then immersed in xylene or other clearing agents to dissolve the wax and to
equilibrate the tissue with the clearing agent itself. Slides are then exposed to a series of graded mixtures of alcohol and water before the stain of interest is applied.

**Hematoxylin and eosin (H&E) stain**

Five \( \mu \text{M} \) sections were mounted onto glass slides, dried in warm air, and placed on a Leica Autostainer for automated H&E staining. The slides were placed in two changes of xylene for approximately four minutes each followed by two changes of 100% ethanol for four minutes each. Slides then were rinsed in tap water for two minutes, placed in Harris Hematoxylin for 6 minutes and washed in running tap water for two minutes. They were then dipped twice in decolorizing acid alcohol solution, washed in running tap water for two minutes, placed in 1% ammonia water for 10 seconds, washed in running water for another two minutes, and placed in 95% ethanol for one minute. The final stage involved placing the slides in eosin stain for three minutes, followed by four changes of 100% ethanol for one minute each, and two changes of xylene for one minute each. Lastly, a coverslip with synthetic resin was placed on the slides. The cellular components can be identified based on the color imparted by the H&E; all nuclei are stained blue and the cytoplasm is colored in various shades of pink.

**Trichome stain**

The term ‘trichome’ indicates a staining method where two or more anionic dyes are used in combination with a heteropolyacid, which is a water- and alcohol-soluble crystalline compound. There are several variations of trichome staining, notably
Masson’s and Mallory’s techniques, all of which enable the identification of collagenous and reticular fibers, basement membranes, and secretory granules.

Five µM sections from paraffin-embedded liver blocks were mounted on glass slides then deparaffinized and hydrated in distilled water. Hydrated sections were then placed in Bouin’s solution overnight at room temperature. Following overnight incubation, slides were rinsed in tap water until colorless and then placed in working Weigert’s iron hematoxylin solution (2.5g hematoxylin, 250 mL 95% alcohol, 4 mL 29% aqueous ferric chloride, 95 mL distilled water, 1 mL HCL) for 10 min. Slides were then rinsed in tap water till clear, placed in Gomori’s 1-step trichome with Aniline blue (#1816, Poly Scientific R&D) for 15 minutes and then rinsed again in tap water till clear. Slides were placed in 5% acetic acid (5 mL glacial acetic acid, 100 mL distilled water) for 2 minutes and then dehydrated in 95% ethanol followed by 100% ethanol and finally cleared in xylene and covered with a coverslip with synthetic resin. With the trichome stain, collagen fibers are recognized as distinct bright blue colorations against a background of red-colored cytoplasm and black nuclei.

**Oil Red O (ORO) stain**

ORO is a member of the solvent (azo) class of dyes which are characterized by their largely non-polar groups and reduced capacity for hydrogen bonding. These dyes function by moving from polar solvents into somewhat less polar (more hydrophobic) domains such as lipids with typical examples of solvent dyes including Sudan III, IV, and ORO. ORO is more hydrophobic and intensely colored than the Sudan dyes thereby allowing for greater resolution of microvesicular lipid droplets.
The preparation of livers for ORO staining differs from those sections subjected to H&E or trichome staining in that the samples require freezing. Following removal of the whole liver, approximately .1-.5 g of freshly excised liver tissue from the left lobe or the medial sublobe of the right lobe were immediately placed in 24x24x5 mm cryomolds (American MasterTech) and filled with optimal cutting temperature (OCT) compound. The cryomolds were then placed in a metal bowl containing a small amount of 2-methylbutane (#270342-IL, 99.5% HPLC grade) for 1-2 minutes atop dry ice. Cryomolds were wrapped in parafilm and aluminum foil, and placed immediately in -80°C until sectioning.

A saturated solution of ORO was developed by adding excess dye powder to a 99% isopropyl alcohol solution and allowed to stand for 2-3 days prior to use. Two volumes of a 1% dextrin in water solution was added to 3 volumes of the ORO stock solution, mixed, and allowed to stand for two days before being filtered through Whatman #4 filter paper, thereby forming the working solution.

Frozen sections were sliced at 4 µM using a cryostat and collected onto slides and then rinsed in 60% ethanol, stained with the working solution of ORO for 10-20 minutes, rinsed in 60% ethanol followed by four changes of water, and then counterstained with an alum-hematoxylin solution. The sections were finally washed in one more exchange of water and mounted in an aqueous medium.

Ultimately the process results in hydrophobic lipids, especially the neutral triglyceride species, being stained red with small intracellular microvesicular droplets.
also clearly visible. The more hydrophilic lipids are also stained, albeit weaker and orange-red in color.

**Parameters of hepatic steatosis**

Histological evaluation of hepatic steatosis for H&E-stained guinea pig liver sections from L-Chol \( (n = 10) \) and H-Chol \( (n = 10) \) was performed by a board-certified pathologist, Joan A Smyth (JAS) of the Department of Pathobiology and Veterinary Science, according to the scoring system originally proposed by Brunt et al (30) and validated by Kleiner et al (31). The grading system of hepatic steatosis is based on the percentage of lobule and parenchyma involved as follows (31):

- **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.
- **Grade 3:** *Severe* fat accumulation in >66% of all hepatocytes.

Additionally, steatosis was evaluated with regard to the pattern-type of lipid droplets and noted as either microvesicular, macrovesicular, or mixed macro and microvesicular steatosis. All photomicrographs of liver sections stained with H&E, trichome, and ORO are the courtesy of JAS.

**Statistical analysis:**

All data were analyzed using the Prism statistical program from GraphPad 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 are expressed as the mean ± standard error of the mean (SEM). An $\alpha$-level of $p < 0.05$ was chosen to denote statistical significance.
**STUDY 1: Results**

*Increased dietary cholesterol does not impact body weight or adiposity*

Following a one week acclimatization period, guinea pigs were randomly assigned to consume either a low-cholesterol diet containing .04% dietary cholesterol (L-Chol) or a high-cholesterol diet containing .25% cholesterol (H-Chol) for approximately 6 weeks. Both diets were identical in macronutrient and micronutrient composition with the sole difference being the cholesterol content; .04% is equivalent to 300 mg cholesterol/day for humans whereas .25% is equivalent to the consumption of 1800 mg cholesterol/day for humans (32). Thus the H-Chol constitutes an exorbitant source of cholesterol and represents a true cholesterol challenge. A complete composition of the diets is listed in Table 1.1.

Initial body weights (in grams, g) at week one did not significantly differ between groups (369 ± 4.5 vs 372 ± 15, \( p = 0.86 \); Figure 1.1) with the heaviest animal (490g) being in the H-Chol group. Both groups gained weight over the course of six weeks yet surprisingly the L-Chol guinea pigs weighed significantly more at week 6 compared to the H-Chol group (639 ± 17 vs 555 ± 17, \( p = 0.002 \); Figure 1.1) with the heaviest guinea pig weighing 749g and 663g in L-Chol and H-Chol respectively. 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 ± 1.8 vs 45 ± 1.9, \( p = <0.0001 \); Figure 1.2). 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 ± 1.2 vs 7 ± 1.2, \( p = .085 \)). In the L-Chol group, the heaviest animal had the second heaviest...
adipose weight whereas in the H-Chol group, the greatest body weight and adipose mass corresponded to the same guinea pig.

**H-Chol feeding induces hypercholesterolemia but does not affect other plasma metabolic parameters**

Of the various plasma parameters measured in the current study the most noticeable difference between the L-Chol and H-Chol groups resided in total cholesterol. H-Chol guinea pigs displayed significantly 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 1.3). 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 most conspicuous features of guinea pigs that mimics human lipoprotein metabolism. Accordingly, linear regression analysis demonstrated a strongly positive correlation between plasma total cholesterol and LDL-C in L-Chol ($r = 0.9$, $p = 0.03$), H-Chol ($r = 0.96$, $p < 0.0001$), and both L-Chol and H-Chol together ($r = 0.98$, $p < 0.0001$; figure 1.4).

Interestingly, H-Chol feeding appeared to have little to no effect on circulating levels of triglycerides ($p = 0.75$), non-esterified fatty acids ($p = 0.84$), or glucose ($p = 0.86$) compared to the L-Chol controls. **Table 1.2** provides the results for all plasma metabolites measured in this study.

**Feeding H-Chol for six weeks increases biochemical indices of liver injury**
Plasma concentrations of liver transaminase enzymes, in particular ALT and AST, serve as clinical markers of hepatic injury and are often referred to as liver function tests (LFTs). The rationale for measuring ALT and AST (along with alkaline phosphatase, ALP) is that when hepatocytes are exposed to some injurious insult they become damaged and leak these enzymes into the hepatic and eventually, general circulation. In general, the greater plasma concentration of these enzymes indicates more severe liver injury, although several (valid) concerns have been raised about their clinical accuracy and reliability.

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 (table 1.2, figure 1.5) indicating that excessive cholesterol feeding does induce some form of hepatic injury. In support of 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 1.6).

**H-Chol does not impact liver weight but increases the liver to body weight ratio**

To determine whether there were differences in liver size and gross morphology whole livers were excised, immediately weighed on a digital scale, and photographed. Surprisingly the livers from H-Chol guinea pigs were similar to L-Chol livers in wet weight ($p = 0.103$; figure 1.7), yet there were striking differences morphologically. Whereas L-Chol livers were smooth and displayed a dark red-brown complexion H-Chol livers were paler, with an almost cloudy white appearance and a rough, tumescent texture (see histological figures).
While organ wet weight does provide important information, a much more reliable measurement is the ratio of the organ to body weight as a whole. Indeed, despite no differences in total liver weight, the liver to body weight (liver/body weight) ratio was significantly greater in the H-Chol group ($p = 0.001$), constituting almost 4% compared to 3% in L-Chol (figure 1.8). Thus H-Chol livers constitute a greater proportion of total body weight following the six week dietary period. Table 1.3 provides the results for all the hepatic parameters assessed in the current study.

*Six week dietary cholesterol challenge promotes accrual and storage of triglycerides and cholesterol in liver*

A well-documented, yet incompletely understood phenomenon in rodents, including guinea, pigs fed cholesterol-enriched diets is the accumulation of hepatic triglycerides (TG), often resulting in fatty liver. Why excessive dietary cholesterol promotes hepatocellular storage of TG has still not been sufficiently explained at the molecular level, although it is tempting that a cooperative relationship exists between TGs and cholesteryl esters (CEs) in governing lipid accumulation.

We also observed an increase in liver TG content of H-Chol livers compared to L-Chol controls ($p = 0.002$; figure 1.9) and this occurred with a concomitant accumulation of hepatic cholesterol, both total ($p = <0.0001$; figure 1.10) 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 equaled 14 mg/g whereas liver TG was around 12 mg/g while L-Chol livers contained similar quantities of these two lipids (table 1.3).
Lastly hepatic phospholipids were shown to be similar between L-Chol and H-Chol groups ($p = 0.21$). It was interesting that total phospholipid concentration was slightly higher in the L-Chol group (table 1.3) suggesting possible disturbances in hepatic phospholipid balance (see study 3 of this dissertation).

**Six weeks of H-Chol induces moderate steatosis and successfully provokes development of hepatic lesions characteristic of NASH**

A trained pathologist (JAS) who was blinded to the study evaluated liver sections from each guinea pig to assess the degree of fatty infiltration, inflammatory features, and fibrosis. Relevant here is that all sections were examined twice and at different time points, thereby ensuring an unbiased evaluation. Additionally, only steatosis was quantified and assigned a score according to the system of Kleiner et al. (31), while features such as inflammatory infiltration and fibrosis were noted and photographed during the course of analysis. 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 presented with fine microvesicular steatosis of unknown origin 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 1.11). All H-Chol livers displayed a pattern of mixed microvesicular and macrovesicular steatosis and there was little variation in the distribution of fatty change throughout zones 1, 2, or 3 of the lobule as evidenced by both H&E and ORO staining (figure 1.12 B,D,F).
Mild lobular inflammation was a feature common to all H-Chol livers and several displayed pronounced inflammatory foci scattered throughout the parenchyma. In addition to inflammatory infiltrates NASH diagnosis relies on the simultaneous presence of more insidious forms of liver injury, with the cardinal feature being ballooned or degenerated hepatocytes. As the description suggests, hepatocytes that undergo this morphological change typically become swollen and disfigured such that they lose their classic hexagonal shape and contain rarified, foamy cytoplasm, presumably the result of increased accumulation of intracellular fluid. We noted ballooning change in several H-Chol livers, though not surprisingly the extent of this lesion throughout the lobule was considerably variable and there were some sections devoid of any ballooned hepatocytes, consistent with the results of many rodent models of NAFLD.

Aside from ballooning, hepatic injury in NASH is characterized by hepatocellular death which presents histologically as acidophil (apoptotic) bodies or spotty necrosis (6). Single-cell necrosis was evident in livers of H-Chol guinea pigs (figure 1.13 A, arrowhead) and this likely contributed to the emergence of the observed inflammatory infiltrates given the invariable immune response that the presence of necrotic cells initiates. One other notable histological lesion that was observed in H-Chol livers was the presence (albeit relatively minor) of glycogenated nuclei (figure 1.13 B, arrow), which are vacuolated hepatocytes with diffuse cytoplasmic glycogen accumulation typically observed in individuals with glycogen storage disease or with poorly controlled type 1 diabetes (7). Like megamitochondria, Mallory-Denk bodies (MDB), or acidophil bodies, glycogenated nuclei are unique lesions indicative of some underlying liver injury,
which, in this particular study, seems to be the bombardment of the liver with excessive dietary cholesterol.

Overall these histological findings support our study hypothesis that dietary cholesterol promotes not only fatty liver, but also is capable of provoking the transition to NASH.
Table 1.1: Dietary composition of .04% low-cholesterol (L-Chol) and .25% high-cholesterol (H-Chol) diets

<table>
<thead>
<tr>
<th>Macronutrient</th>
<th>L-Chol</th>
<th>H-Chol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% gram</td>
<td>% kcal</td>
</tr>
<tr>
<td>Protein</td>
<td>22.3</td>
<td>23</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>40.6</td>
<td>42</td>
</tr>
<tr>
<td>Fat</td>
<td>15.1</td>
<td>35</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>L-Chol</th>
<th>H-Chol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>grams</td>
<td>grams</td>
</tr>
<tr>
<td>Soy protein</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>L-methionine</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Corn starch</td>
<td>125</td>
<td>125</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Sucrose</td>
<td>214</td>
<td>214</td>
</tr>
<tr>
<td>Cellulose</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Guar gum</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Olive oil</td>
<td>33.36</td>
<td>33.36</td>
</tr>
<tr>
<td>Palm kernel oil</td>
<td>68.11</td>
<td>68.11</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>37.53</td>
<td>37.53</td>
</tr>
<tr>
<td>Mineral mix(^1)</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>Vitamin mix(^1)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.37 ((.04%)(^2))</td>
<td>2.3 ((.25%)(^3))</td>
</tr>
</tbody>
</table>

Caloric density (kcal/g) | 3.87 | 3.88 |

\(^1\)Mineral and vitamin mixes were formulated to meet the National Research Council requirements for guinea pigs.

\(^2\).04% is equivalent to approximately 300 mg cholesterol/day for humans.

\(^3\).25% is equivalent to approximately 1800 mg cholesterol/day for humans.
**Figure 1.1:** Initial and final body weights along with average body weight change of L-Chol and H-Chol guinea pigs

![Graph showing initial and final body weights and average body weight change.](image)

**Figure 1.2:** Average daily food consumption of guinea pigs fed L-Chol or H-Chol

![Graph showing average daily food consumption.](image)

**Figure 1.3:** Plasma cholesterol profile of L-Chol and H-Chol fed guinea pigs

![Graph showing plasma cholesterol profile.](image)
**Figure 1.4:** Plasma total cholesterol as a function of LDL-C

- **L-CHOL**
- **H-CHOL**

- **Figure 1.4:** Plasma total cholesterol as a function of LDL-C

- **L-Chol & H-Chol**

- **TC** (mg/dL)
  - $r = 0.98$
  - $r^2 = 0.97$
  - $p < 0.0001^*$

- **LDL-C** (mg/dL)
Table 1.2: Plasma characteristics of guinea pigs fed .04% and .25% cholesterol for six weeks

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>L-Chol (n = 5)</th>
<th>H-Chol (n = 10)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>48 ± 4.8</td>
<td>284 ± 36.6</td>
<td>0.0001*</td>
</tr>
<tr>
<td>Free cholesterol (mg/dL)</td>
<td>21 ± 1.4</td>
<td>99 ± 10</td>
<td>&lt; 0.0001*</td>
</tr>
<tr>
<td>*LDL-C (mg/dL)</td>
<td>32 ± 6.2</td>
<td>201 ± 29</td>
<td>0.0003*</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>4 ± 1.4</td>
<td>72 ± 11.6</td>
<td>0.0003*</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>58 ± 9.8</td>
<td>61 ± 6</td>
<td>0.75</td>
</tr>
<tr>
<td>Non-esterified fatty acids (mEq/L)</td>
<td>0.5 ± 0.03</td>
<td>0.5 ± 0.04</td>
<td>0.84</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>186 ± 12</td>
<td>184 ± 9.4</td>
<td>0.86</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>86 ± 23.2</td>
<td>196 ± 30.8</td>
<td>0.042*</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>213 ± 71.7</td>
<td>472 ± 72.6</td>
<td>0.049*</td>
</tr>
</tbody>
</table>

All data presented as mean ± standard error of the mean (SEM)

Abbreviations: LDL-C; low-density lipoprotein cholesterol, HDL-C; high-density lipoprotein cholesterol, ALT; alanine aminotransferase, AST; aspartate aminotransferase

*LDL-C calculated according to Friedwald equation.
Figure 1.5: Plasma activity level of liver enzymes in L-Chol and H-Chol guinea pigs

![Graph showing plasma activity level of liver enzymes in L-Chol and H-Chol guinea pigs.]

- ALT: L-CHOL (100), H-CHOL (300)
- AST: L-CHOL (200), H-CHOL (600)

Figure 1.6: Plasma ALT as a function of plasma total cholesterol in H-Chol fed guinea pigs

![Graph showing plasma ALT as a function of plasma total cholesterol in H-Chol fed guinea pigs.]

- Correlation: $r = 0.78$, $r^2 = 0.61$, $p = 0.02^*$
**Figure 1.7:** L-Chol and H-Chol guinea pig liver weights

![Graph showing liver weight comparison between L-CHOL and H-CHOL with p = 0.103](image)

**Figure 1.8:** Ratio of liver to final body weight in L-Chol and H-Chol

![Graph showing liver weight percentage comparison between L-CHOL and H-CHOL with p = 0.001*](image)
Table 1.3: Hepatic characteristics of guinea pigs fed .04% and .25% cholesterol for six weeks

<table>
<thead>
<tr>
<th>Liver parameter</th>
<th>L-Chol ($n = 10$)</th>
<th>H-Chol ($n = 10$)</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weight (g)</td>
<td>$19.3 \pm .8$</td>
<td>$21.9 \pm 1.3$</td>
<td>0.103</td>
</tr>
<tr>
<td>Liver/body weight (%)</td>
<td>$3 \pm .1$</td>
<td>$3.9 \pm .2$</td>
<td>0.001*</td>
</tr>
<tr>
<td>Triglycerides (mg/g)</td>
<td>$6 \pm .4$</td>
<td>$11.6 \pm 1.3$</td>
<td>0.002*</td>
</tr>
<tr>
<td>Phospholipids* (ug/g)</td>
<td>$29557 \pm 1359$</td>
<td>$27026 \pm 1216$</td>
<td>0.21</td>
</tr>
<tr>
<td>Total cholesterol (mg/g)</td>
<td>$5.1 \pm .4$</td>
<td>$14 \pm 1.3$</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Steatosis score</td>
<td>$.2 \pm .2$</td>
<td>$1.8 \pm .2$</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>

All data presented as mean ± standard error of the mean (SEM)

* For phospholipid analysis, $n = 6$ for L-Chol and $n = 5$ for H-Chol
Figure 1.9: Hepatic triglyceride content of L-Chol and H-Chol guinea pigs

\[ p = 0.002^* \]
Figure 1.10: Hepatic total cholesterol content of L-Chol and H-Chol guinea pigs
Figure 1.11: Liver steatosis scoring for L-Chol and H-Chol fed guinea pigs

Figure 1.12: Gross morphology and histology of livers from L-Chol and H-Chol guinea pigs (see following page)
Figure 1.13: Features of liver injury characteristic of NASH in H-Chol guinea pigs
STUDY 1: Discussion

In the current study we have presented evidence that supports a causative relationship between cholesterol-enriched dietary feeding and the emergence and progression of NAFLD in non-genetically modified guinea pigs. Over the course of six weeks guinea pigs fed a diet containing 0.25% cholesterol (H-Chol) developed marked hypercholesterolemia, displayed biochemical evidence of hepatic injury, and accumulated copious amounts of triglyceride and cholesterol species in the liver causing moderate steatosis. Accompanying this fatty infiltration was the presence of several histological features characteristic of NASH, including mild lobular inflammation, hepatocyte ballooning, single cell necrosis, and glycogenated nuclei. Altogether, these data demonstrate that manipulating dietary cholesterol content may prove a useful experimental approach in understanding the pathophysiology of NAFLD.

To our knowledge, this is the first study to investigate the impact of high cholesterol on liver pathology in guinea pigs, which may serve as a possible alternative to the mouse or rat models that have long commandeered the field of NAFLD research. Previous studies in our lab have demonstrated that guinea pigs accumulate significant amounts of triglyceride and total, free, and esterified cholesterol when fed cholesterol-enriched diets (32). This same study also demonstrated that feeding 0.25% cholesterol for 12 weeks impacted several key regulatory mechanisms involved in hepatic cholesterol metabolism. Specifically, guinea pigs fed the high cholesterol diet had lowered hepatic mRNA abundance of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) and increased activity of acyl CoA cholesterol acyltransferase (ACAT) when
compared to low-cholesterol fed guinea pig controls (32). 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 HMGR, a
major regulatory point in cholesterol synthesis (33). 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 (22,34). 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 (35). 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 dependant (36).

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 species (MUFA) (37). 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 (38).

One of the features that makes guinea pigs attractive for studying cholesterol’s involvement in NAFLD is that they, like humans, 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 nor 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 as well as 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.

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.
References:


Abstract:

Cholesterol-induced non-alcoholic fatty liver disease (NAFLD) occurs in guinea pigs when fed diets containing 0.25% dietary cholesterol, yet whether or not dietary manipulation can improve the state of disease in guinea pigs is not yet well known. Because guinea pigs respond to dietary intervention such as carbohydrate restriction in a manner resembling humans, we sought to explore the metabolic effects of manipulating dietary fat and carbohydrate content in guinea pigs with pre-existing NAFLD and determine whether these interventions could improve histological features characteristic of this disease. Accordingly, after a six week period of a high-cholesterol diet, guinea pigs were randomized to consume either a diet enriched in both fat and carbohydrate (herein referred to as a high-carbohydrate diet, HCD) or a diet restricted in carbohydrate but plentiful in fat (a low carbohydrate diet, LCD). Both diets contained equivalent cholesterol (0.04% w/w). Both HCD and LCD were consumed for six weeks and guinea pigs were evaluated for metabolic parameters and liver histology to determine any dietary effects. Plasma cholesterol species were elevated in LCD compared to HCD guinea pigs, yet only cholesterol associated with HDL (HDL-C) reached statistical significance ($p = 0.044$). Circulating triglyceride (TG) was slightly though not significantly ($p = 0.28$) greater in LCD versus HCD, while there were no differences in circulating levels of glucose ($p = 0.85$), non-esterified fatty acids ($p =$ 125
0.97), nor plasma activity of the liver enzymes ALT ($p = 0.59$) or AST ($p = 0.75$). Plasma ketone bodies were only slightly elevated in the LCD group as compared to the HCD ($p = 0.07$), though this may be partially explained by the variable responses of guinea pigs fed the LCD. Levels of hepatic TG ($p = 0.01$), total cholesterol ($p = 0.034$), and free cholesterol ($p = 0.02$), but not cholesterol ester ($p = 0.45$) were increased in guinea pigs fed LCD compared to HCD, indicating increased retention of these lipids under a LCD. Remarkably, despite the increased lipid content of LCD livers, there were similar histological findings between both groups, with guinea pigs fed LCD or HCD displaying evidence of bridging fibrosis. There was also evidence of biliary hyperplasia in the HCD group, although this response was not uniform throughout the group. These findings collectively suggest that feeding either a LCD or HCD following a dietary cholesterol challenge fails to improve liver histopathology, possibly indicating that dietary cholesterol loading renders livers unresponsive to dietary macronutrient manipulation.
STUDY 2: Introduction

To date, there have been relatively few long-term controlled studies investigating the role of dietary composition, in particular, carbohydrate and carbohydrate restriction on NAFLD although several lines of evidence suggest that limited carbohydrate consumption may be beneficial towards improved liver function.

Several investigators have observed a strong association between excessive carbohydrate intake and an increased severity of hepatic steatosis and the inflammatory NASH (1,2) in humans. In a recent prospective study, Kang et al (2) evaluated 91 patients with clinically suspected NAFLD and characterized 31 patients (34%) as having metabolic syndrome. Those patients with metabolic syndrome were shown to consume significantly more carbohydrate (p =0.03) and less fat (p =0.01) and displayed higher histological severity scores for steatosis (p =0.004) and NASH (p =0.0006) compared to patients without metabolic syndrome. This particular study is noteworthy as it recapitulates current evidence which demonstrates a potent link between excessive carbohydrate consumption and the development of physiological factors associated with metabolic syndrome as elegantly described by Volek et al (3). A carbohydrate restricted dietary intervention was recently conducted in an obese female patient diagnosed with NAFLD (4) that presented with abnormal plasma ALT levels and a hepatic triglyceride content of 44.6% as assessed by $^1$H MRS. The patient was instructed to follow for 2 weeks a ketogenic diet composed of 5: 41: 54 % carbohydrate: fat: protein respectively. At 5 weeks after the initiation of the diet, there was a complete correction of plasma ALT values and remarkably, a dramatic four-fold reduction in hepatic triglyceride content to
11.9%. While long-term controlled human studies designed to evaluate the role of carbohydrate restriction on improvements in NAFLD are sparse, the available evidence does indeed support a beneficial role for this dietary intervention in ameliorating the associated clinical and histological abnormalities inherent in this disease. In an interesting animal study (5) two groups of mice were fed diets high in carbohydrate (68%) that differed in starch composition in order to examine the effects of rapidly absorbed carbohydrate on hepatic steatosis. One group consumed a diet composed of 100% amylopectin, a highly branched chain glucose polymer which is rapidly metabolized within the small intestine, whereas the other group consumed 60% amylose and 40% amylopectin; amylose due to its linear structure, is hydrolyzed at a slower rate and therefore predicted to contribute to less severe hepatic steatosis. Indeed, histological examination indicated the presence of mixed hepatic steatosis in mice fed the rapidly absorbed carbohydrate and a two-fold greater hepatic triglyceride content (20.7 ± 9.4 mg/g vs 9.6 ± 4.9 mg/g; p =0.01) when compared to the mice that consumed the slower absorbed carbohydrate. Again, long term clinical studies which examine the relationship between carbohydrate and NAFLD in humans are warranted, however this study clearly points to the potential benefit(s) of reduced carbohydrate or low glycemic index diets as a novel dietary treatment for reversing the progression of hepatic steatosis.

Dietary fat, in particular PUFAs are known to exert hypotriglyceridemic effects and are a potentially important dietary treatment in reducing cardiovascular disease risk. Evidence is also accumulating that suggests they may also exert various
hepatoprotective effects. These fatty acids are well known regulators of hepatic gene
expression with regard to carbohydrate and lipid metabolism, suppressing L-PK in the
glycolytic pathway as well as several key lipogenic enzymes (see (6) for a brief review).
Impressively, SREBP-1c is potently inhibited by PUFAs and although the mechanistic
details require further studies, it has been demonstrated that PUFAs reduce SREBP-1c
transcription, enhance SREBP-1c mRNA turnover and directly interfere with the
proteolytic cleavage of the transcriptionally mature SREBP-1c protein (6). It has also
been recently demonstrated that PUFAs, specifically linoleate (18:2), eicosapentanoic
acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) effectively inhibit ChREBP
activity in mice, thereby decreasing expression of both L-PK and FAS (7). A recent
study investigating the potential therapeutic effects of omega-3 FAs in patients clinically
diagnosed as having hepatic steatosis (8) found that those receiving 2.7 grams daily of
concentrated EPA for twelve months had significantly reduced plasma values of ALT
and AST and a regression of steatosis as assessed by histological analysis. An eight
week trial in patients with elevated hepatic TG content (as assessed by proton magnetic
resonance spectroscopy) who received 9 grams/day of fish oil showed a 46% reduction
in plasma TG, a 21% reduction in plasma VLDL and IDL cholesterol and a 15%
decrease in plasma apolipoprotein B, yet no improvement in hepatic TG content (9). It
must be mentioned that the sample population for this study was fairly small (n=17) and
included mainly African-Americans, who tend to accumulate less intraperitoneal fat and
differ from other ethnic groups such as Hispanics and Caucasians in terms of the
prevalence of steatosis (10).
Lastly, feeding ketogenic diets that are very low in carbohydrate content have been shown to induce a unique metabolic state in mice by improving glucose homeostasis, insulin sensitivity, plasma liver enzyme levels, and significantly reducing weight gain (11). They have also been demonstrated to prevent hepatic steatosis by reducing hepatic levels of palmitate (16:0), the end product of DNL (12). Therefore an intervention based on manipulating the macronutrient content may prove a valuable therapeutic modality in NAFLD. This possibility is the subject of investigation in the current study, in which guinea pigs were assigned to a diet either enriched in fat and low in carbohydrate (LCD) or a diet enriched with carbohydrate and fat (HCD). Importantly these diets were administered after six weeks of high-cholesterol feeding to determine the metabolic and pathological impact of each diet on guinea pigs with established liver damage.
STUDY 2: Materials and experimental methodology

Study design:

As demonstrated in study 1, guinea pigs fed H-Chol for six weeks are hypercholesterolemic and develop hepatic steatosis and histological features characteristic of NASH. To ascertain whether manipulating dietary macronutrient composition could potentially improve these metabolic and pathological abnormalities, we randomly selected nineteen guinea pigs after six weeks of H-Chol feeding and further randomized them to consume either a low-carbohydrate diet (LCD, \( n = 9 \)) or a high-carbohydrate diet (HCD, \( n = 10 \)). Both diets were prepared by Research Diets (New Brunswick, NJ: LCD, D10030306; HCD, D10030305) and diets and water were consumed \textit{ad libitum} for a total of six weeks. Importantly, both the LCD and HCD contained similar amounts of dietary cholesterol (0.04% and 0.05% for LCD and HCD respectively) which was equivalent to an intake of 300 mg/day for humans (Table 2.1).

All guinea pigs were housed in the biology vivarium in the School of Pharmacy under identical conditions to those as study 1. Weekly body weight measurements and assessment of food intake were performed identical to study 1. 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 (IACUC) at UConn.

At the conclusion of the six week study period all guinea pigs were fasted for 12 hours overnight and then anesthetized under isoflurane vapors. Following cardiac puncture, whole blood and livers were collected for analysis as described in study 1.
Our original power calculations determined that a sample size of 10 guinea pigs/group would be sufficient to determine statistical significance, however one animal from the LCD had to be euthanized due to focal necrosis, colon rupture, and acute peritonitis of the abdominal cavity. These morphologic findings were unrelated to any effects from the diet itself. Thus all analyses of the LCD group were performed with a sample size of nine guinea pigs.

**Plasma metabolite analysis:**

Whole blood and plasma separation was performed identical to study 1. Plasma analytes were identical to those measured in study 1 and will not be described here (see materials and experimental methodology section of study 1 for assay descriptions and principles). An additional plasma metabolite that was measured in the current study was ketone bodies, the details of which are provided below.

*Ketone bodies*

The plasma ketone bodies acetoacetone (AcAc) and 3-hydroxybutyrate (3-HB) were measured using a commercially available kit (Wako Diagnostics, Richmond, VA) according to the manufacturer’s instructions. The assay is based on a series of cyclic enzymatic reactions in which AcAc and 3-HB are converted to 3-HB and AcAc respectively. The reactions proceed in the presence of 3-hydroxybutyrate dehydrogenase, NADH, and thio-NAD and by measuring the rate of thio-NADH production spectrophotometrically it is possible to determine the concentration of ketone bodies in plasma. After mixing sample plasma with the appropriate reagents, readings were taken every 30 seconds for two minutes at 405nm. The plasma concentration of
AcAc and 3-HB were calculated by multiplying the concentration of a calibrator by the per minute change in optical density of the sample and dividing by the change in calibrator optical density per minute. Results are expressed as µmol/L. A schematic of the reaction sequence is shown here:

Hepatic lipid analysis:

Liver extraction and selection of tissue samples was conducted as described in study 1. Protocols for lipid extraction and biochemical measurements of lipid species is identical to study 1.

Hepatic glycogen:

To determine the metabolic impact of a LCD or HCD on liver glycogen storage, approximately 1 g of liquid nitrogen frozen liver was homogenized in a small amount of distilled water (dH2O) using a Tissue Tearor homogenizer (model 985370, Biospec Products Inc). Each homogenized liver sample was then taken to a final content of 30-50% in 30% potassium hydroxide (KOH), heated at 100°C for 2 hours, and cooled. Next, two volumes of 95% ethanol was added to precipitate the crude glycogen. Samples were then centrifuged and the precipitates collected. Precipitates were then suspended in a minimal amount of dH2O and acidified to a pH of 3 with 5N hydrochloric acid (HCL), followed by re-precipitation with 1 volume of ethanol. The
wash/acidification/precipitation process was repeated a total of three times and the precipitates were finally washed with ethanol and dried in a speed vac. Treating the precipitates in this manner ultimately removes a great majority of glucose with minimal effect on tissue glycogen.

After weighing the dried material, samples were ready to be analyzed with a direct enzymatic procedure in accordance with the manufacturer’s instructions (Abcam Inc, Cambridge, MA). The assay is based on an enzymatic procedure in which glucoamylase first hydrolyzes glycogen to free glucose. Next, glucose oxidase and peroxidase act on the free glucose, generating a product capable of reacting with a color probe that, when measured spectrophotometrically at 570 nm, provides the glycogen concentration present in the liver sample. To correct for any potential background concentration of glucose, an aliquot of each sample was used as a glucose control in which no hydrolysis enzyme mix was added. The level of glucose background in each sample was then subtracted from the glycogen readings. In addition to measuring hepatic glycogen in LCD and HCD guinea pigs, a small sample (n = 4) of L-Chol livers were included as controls to observe how glycogen content changes under different dietary conditions. Results were obtained as µg/µL and then converted to µg/g liver.

Liver pathology:

Selection of liver samples for histological staining and pathological evaluation followed the same procedure as described for study 1. Parameters for grading hepatic steatosis were also identical to study 1. A trained pathologist (JAS) was responsible for
histological evaluation of all liver sections as well as photomicrographs of representative lesions.

**Statistical analysis:**

All data were analyzed using the Prism statistical program from GraphPad Software version 5.0c (San Diego, CA). Two-tailed independent student’s t-tests were performed when comparing the differences between LCD and HCD groups. In the case of unequal variances, an unpaired t-test with Welch’s correction was used. To compare differences between H-Chol, LCD, and HCD, a one-way analysis of variance (1-way ANOVA) was used followed by Tukey’s *post-hoc* multiple comparisons test. In the case of unequal variances as determined by Bartlett’s test, data were analyzed with Kruskal-Wallis test followed by Dunn’s *post-hoc* multiple comparisons test All data are expressed as the mean ± standard error of the mean (SEM). An α-level of $p < 0.05$ was chosen to denote statistical significance.
**STUDY 2: Results**

*LCD and HCD guinea pigs show similar weight gain despite increased daily food consumption in HCD group*

Following a one week acclimatization period, guinea pigs who had previously been fed a H-Chol diet for six weeks were randomized to consume either a LCD or HCD with normal dietary cholesterol (0.04%) for an additional six week period. Nutritionally, the LCD consisted primarily of dietary fat and protein, with the least amount of calories coming from carbohydrate sources (65:25:9 % kCal from fat, protein, and carbohydrate respectively) while the HCD consisted primarily of carbohydrate, followed by fat and protein (54:30:15 % kCal). **Table 2.1** provides the complete composition and **table 2.2** lists the percentage distribution of dietary fatty acid classes for both diets.

There were no significant differences in initial body weights between LCD and HCD (613 ± 37 vs 643 ± 22; \( p = 0.48 \)), nor were there any differences in the final body weights at week 6 (707 ± 45 vs 748 ± 29, \( p = 0.45 \); **figure 2.1 a**). It is possible that a significant difference could exist between final body weights of LCD and HCD were it not for one guinea pig that weighed over 1 kilogram (**figure 2.1 b**). Accordingly, both groups displayed a similar change in body weight (\( p = 0.77 \)) over the course of six weeks. These changes occurred despite HCD animals being more hyperphagic, as they had significantly greater daily food consumption (\( p = 0.0002 \)). This effect is likely due to the greater overall caloric density of the LCD compared to the HCD (**table 2.1**). These data indicate that LCD and HCD have similar effects on body weight over the course of six weeks. Typically LCDs initiate greater weight loss in guinea pigs.
compared to HCDs, however these results were observed with longer feeding durations such as 12 weeks (13), thus the current six week study may have been insufficient for these changes to occur.

**Carbohydrate restriction tends to increase plasma cholesterol**

Reducing dietary carbohydrate is known to have several profound effects on lipoprotein metabolism. For example greater dietary consumption of saturated fats and cholesterol that can occur on a LCD raises plasma total cholesterol, but interestingly, much of this increase is attributed to cholesterol contained within the HDL particles.

In the current study we observed a trend for higher concentrations of plasma TC, FC, and LDL-C in LCD compared to HCD (figure 2.3). Interestingly the only parameter that differed significantly was plasma HDL-C and this was greatest in LCD compared to HCD ($p = 0.044$). A clue to understanding these data may be the degree of variability observed within the LCD. Figure 2.4 depicts the individual plasma TC responses, which in the HCD are closely centered around the mean while values are much more variable in the LCD group. Similar variability was seen in the individual plasma FC, LDL-C, and HDL-C responses (data not shown). The cholesterol profile of both LCD and HCD demonstrated that LDL particles transport the majority of cholesterol, which is unique amongst other experimental rodent models such as the rat or mouse that maintain very elevated concentrations of HDL particles. Linear regression analysis revealed a strong positive correlation between plasma TC and LDL-C in LCD ($r = .99, p = < 0.0001$), HCD ($r = .98, p = < 0.0001$), and LCD and HCD together ($r = .99, p < 0.0001$; figure 2.5 a).
Finally, this relationship also persisted when L-Chol, H-Chol, LCD, and HCD groups were analyzed together ($r = .99, p < 0.0001$; figure 2.5 b).

The average plasma TC of both LCD and HCD it should be noted, were significantly lower as compared to the H-Chol group according to ANOVA ($p < 0.0001$). In fact, all plasma cholesterol parameters of LCD and HCD differed significantly from H-Chol (see table 2.5 at the end of this section which lists the ANOVA results of all plasma and hepatic parameters between H-Chol, LCD, and HCD). This implies that removal of exaggerated amounts of dietary cholesterol improves hypercholesterolemia whether guinea pigs were on a LCD or HCD.

**LCD and HCD produced similar effects on plasma metabolic parameters**

One of the most commonly observed features of either very-low or moderate LCDs is a pronounced reduction in VLDL-associated triglycerides. In the present study however this was not observed as both LCD and HCD had similar levels ($p = 0.28$). Furthermore, we could not detect any statistical differences in other routine plasma parameters such as NEFA ($p = 0.97$) and glucose ($p = 0.85$) between LCD and HCD. Table 2.3 provides the results for all the plasma metabolites measured and figure 2.6 depicts the individual responses for plasma triglyceride (a), NEFA (b), glucose (c), and ketone bodies (d). One of the most interesting results concerned plasma ketone bodies, which tended to be higher in LCD but did not reach statistical significance at the 0.05 level. This result was particularly surprising given the overwhelming reliance of dietary fat as opposed to carbohydrate in the LCD group, conditions that favor ketone body synthesis and utilization. This may partially be explained by the individual
responses of LCD guinea pigs which are highly variable compared to those of the HCD (figure 2.6, d). Likely, carbohydrate restriction elicits unique individual metabolic responses whose importance may lie in their variability rather than the totaled group average of certain plasma parameters.

**Feeding a LCD or HCD for six weeks fails to improve biochemical indices of liver injury**

Both LCD and HCD guinea pigs displayed similar plasma activity levels of the liver enzymes ALT ($p = 0.59$) and AST ($p = 0.75$), indirectly indicating a similar degree of hepatic injury (table 2.3, figure 2.7). Interestingly, despite a trend for lower plasma FC in the HCD group, AST activity level was positively associated with this plasma variable ($r = .8$, $p = 0.009$). This correlation hints at a relationship between plasma FC and liver injury that occurs only in the context of a HCD. Most importantly neither plasma ALT ($p = 0.82$) or AST ($p = 0.66$) differed significantly from those of the H-Chol group as determined by ANOVA (table 2.5). These results imply that a LCD or HCD is unable to improve these markers of biochemical injury following a period of H-Chol feeding, a finding which conflicts with our original study hypothesis.

**Feeding a LCD for six weeks does not significantly reduce hepatic glycogen compared to HCD**

Liver glycogen depletion is a well known metabolic effect of carbohydrate restriction while feeding a carbohydrate-enriched diet is known to stimulate glycogenesis thereby increasing hepatic glycogen stores. In the current study however, both LCD and HCD livers contained similar concentrations of glycogen ($p = 0.12$; figure 2.8 a, table 2.4). The lack of statistical significance could be the result of the
considerable variability of the HCD group. Contrary to other experimental measures, liver glycogen was fairly consistent between LCD animals and centered around the mean. There is also the possibility that this particular carbohydrate-restricted diet formulation was not low enough in carbohydrate content to elicit glycogenolysis, or glycogen breakdown. For a comparison, we also measured the glycogen content of a small sample ($n = 4$) of L-Chol controls and found that both LCD and HCD displayed greater glycogen storage. This is the first study that we are aware of that has measured this parameter in guinea pigs therefore additional studies need to be conducted to gain insight as to how glycogen content changes according to diet.

Despite similar concentrations, glycogen content was shown to be positively correlated with liver weight ($r = .63$, $p = 0.05$) in HCD animals only. Even more intriguing was the positive correlation between glycogen and plasma ALT activity ($r = .7$, $p = 0.04$; figure 2.8 b) in the HCD group—although one cannot infer causality from regression analysis, this relationship suggests that increased glycogen content may be an important contributor to plasma ALT levels, at least in the context of a HCD.

**LCD livers show increased retention and storage of hepatic triglycerides, total, and free cholesterol without any difference in organ weight**

Previously it was observed that guinea pigs fed a LCD in conjunction with 0.25% dietary cholesterol develop severe hepatomegaly compared to guinea pigs fed the same cholesterol concentration on a HCD (13). In the present study where both the LCD and HCD have normal cholesterol content, there was no difference in organ weight between the groups ($p = 0.36$; figure 2.9). The results for all hepatic parameters is
provided in table 2.4. Liver weight was shown to be correlated with final body weight for both LCD and HCD (figure 2.10 a,b), although the relationship appears strongest in the LCD group. Furthermore, the liver to body weight ratio tended to be higher in LCD, constituting around 4% compared to about 3.5% in HCD, though this did not reach statistical significance ($p = 0.055$; figure 2.11). Given the correlation between liver and final body weight in the LCD group, it is not surprising that the individual ratios varied in a manner similar to those for final body weight (figure 2.1 b), which may explain the lack of statistical significance.

Increased cytoplasmic accumulation of fatty acids, triglycerides, and cholesterol results in hepatic steatosis, the *sine qua non* of NAFLD. Implementing a LCD or HCD following a H-Chol challenge was hypothesized to reduce hepatic lipid content, albeit through different mechanisms. By limiting the amount of dietary carbohydrate (as glucose and fructose) delivered to the liver, a LCD would in effect limit pancreatic insulin release, thereby reducing insulin's potent stimulatory effect on DNL and simultaneously increasing the oxidation and removal of fatty acids to the circulation as ketone bodies. In contrast, a HCD, by providing more carbohydrate and less fat to the liver, could reduce hepatic lipid content by increasing the production of fatty acids and triglycerides which in turn would be incorporated into VLDL particles for eventual egress to the circulation (see literature review for detailed discussion).

Interestingly, liver TG content was significantly greater in LCD livers ($p = 0.01$; figure 2.12) compared to HCD. There was however, a significant reduction of liver TG in both LCD and HCD compared to the H-Chol group ($p = 0.0005$; table 2.5), indicating
that indeed both groups were capable of decreasing cellular TG stores. A particularly intriguing observation was that in LCD animals, lower levels of liver TG tended to be correlated with higher plasma ketone bodies ($r = -.65, p = 0.056$) while a positive correlation, though not significant, was observed in the HCD group ($r = .45, p = 0.19$). This could imply that the microsomal and mitochondrial oxidative systems responsible for generating fatty acyl-CoAs may be impaired or overwhelmed in HCD livers. Hepatic levels of both TC ($p = 0.034$; figure 2.13 a,b) and FC ($p = 0.02$; figure 2.13 c,d) were also increased in LCD guinea pigs compared to HCD, however there was no difference in cholesterol ester content ($p = 0.45$; figure 2.13 e,f). One should also note the considerable variability of individual responses in the LCD group with regard to hepatic cholesterol measurements when interpreting these data. Together, these findings indicate that LCD livers are prone to increased retention of both cholesterol and TG following a H-Chol challenge. It also appears that liver cholesterol content is a more important contributor to overall liver weight, at least in LCD livers as we observed a positive correlation between liver weight and both TC ($r = .82, p = 0.007$) and FC ($r = .71, p = 0.04$), but not TG ($r = .38, p = 0.31$). There was no correlation between liver weight and hepatic TC ($r = -.19, p = 0.6$), FC ($r = .024, p = 0.95$), or TG ($r = -.42, p = 0.23$) in the HCD group.

Overall, hepatic TC content was significantly lower in both LCD and HCD when compared to H-Chol ($p = 0.0001$; table 2.5). It should be mentioned here that in LCD livers TC was present in higher concentrations than TG, a finding that was also present in the H-Chol group, but absent in the HCD group (table 2.5). The importance of this
observation is currently unclear. Lastly, the concentration of hepatic phospholipids did not differ significantly between either LCD or HCD ($p = 0.64$), however there was a consistent reduction of this lipid when compared to the H-Chol group ($p = 0.001$; table 2.5). The consequences of this overall reduction in PL content are as of yet unknown, although it is feasible that it may play a role in worsening liver pathology (see below). More detailed analysis of hepatic PL under these different dietary schemes is provided in study 3 of this dissertation.

**Six weeks of LCD or HCD resulted in similar histological grading of steatosis**

As described in study 1, liver sections were evaluated for fatty infiltration and assigned a score based on the percentage of hepatocytes that show fatty change. There were no overt differences in the gross morphology of LCD or HCD livers (figure 2.14 a) with each group containing a range of normal reddish, brown as well as swollen, pale livers indicative of increased lipid accumulation. Surprisingly there was no statistical difference between the group scores of LCD and HCD ($p = 0.13$; table 2.4, figure 2.14 b) although the overall score was slightly higher in the LCD group. Three LCD livers (33%) were designated as having severe steatosis (grade 3), while zero guinea pigs in the HCD group displayed steatosis to this extent. Instead, the majority of HCD livers displayed either minimal, grade 0 (40%) or mild, grade 1 (40%) fatty infiltration. Additionally, the pattern of steatosis for both groups was predominantly macrovesicular with mixed microvesicular droplets present as well. It seems likely that increased cholesterol deposition may also be a more important determinant of steatosis in the LCD group as we observed a strong positive correlation between steatosis score
and liver TC content ($r = .75, p = 0.02$). This relationship was not present in the HCD group ($r = .44, p = 0.205$). No statistically significant correlations were observed between fat score and other lipids, particularly TG, in either LCD ($r = .52, p = 0.15$) or HCD ($r = -.03, p = 0.93$).

One of the original hypotheses for this study was that both a LCD and HCD would successfully reduce the degree of hepatic steatosis from what was observed in H-Chol livers, albeit through different mechanisms. Surprisingly, although the average score for LCD and HCD was lower than the H-Chol group, there was not an overall significant difference between groups as revealed by ANOVA ($p = 0.054$) (table 2.5, figure 2.14 c). This result is somewhat incongruous with the earlier findings that all hepatic lipids were significantly reduced in the LCD and HCD compared to H-Chol (table 2.5). It is currently unclear as to how to interpret this result, although it does highlight some potential discrepancies that likely exist between biochemical data and pathological examination.

**Heterogeneous pathological response of livers from both LCD and HCD fed guinea pigs**

Hepatic steatosis, as we have discussed previously, is the beginning of the NAFLD spectrum and sets the stage for potential development of more insidious forms of liver injury, including hepatic inflammation and ballooning which comprises NASH, as well as bridging fibrosis. Therefore reducing the amount of lipid deposition in the liver is seen as potentially beneficial for improving hepatic architecture and reducing the possibility of various ‘second hits’ that can trigger disease progression. We noted
earlier the progressive decrease of liver TG, TC, and FC (table 2.5) along with the trend for decreased pathological scoring of steatosis as guinea pigs were transitioned from H-Chol to LCD or HCD (figure 2.14 c). Therefore it is all the more surprising that there was actually a worsening of liver pathology in some guinea pigs of both the LCD and HCD groups. While there were indeed some sections that were histologically normal, there were also sections that presented with more advanced histological lesions such as inflammation and hepatocyte ballooning (figure 2.15). Perhaps the most striking observation of this study centered around the presence of advanced lesions in livers with mild or minimal steatosis as shown in figure 2.16. This figure depicts a LCD liver with grade 1 steatosis and bridging fibrosis (panels A,C) and a HCD liver with grade 0 steatosis and biliary hyperplasia (panels B,D). Interestingly, there were no conspicuous metabolic parameters in this LCD animal that would suggest possible mechanisms for fibrotic development. On the other hand, the HCD animal that presented with biliary hyperplasia had the highest levels of all plasma cholesterol species and plasma AST in the HCD group. Another example is shown in figure 2.17 which demonstrates a pattern of bridging fibrosis in a HCD guinea pig with grade 0 steatosis. Taken together these results may imply an inverse relationship between more advanced hepatic lesions and steatosis.

Overall, administering a six week LCD or HCD intervention following a six week H-Chol challenge resulted in a heterogeneous pathological response in the liver; that no clear cut separation between LCD or HCD could be made suggests that both groups were equally affected by the H-Chol challenge. It is likely that bombarding the liver with
copious amounts of dietary cholesterol produces some lingering cellular effects that proceed even when a LCD or HCD is administered.
Table 2.1: Dietary composition of low-carbohydrate diet (LCD) and high-carbohydrate diet (HCD)

<table>
<thead>
<tr>
<th>Macronutrient</th>
<th>LCD</th>
<th>HCD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% gram</td>
<td>% kcal</td>
</tr>
<tr>
<td>Protein</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Fat</td>
<td>34</td>
<td>65</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>LCD grams</th>
<th>HCD grams</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy protein</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>L-methionine</td>
<td>6.25</td>
<td>3.75</td>
</tr>
<tr>
<td>Corn starch</td>
<td>-</td>
<td>239.5</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>90.5</td>
<td>35</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>274</td>
</tr>
<tr>
<td>Cellulose</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Guar gum</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>194</td>
<td>89</td>
</tr>
<tr>
<td>Coconut oil, hydrogenated</td>
<td>60</td>
<td>28</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>39</td>
<td>18</td>
</tr>
<tr>
<td>Mineral mix¹</td>
<td>13.88</td>
<td>13.88</td>
</tr>
<tr>
<td>Vitamin mix¹</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.42 (.05%)</td>
<td>0.42 (.04%)</td>
</tr>
<tr>
<td>Caloric density (kcal/g)</td>
<td>4.77</td>
<td>3.87</td>
</tr>
</tbody>
</table>

¹Mineral and vitamin mixes were formulated to meet the National Research Council requirements for guinea pigs.

Table 2.2: Distribution of fatty acid classes in LCD and HCD

<table>
<thead>
<tr>
<th>Fatty Acid Class</th>
<th>LCD % kcal</th>
<th>HCD % kcal</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA</td>
<td>17.6</td>
<td>8.2</td>
</tr>
<tr>
<td>MUFA</td>
<td>35.8</td>
<td>16.4</td>
</tr>
<tr>
<td>PUFA</td>
<td>11.4</td>
<td>5.2</td>
</tr>
</tbody>
</table>
Figure 2.1: Average initial and final body weights (a) and individual final body weights (b) of LCD and HCD fed guinea pigs

![Graph showing body weight changes and final body weights for LCD and HCD fed guinea pigs.]

Figure 2.2: Average body weight change (a) and daily food consumption (b) of LCD and HCD guinea pigs

![Graph showing body weight change and food consumption for LCD and HCD fed guinea pigs.]
Figure 2.3: Plasma cholesterol profile of LCD and HCD fed guinea pigs

Figure 2.4: Individual TC values of LCD and HCD guinea pigs
Figure 2.5: Correlation between plasma TC and LDL-C in LCD and HCD (a) and in L-Chol, H-Chol, LCD, and HCD (b)

(a)

(b)
Table 2.3: Plasma characteristics of guinea pigs fed LCD or HCD for six weeks

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>LCD (n = 9)</th>
<th>HCD (n = 10)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>128 ± 24</td>
<td>73 ± 9</td>
<td>0.059</td>
</tr>
<tr>
<td>Free cholesterol (mg/dL)</td>
<td>43 ± 8.6</td>
<td>23 ± 3.1</td>
<td>0.051</td>
</tr>
<tr>
<td>*LDL-C (mg/dL)</td>
<td>84 ± 17</td>
<td>47 ± 6.6</td>
<td>0.072</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>30 ± 6</td>
<td>15 ± 2.8</td>
<td>0.044*</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>72 ± 9.5</td>
<td>57 ± 9.1</td>
<td>0.28</td>
</tr>
<tr>
<td>Non-esterified fatty acids (mEq/L)</td>
<td>0.5 ± .03</td>
<td>0.5 ± .04</td>
<td>0.97</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>162 ± 5.6</td>
<td>160 ± 9.1</td>
<td>0.85</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>180 ± 52.5</td>
<td>228 ± 68.8</td>
<td>0.59</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>424 ± 114.7</td>
<td>500 ± 203.4</td>
<td>0.75</td>
</tr>
<tr>
<td>Ketones (µmol/L)</td>
<td>186 ± 36</td>
<td>107 ± 12</td>
<td>0.07</td>
</tr>
</tbody>
</table>

All data presented as mean ± standard error of the mean (SEM)  
Abbreviations: LDL-C; low-density lipoprotein cholesterol, HDL-C; high-density lipoprotein cholesterol, ALT; alanine aminotransferase, AST; aspartate aminotransferase  
*LDL-C calculated according to Friedwald equation: LDL-C = TC − [HDL-C + (TG/5)]
Figure 2.6: Individual plasma TG (a), NEFA (b), glucose (c), and ketones (d)
Figure 2.7: Plasma activity level of liver enzymes in LCD and HCD guinea pigs
Figure 2.8: Liver glycogen content (a) and correlation with ALT in HCD (b)

a)

![Liver glycogen content bar graph](image)

Liver glycogen ($\mu$g/$\mu$L)

- L-CHOL
- LCD
- HCD

$p = 0.12$

b)

![Liver glycogen vs. ALT scatter plot](image)

- HCD
- ALT (U/L)
- Liver glycogen ($\mu$g/$\mu$L)

$r = .7$

$r^2 = .5$

$p = 0.04^*$
Figure 2.9: Liver weights of LCD and HCD guinea pigs

![Liver weight comparison between LCD and HCD guinea pigs.](chart1)

Figure 2.10: Correlation between liver weight and final body weight in LCD (a) and HCD (b)

![Correlation plots for LCD and HCD.](chart2)

- **a)** LCD correlation: \( r = 0.8 \), \( r^2 = 0.64 \), \( p = 0.001 \)
- **b)** HCD correlation: \( r = 0.71 \), \( r^2 = 0.51 \), \( p = 0.02 \)
Figure 2.11: Ratio of liver to final body weight in LCD and HCD
Table 2.4: Hepatic characteristics of guinea pigs fed LCD or HCD for six weeks

<table>
<thead>
<tr>
<th>Liver parameter</th>
<th>LCD (n = 9)</th>
<th>HCD (n = 10)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weight (g)</td>
<td>28.6 ± 3.3</td>
<td>25.2 ± 1.6</td>
<td>0.36</td>
</tr>
<tr>
<td>Liver/body weight (%)</td>
<td>3.9 ± .2</td>
<td>3.4 ± .1</td>
<td>0.055</td>
</tr>
<tr>
<td>Triglycerides (mg/g)</td>
<td>7.8 ± .6</td>
<td>5.9 ± .3</td>
<td>0.01*</td>
</tr>
<tr>
<td>Phospholipids (µg/g)</td>
<td>20487 ± 993</td>
<td>21114 ± 859</td>
<td>0.64</td>
</tr>
<tr>
<td>Total cholesterol (mg/g)</td>
<td>9.1 ± 1.2</td>
<td>6 ± .7</td>
<td>0.034*</td>
</tr>
<tr>
<td>Free cholesterol (mg/g)</td>
<td>5.9 ± .8</td>
<td>3.4 ± .6</td>
<td>0.02*</td>
</tr>
<tr>
<td>Cholesterol esters (mg/g)</td>
<td>3.2 ± .8</td>
<td>2.5 ± .3</td>
<td>0.45</td>
</tr>
<tr>
<td>Steatosis score</td>
<td>1.6 ± .4</td>
<td>.8 ± .2</td>
<td>0.13</td>
</tr>
<tr>
<td>Glycogen (µg/µL)</td>
<td>0.011 ± .003</td>
<td>0.022 ± .006</td>
<td>0.12</td>
</tr>
</tbody>
</table>

All data presented as mean ± standard error of the mean (SEM)
Figure 2.12: Hepatic triglyceride content of LCD and HCD guinea pigs
Figure 2.13: Hepatic TC (a,b), FC (c,d), and CE (e,f) in LCD and HCD

(a) Liver TC (mg/g)

(b) Liver TC (mg/g)

(c) Liver FC (mg/g)

(d) Liver FC (mg/g)

(e) Liver CE (mg/g)

(f) Liver CE (mg/g)

*p = 0.034

*p = 0.02

*p = 0.45
Figure 2.14: Livers (a) and steatosis scoring for LCD and HCD (b) and L-Chol, H-Chol, LCD, and HCD fed guinea pigs (c)
Figure 2.15: Example of inflammatory foci situated between normal hepatocytes (right) and steatotic hepatocytes with ballooning change (left)
**Figure 2.16:** Example of bridging fibrosis with mild steatosis (LCD) and biliary hyperplasia with minimal steatosis (HCD)

![Image of LCD and HCD examples](image)

**Figure 2.18:** Example of bridging fibrosis in HCD fed guinea pig (see following page)
<table>
<thead>
<tr>
<th>Parameter</th>
<th>H-Chol</th>
<th>LCD</th>
<th>HCD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>284 ± 36.6</td>
<td>128 ± 24</td>
<td>73 ± 9*</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>(mg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>99 ± 10</td>
<td>43 ± 8.6</td>
<td>23 ± 3.1*</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>(mg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>201 ± 29</td>
<td>84 ± 17</td>
<td>47 ± 6.6*</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>72 ± 11.6</td>
<td>30 ± 6</td>
<td>15 ± 2.8*</td>
<td>0.0002</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>61 ± 6</td>
<td>72 ± 9.5</td>
<td>57 ± 9.1</td>
<td>0.45</td>
</tr>
<tr>
<td>(mg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEFA (mEq/L)</td>
<td>0.5 ± .04</td>
<td>0.5 ± .03</td>
<td>0.5 ± .04</td>
<td>0.99</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>184 ± 9.4</td>
<td>162 ± 5.6</td>
<td>160 ± 9.1</td>
<td>0.11</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>196 ± 30.8</td>
<td>180 ± 52.5</td>
<td>228 ± 68.8</td>
<td>0.82</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>472 ± 72.6</td>
<td>424 ± 114.7</td>
<td>500 ± 203.4</td>
<td>0.66</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>21.9 ± 1.3</td>
<td>28.6 ± 3.3</td>
<td>25.2 ± 1.6</td>
<td>0.15</td>
</tr>
<tr>
<td>Liver/body weight</td>
<td>3.9 ± .2</td>
<td>3.9 ± .2</td>
<td>3.4 ± .1</td>
<td>0.08</td>
</tr>
<tr>
<td>(%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>11.6 ± 1.3</td>
<td>7.8 ± .6*</td>
<td>5.9 ± .3*</td>
<td>0.0005</td>
</tr>
<tr>
<td>(mg/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipids</td>
<td>27026 ± 1216</td>
<td>20487 ± 993*</td>
<td>21114 ± 859*</td>
<td>0.001</td>
</tr>
<tr>
<td>(ug/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>14 ± 1.3</td>
<td>9.1 ± 1.2*</td>
<td>6 ± .7*</td>
<td>0.0001</td>
</tr>
<tr>
<td>(mg/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steatosis score</td>
<td>1.8 ± .2</td>
<td>1.6 ± .4</td>
<td>.8 ± .2</td>
<td>0.054</td>
</tr>
</tbody>
</table>
STUDY 2: Discussion

Contrary to our initial hypothesis, the current study revealed that despite implementing two diets (LCD and HCD) with distinct macronutrient compositions after a dietary cholesterol challenge period, there was an overall worsening of liver pathology and injury. Because no clear cut differences in the pathological response could be observed between LCD and HCD, this likely indicates that the six week induction phase of NAFLD caused by a 0.25% high-cholesterol diet overrides any potential dietary benefit. This is the first study to our knowledge that implemented a LCD or HCD intervention following cholesterol-induced NAFLD, at least in phenotypically normal guinea pigs. Previous studies from our laboratory have demonstrated that a LCD with similar macronutrient profile as the current study results in improved atherosclerosis and decreased arterial inflammation (14,15). We have also observed that feeding a LCD in conjunction with a cholesterol-enriched diet lowers hepatic cholesterol and triglyceride, along with mRNA levels of hepatic LDLR and HMGCR when compared to guinea pigs fed a HCD/cholesterol-enriched diet (16). In the first study from our lab to document changes in liver histology, guinea pigs fed a LCD with 0.25% cholesterol had a similar extent of mixed macro- and microvesicular steatosis when compared to guinea pigs fed a HCD and 0.25% cholesterol (13). This study noted no differences in hepatic lipid concentrations of cholesterol species, free fatty acids, or triglycerides, although LCD livers were 40% larger overall. This finding was attributable to an increased expression of proliferating cell nuclear antigen (PCNA), a marker of hepatocyte proliferation; these results suggested that during a LCD, the excess cholesterol stimulated a proliferative
process to accommodate the incoming cholesterol. The significance of hepatic cholesterol in liver regeneration has also recently been observed in more in-depth experiments (17).

A key difference in the current study as compared to previous ones using guinea pigs and dietary intervention is that here the LCD and HCD were consumed in conjunction with a low level of dietary cholesterol after being crossed over from a purely cholesterol-enriched diet. This may provide some insight into the results observed here. There were no significant differences in plasma lipids, with the exception of HDL-C, which was increased in LCD. This is a well-known result of carbohydrate restriction in both humans and guinea pigs (3). Also both groups had similar biochemical liver injury evidenced by circulating ALT and AST- these values were also similar statistically to those obtained from a H-Chol group (table 2.5) indicating a continued degree of liver dysfunction. LCD guinea pig livers stored more cholesterol and triglyceride when compared to the HCD, however no significant differences were observed in liver size or its relation to overall body weight. Most intriguing was that the guinea pigs with the most severe pathological features such as bridging fibrosis and biliary hyperplasia had some of the lowest steatosis scores. It seems that an inverse relationship between the extent of pathological lesions and steatosis exists, at least in the current study, although it is well understood that hepatic lipid content tends to decrease with more advanced liver disease.

Despite some study limitations that include small sample sizes, the findings of the current study provide the first step in understanding the contribution of dietary
intervention to NAFLD in guinea pigs. It also underscores the role of individual responses to macronutrient manipulation as the LCD group was noted to have pronounced variability in several parameters (see results). In fact, guinea pigs that responded well to LCD intervention displayed all of the features typically associated with carbohydrate restriction including reduced plasma triglycerides, increased HDL-C, reduced weight gain and decreased daily food consumption. Therefore, guinea pigs, similar to humans, may respond differentially to carbohydrate restriction. The fact that responders and non-responders can occur in the same strain of guinea pigs indicates some underlying genetic predisposition that has yet to be defined.
References:


3. Volek JS and Feinman RD. **Carbohydrate restriction improves the features of metabolic syndrome.** Metabolic syndrome may be defined by the response to carbohydrate restriction. *Nutr Metab (Lond)* 2005; 2:31.


STUDY 3: Assessment of hepatic phospholipid and triglyceride fatty acid composition in response to dietary manipulation

Abstract:

Livers from guinea pigs fed either low (L-Chol, 0.04%) or high dietary cholesterol (H-Chol, 0.25%) \((n = 5-6 \text{ per group})\) were quantified for fatty acids (FA) in liver triglycerides (TG) and phospholipids (PL). H-Chol decreased the percentage of saturated fatty acid (SFA) \((p = 0.005)\), particularly stearate \((p = 0.003)\) and increased monounsaturated fatty acid (MUFA) \((p = 0.012)\), particularly oleate \((p = 0.014)\) in PL fractions. Similar changes were observed in the TG fraction. Despite no differences in the PL content of linoleic acid \((p = 0.6)\), H-Chol PLs were significantly depleted of arachidonic acid both in absolute \((p = 0.001)\) and relative terms \((p = 0.004)\). A second aim was to investigate these lipid fractions in animals randomized to diets either low (LCD) \((n = 9)\) or high in carbohydrate (HCD) \((n = 10)\) following the H-Chol phase. There were no differences in the fatty composition of TG or PL with the exception of palmitate, which was increased in HCD PL fractions \((p = 0.005)\). When compared to the H-Chol group, both LCD and HCD groups had a pronounced depletion of total phospholipid content \((p = 0.001)\) and this was shown to be negatively correlated with plasma ALT \((r = -0.7, p = 0.0004)\) and AST \((r = -0.8, p = 0.0002)\). Compared to the H-Chol, both LCD and HCD also demonstrated increases in the percent of MUFA and decreases in PUFA in both PLs and TGs. The percent composition of omega 6 was also reduced while omega 9 was increased in both lipid fractions compared to H-Chol. The differences in omega 6 can be explained by a decrease in PL and TG content of linoleic acid while the increase
in omega 9 can be explained by increased oleate content of PL and TG. These data demonstrate that high dietary cholesterol increases the incorporation of unsaturated fatty acids in liver PL and TG, while also decreasing PL arachidonic acid. They also show that manipulating macronutrients has no impact on liver fatty acid composition and this is likely due to the lingering effects of the dietary cholesterol overload in these guinea pigs.
Phospholipids represent a specialized class of amphiphilic lipids that are crucial to an array of cellular functions; they provide structure and order to lipid bilayers of organelle membranes, serve as components of lipid signaling cascades, shelter hydrophobic lipids in lipoproteins and lipid droplets (LD) from the aqueous environment, and more recently have been shown to lend themselves towards the formation of triglycerides (1).

The landscape of cellular membranes can be envisioned as a patchwork-like quilt of protein and lipid molecules with the latter comprising approximately 50% of total membrane mass. Phospholipids, cholesterol, and fatty acids dot lipid bilayers in a bewildering array of combinations that reflect variations in polar head groups, hydrocarbon chain lengths and degrees of saturation; it has been estimated that membranes contain more than 1,000 different phospholipid species alone (2). Phosphatidylcholine (PC) represents the bulk of membrane phospholipids totaling 40-50% followed by phosphatidylethanolamine (PE) at 20-50% and phosphatidylserine (PS) at 10-20% (2). A responsible discussion detailing the functions and interactions of all membrane phospholipids is daunting, though fortunately several insightful commentaries exist, many of which are the prodigious work of Jean and Dennis Vance (3-5).

PC commands special attention amongst other phospholipids, not least because it prevails as the major membrane phospholipid (40-50% of total phospholipids), especially in both mitochondrial (44%), and endoplasmic reticulum (40%) membranes,
and predominates in plasma (24% total lipid by weight). The monolayer of LDs is also largely composed of PC, which provides key stabilizing properties that other phospholipid species lack (6,7). Not surprisingly, PC is known to influence several aspects of hepatic lipid metabolism and is crucial for normal liver functioning. This is reflected in the biosynthesis of PC, which principally occurs through the Kennedy (CDP-choline) pathway via the rate limiting enzyme, CTP:phosphocholine cytidylyltransferase (CT) (8). Alternatively, another pathway for PC synthesis involves the sequential methylation of PE via PE \( N \)-methyltransferase (PEMT). This secondary pathway is only utilized by hepatocytes, providing 30% of hepatic PC whereas the CT pathway generates the remaining 70% (1). The existence of both pathways likely ensures a consistent availability of PC for incorporation into membrane lipid bilayers as well as budding very-low density lipoproteins (VLDL) and lipid droplets, where it serves as a component of the phospholipid monolayer.

As phospholipids are essential components of the milieu of cellular membranes, conditions in which their availability is limited may trigger cell or organ dysfunction. For instance, in non-alcoholic fatty liver disease (NAFLD), the liver experiences a spectrum of histological changes that begins with increased triglyceride accumulation (hepatic steatosis) and can transition to a more inflamed state known as non-alcoholic steatohepatitis (NASH) (9,10). One manner in which steatosis can arise is from an insufficiency of hepatic phospholipid availability which impairs the lipidation of apolipoprotein B-100 (ApoB), the major apoprotein of VLDL particles. Lipidation of apoB with phospholipids is a critical step for the assembly and eventual egress of VLDL.
through hepatic sinusoids and out of the liver (11), thus under conditions of limited availability, improperly formed VLDL particles accumulate within the secretory pathway and the contained lipid accrues in the cytoplasm, triggering steatosis. In fact, one of the most commonly employed experimental techniques for inducing steatosis in animals is through the administration of a methionine and choline deficient diet (MCD), which limits the dietary availability of choline, the precursor to PC, thereby reducing hepatic VLDL secretion and causing increased hepatic triglyceride content (12). Though the MCD diet provides useful information with regard to intrahepatic trafficking of VLDL and produces very consistently pathological features of NASH, it represents a physiologically irrelevant scenario as limited dietary choline is a very rare occurrence to confront humans. Moreover, the metabolic context in which the MCD diet takes place is also very different from humans with NAFLD (13), as mice fed MCD do not exhibit any features of insulin resistance or impaired glucose metabolism (14).

Insights into the diverse roles phospholipid metabolism plays in liver function have been gained through studies using genetically-targeted mouse models (15). Collectively the studies to date have shown fairly convincingly that PC and PE are involved in both steatosis as well as the transition to NASH. For example mice with a liver specific deletion of CT, the rate limiting enzyme of PC synthesis, were shown to develop steatosis on a chow-diet and when fed a high-fat diet, rapidly developed NASH within one week (16). Furthermore, mice genetically manipulated to lack PEMT develop liver failure when fed a choline-deficient diet and this is attributable to a greater than 50% reduction in the hepatic ratio of PC to PE (17). Alterations in this ratio caused loss
of membrane integrity and the formation of ballooned hepatocytes, which is a seminal feature of NASH, demonstrating that maintenance of the PC to PE ratio may be involved in dictating the transition of steatosis to NASH. The authors also observed a similar occurrence in humans with NASH, adding to the growing list of studies that implicate phospholipid composition as another determinant of NAFLD severity (18). A well-conducted lipidomics study of human livers found a stepwise reduction in hepatic PC content as NAFLD severity increased. There was also a concomitant increase in the free cholesterol to phospholipid ratio which is significant given that in membranes the free cholesterol molecule situates itself between neighboring phospholipids, forming so-called condensed complexes (19).

The growing awareness that NAFLD occurs in the midst of numerous alterations to normal lipid metabolism prompted us to investigate some of the finer changes that might occur in specific lipid species when liver injury is present. To do this, we sought to identify changes in the fatty acid composition of both phospholipids as well as triglycerides from livers of guinea pigs with varying degrees of NAFLD. We used four groups of animals based on diet; a low-cholesterol (L-Chol) control group, a high-cholesterol group (H-Chol), a carbohydrate restricted (LCD) group, and a carbohydrate-enriched (HCD) group. Because we observed that H-Chol feeding promotes steatosis as well as inflammation and evidence of hepatic injury, we wanted to determine how changes in these two lipid fractions might help explain the degree of liver damage. We also observed that when guinea pigs were fed the H-Chol and then assigned to either a LCD or HCD, both groups had more advanced hepatic dysfunction than that observed
for the H-Chol. As no obvious differences were observed between the LCD and HCD in terms of liver injury, we focused on changes that occurred in both lipid fractions as potential explanations for these pathological changes. In short, we find that H-Chol feeding causes liver PL and triglycerides to become more unsaturated in terms of fatty acid composition, and also triggers a reduction in PL arachidonic acid content despite having similar linoleic acid content as L-Chol controls. There were no major differences between LCD and HCD, however both experienced a pronounced depletion of total PL content following the H-Chol challenge, which may help to explain the worsened liver pathology of these two dietary groups.

STUDY 3: Methods and experimental methodology

Fatty acid profile of liver PL and TG:

How certain dietary manipulations impact liver fatty acid metabolism in guinea pigs has never been sufficiently investigated. To that end we sought to analyze the composition of fatty acids present in liver triglycerides (TG), a predominant storage lipid,
and phospholipids (PL), the major lipid constituent of membrane lipid bilayers, in guinea pigs fed either L-Chol \((n = 6)\), H-Chol \((n = 5)\), LCD \((n = 9)\), and HCD \((n = 10)\) for six weeks. For analysis approximately 1 g of tissue from the left lobe or medial sublobe of the right lobe was sliced with a razor, placed in a 1.5 mL snap tube, immediately submerged in liquid nitrogen, and stored at -80°C until samples were ready for analysis.

Samples were tightly wrapped and sealed to limit exposure to light and packaged in dry ice on the day of shipment. All samples were analyzed using capillary gas chromatography at Lipid Technologies LLC (Austin, MN).

**Capillary gas chromatography**

Lipids were extracted from livers based on the methodology of Bligh and Dyer (20). Livers were homogenized and mixed with methanol, chloroform, and water and lipids were extracted from the chloroform layer. The extracts were maintained under an atmosphere of nitrogen and kept frozen until additional processing took place. Prior to the separation of lipid classes samples were dried under a steady stream of nitrogen and rediluted in 50 µL of chloroform. Next, lipid classes including total TG and PL were separated on commercial silica gel G plates (AnalTech, Newark, DE) with development of the plates occurring in a solvent system consisting of distilled petroleum ether: diethyl ether: acetic acid (80:20:1 by volume). Silica gel plates were then sprayed with a methanolic solution (0.5% 2,7-dichlorofluorescein) in order to visualize the lipid bands under ultraviolet light exposure. Desired bands were scraped into Teflon-lined screw cap tubes and samples were then transesterified with 10% boron trifluoride in the presence of excess methanol in a water bath (80°C) for approximately 90 minutes. The
resultant fatty acid methyl esters were then extracted with water and petroleum ether and stored frozen prior to gas chromatographic analysis.

Fatty acid methyl ester composition was subsequently determined by capillary gas chromatography. First, methyl esters were blown to dryness under nitrogen and resuspended in hexane. Next, fatty acid methyl esters were separated and quantified using a Shimadzu capillary gas chromatograph with a programmed temperature from 190° to 240°C at 7°C/ min with a final hold of 10 min, thereby separating and measuring fatty acid methyl esters ranging from 12:0 to 24:1 at a detector temperature of 250°C. All chromatographic data was collected and processed using EZChrom software (Scientific Products, CA) and fatty acids were identified by comparison to authentic fatty acid standards and quantitated with peak area and internal standard. Individual peaks that represented as little as 0.05% of fatty acid methyl esters were distinguished. Data is expressed as both percent composition and absolute composition (µg/g liver).

Statistical analysis:

All data were analyzed using the Prism statistical program from GraphPad 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 and between LCD and HCD groups. An unpaired t-test with Welch’s correction was used when unequal variances were detected. To compare differences between H-Chol, LCD, and HCD, a one-way analysis of variance (1-way ANOVA) was used followed by Tukey’s post-hoc multiple comparisons test. In the case of unequal variances as determined by Bartlett’s test, data were analyzed with Kruskal-Wallis test followed by Dunn’s post-hoc
multiple comparisons test. All data are expressed as the mean ± standard error of the mean (SEM). An α-level of $p < 0.05$ was chosen to denote statistical significance. Graphical data represents the % fatty acid composition of either phospholipids or triglycerides. The $p$ value represents the ANOVA results comparing H-Chol, LCD, and HCD.

**STUDY 3: Results**

*H-Chol alters fatty acid profile of liver phospholipids and triglycerides by decreasing SFA and PUFA while increasing MUFA*
In this study we used capillary gas chromatography to analyze the fatty acid composition of liver phospholipids and triglycerides in guinea pigs fed four distinct diets; L-Chol, H-Chol, and LCD and HCD following H-Chol.

Looking first at hepatic phospholipids, it was evident that feeding H-Chol for six weeks dramatically alters the fatty acid profile from that observed in the L-Chol group. Table 3.1 contains the results for L-Chol and H-Chol in the phospholipid fraction and data is expressed as both percent composition and absolute concentration (µg/g liver). H-Chol feeding significantly reduced the percentage of SFA (p = 0.005) while at the same time increasing the percentage of MUFA (p = 0.012). There was no difference in the percent PUFA content (p = 0.2), however there was a significant reduction in the absolute amount (p = 0.03) in H-Chol livers. Intriguingly, there was also a marked reduction in highly unsaturated fatty acids (HUFA) in H-Chol samples, both in terms of percent (p = 0.004) and absolute levels (p = 0.001). Overall, phospholipids in both L-Chol and H-Chol appear to be composed primarily of SFA and PUFA as both species each represented around 40% of the total, with MUFA comprising between 14-17%. The fact that MUFA content increased with H-Chol feeding likely indicates an increase in hepatic SCD-1 activity to provide increased substrate for formation of certain phospholipid molecules. Further substantiating the likelihood of increased SCD-1 action is the reduced PUFA concentration, as PUFA, specifically n3 and n6 species (see below) are potent inhibitors of SCD-1 expression (21).

H-Chol phospholipids and triglycerides show decreased stearate but increased oleate compared to L-Chol
Between L-Chol and H-Chol there were no significant differences in the composition of shorter chain SFA such as myristic acid. Both were equally composed of palmitate and palmitoleate, two key membrane fatty acids; there was actually very little palmitoleate present in both groups, making up only around .5%, which indicates that liver phospholipids of guinea pigs do not contain notable amounts of this fatty acid. There was considerable accumulation of stearate within this lipid fraction and interestingly, this accumulation was greatest in L-Chol as feeding H-Chol resulted in less stearate, both as percent composition \((p = 0.003)\) and absolute amount \((p = 0.024)\). The decreased stearate in H-Chol is likely a consequence of increased hepatic SCD-1 activity as there was evidence of increased oleate concentration in H-Chol phospholipids, constituting 15% compared to 13% in L-Chol \((p = 0.014)\).

Without directly measuring the enzymatic activity of SCD-1 in liver microsomes, many of these observations are speculative in nature. However, given the preponderance of evidence detailing cholesterol's regulation of this lipogenic enzyme \((21,22)\) it is plausible, indeed, exceedingly likely that H-Chol increased the endogenous production of MUFAs, in particular oleate, in liver phospholipids. Reflecting this possibility is the observed reduction of the stearate to oleate ratio \((18:0/18:1)\) in H-Chol livers compared to L-Chol \((p = 0.005)\). Alterations in this ratio are known to have profound effects on membrane integrity and fluidity and a reduced ratio in H-Chol may help to, in part, explain some of the changes to liver microanatomy observed in study 1.

**H-Chol reduces phospholipid arachidonic acid content and causes a reduction in overall hepatic omega 6 concentration**
PUFAs consist of primarily omega 3 and omega 6 species and in liver tissue, three fatty acids predominate: docosahexaenoic acid (DHA, 22:6n3), linoleic (18:2n6), and arachidonic (20:4n6). This was true in the current study with linoleic acid being the major PUFA in both L-Chol and H-Chol, followed by much lower amounts of arachidonic, and DHA (Table 3.1). Overall, the omega 6 content represented between 37-39% of total phospholipid PUFA content with H-Chol displaying significantly lower absolute concentration compared to the L-Chol ($p = 0.025$). Closer inspection revealed that this difference was due to lower hepatic levels of arachidonic ($p = 0.001$) and its immediate precursor, dihomo-γ-linolenic acid (DGLA, 20:3n6) ($p = 0.001$). Similar statistical differences were also evident when the data were expressed as percent composition (Table 3.1). Moreover, we noted a significant reduction in the arachidonic to linoleic ratio (20:4n6/18:2n6) ($p = 0.01$) in H-Chol compared to L-Chol. The overall omega 3 content was similar between both groups, however we did note an increase in DHA within phospholipids in H-Chol ($p = 0.01$), the relevance of which is not currently clear. There was a similar ratio of omega 6 to omega 3 between groups ($p = 0.14$), although it should be noted that it was reduced in the H-Chol group. Thus, it appears that H-Chol feeding somehow depletes hepatic phospholipids of arachidonic acid and thereby reduces the omega 6 PUFA content; whether this depletion of arachidonic acid is due to increased phospholipase A2-mediated release from membranes or enhanced conversion to bioactive eicosanoids is currently unknown and requires further investigation.
**LCD and HCD guinea pigs display identical changes in hepatic phospholipid and triglyceride fatty acid composition**

We had anticipated that feeding a LCD or HCD following a H-Chol challenge would differentially impact the fatty acid composition of various lipid fractions based on the specific metabolic effects elicited by these diets. Remarkably, the fatty acid makeup of phospholipids were almost identical in both groups, with one notable exception being palmitate (table 3.2). In the HCD group, palmitate was significantly increased compared to the LCD group, both when expressed as percent ($p = 0.008$) and absolute amount ($p = 0.005$). This may reflect differences in the DNL pathway between LCD and HCD as palmitate is a major end product of endogenous fatty acid synthesis. Why this difference is observed in the phospholipid fraction and why it occurs with palmitate and not stearate, another end product of DNL, is not presently understood.

**Phospholipid content is markedly depleted in LCD and HCD livers following a H-Chol challenge**

One of the most intriguing findings from this current study was that when compared to the H-Chol group, livers from guinea pigs in either the LCD or HCD had a pronounced reduction in total phospholipid content ($p = 0.001$; see table 2.5 in study 2). Although the relevance of this finding was not further investigated, we speculate that this may be due to an increased membrane cholesterol content, which might disrupt membrane bilayers, resulting in the removal of the phospholipids themselves. Future studies using this same study design should address whether this is the case or if in fact other mechanisms are at play.
Table 3.1: Fatty acid composition of hepatic phospholipids in L-Chol and H-Chol fed guinea pigs
Data are represented as mean ± SEM of \( n = 6 \) (L-Chol) and \( n = 5 \) (H-Chol) animals. \( p \)-value was calculated using an unpaired, two-tailed student’s \( t \)-test with an \( \alpha \)-level of 0.05 considered statistically significant. ND; not detected.

**Table 3.2: Fatty acid composition of hepatic triglycerides in L-Chol and H-Chol fed guinea pigs**
Data are represented as mean ± SEM of \( n = 6 \) (L-Chol) and \( n = 5 \) (H-Chol) animals. \( p \)-value was calculated using an unpaired, two-tailed student’s \( t \)-test with an \( \alpha \)-level of 0.05 considered statistically significant. ND; not detected.

**Table 3.3: Fatty acid composition of hepatic phospholipids in LCD and HCD fed guinea pigs**
Data are represented as mean ± SEM of $n = 9$ (LCD) and $n = 10$ (HCD) animals. $p$-value was calculated using an unpaired, two-tailed student’s $t$-test with an $\alpha$-level of 0.05 considered statistically significant. ND; not detected.

**Table 3.4**: Fatty acid composition of hepatic triglycerides in LCD and HCD fed guinea pigs

<table>
<thead>
<tr>
<th>Fatty acid species</th>
<th>LCD</th>
<th>HCD</th>
<th>p-value</th>
<th>LCD</th>
<th>HCD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:0</td>
<td>ND</td>
<td>ND</td>
<td>--</td>
<td>ND</td>
<td>ND</td>
<td>--</td>
</tr>
<tr>
<td>14:0</td>
<td>.62 ± .04</td>
<td>.59 ± .03</td>
<td>0.61</td>
<td>90 ± 5.5</td>
<td>91 ± 7.1</td>
<td>0.93</td>
</tr>
<tr>
<td>16:0</td>
<td>12 ± .3</td>
<td>14 ± .68</td>
<td>0.008*</td>
<td>1729 ± 55</td>
<td>2137 ± 110</td>
<td>0.005*</td>
</tr>
<tr>
<td>15:1 n-7</td>
<td>.35 ± .01</td>
<td>.43 ± .04</td>
<td>0.073</td>
<td>52 ± 3.4</td>
<td>67 ± 7.3</td>
<td>0.09</td>
</tr>
<tr>
<td>18:0</td>
<td>29 ± 1</td>
<td>28 ± .83</td>
<td>0.3</td>
<td>4252 ± 81</td>
<td>4199 ± 146</td>
<td>0.8</td>
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<tr>
<td>18:1 n-9</td>
<td>20 ± .46</td>
<td>20 ± .52</td>
<td>0.8</td>
<td>2925 ± 168</td>
<td>2994 ± 180</td>
<td>0.8</td>
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<tr>
<td>18:2 n-6</td>
<td>27 ± 1.4</td>
<td>27 ± .82</td>
<td>0.65</td>
<td>4119 ± 395</td>
<td>4082 ± 238</td>
<td>0.93</td>
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<tr>
<td>18:3 n-3</td>
<td>.49 ± .09</td>
<td>.52 ± .06</td>
<td>0.81</td>
<td>76 ± 17</td>
<td>82 ± 16</td>
<td>0.81</td>
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<tr>
<td>20:3 n-6</td>
<td>.11 ± .01</td>
<td>.12 ± .01</td>
<td>0.24</td>
<td>16 ± 1.3</td>
<td>18 ± 1.6</td>
<td>0.3</td>
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<td>20:4 n-6</td>
<td>2.4 ± .3</td>
<td>2.7 ± .28</td>
<td>0.43</td>
<td>361 ± 64</td>
<td>417 ± 53</td>
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<tr>
<td>20:5 n-3</td>
<td>.06 ± .01</td>
<td>.05 ± .01</td>
<td>0.74</td>
<td>8.3 ± 1.7</td>
<td>8.3 ± 1.7</td>
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</tr>
<tr>
<td>22:6 n-3</td>
<td>.65 ± .04</td>
<td>.6 ± .03</td>
<td>0.2</td>
<td>95 ± 3.8</td>
<td>89 ± 5.1</td>
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<td>SFA</td>
<td>43 ± 1.3</td>
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<td>6293 ± 142</td>
<td>6639 ± 206</td>
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<td>MUFA</td>
<td>21 ± .5</td>
<td>21 ± .5</td>
<td>0.8</td>
<td>3125 ± 176</td>
<td>3207 ± 192</td>
<td>0.75</td>
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<tr>
<td>PUFA</td>
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<td>32 ± 1.2</td>
<td>0.87</td>
<td>4875 ± 488</td>
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<td>0.95</td>
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<tr>
<td>HUFA</td>
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<td>5.1 ± .4</td>
<td>0.52</td>
<td>717 ± 91</td>
<td>785 ± 78</td>
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<tr>
<td>n-3</td>
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<td>233 ± 17</td>
<td>221 ± 19</td>
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<td>n-6</td>
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<td>31 ± 1.2</td>
<td>0.91</td>
<td>4635 ± 476</td>
<td>4675 ± 303</td>
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<td>n-9</td>
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<td>20 ± .5</td>
<td>0.82</td>
<td>3028 ± 175</td>
<td>3107 ± 187</td>
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<td>n-6/n-3</td>
<td>20 ± 1.5</td>
<td>22 ± 1.3</td>
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<td>--</td>
<td>--</td>
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<td>16:0/16:1 ratio</td>
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<td>35 ± 3.7</td>
<td>0.71</td>
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<tr>
<td>18:0/18:1 ratio</td>
<td>1.5 ± .07</td>
<td>1.4 ± .07</td>
<td>0.62</td>
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<td>--</td>
<td>--</td>
</tr>
<tr>
<td>20:5/18:3 ratio</td>
<td>.17 ± .05</td>
<td>.11 ± .02</td>
<td>0.33</td>
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<tr>
<td>20:4/18:2 ratio</td>
<td>.08 ± .01</td>
<td>.1 ± .007</td>
<td>0.1</td>
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</table>
Data are represented as mean ± SEM of *n* = 9 (LCD) and *n* = 10 (HCD) animals. *p*-value was calculated using an unpaired, two-tailed student’s *t*-test with an *α*-level of 0.05 considered statistically significant. ND; not detected.

**Figure 3.1:** One-way ANOVA of liver fatty acid classes between H-Chol, LCD, and HCD groups

<table>
<thead>
<tr>
<th>Fatty acid species</th>
<th>LCD</th>
<th>HCD</th>
<th><em>p</em>-value</th>
<th>LCD</th>
<th>HCD</th>
<th><em>p</em>-value</th>
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<tr>
<td>Triglycerides (%)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>12:0</td>
<td>.66 ± .15</td>
<td>.4 ± .05</td>
<td>0.13</td>
<td>307 ± 106</td>
<td>155 ± 34</td>
<td>0.2</td>
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<tr>
<td>14:0</td>
<td>2.7 ± .46</td>
<td>2.3 ± .24</td>
<td>0.42</td>
<td>1067 ± 314</td>
<td>765 ± 136</td>
<td>0.4</td>
</tr>
<tr>
<td>16:0</td>
<td>14 ± 1.4</td>
<td>15 ± 1.1</td>
<td>0.43</td>
<td>5237 ± 1388</td>
<td>4993 ± 732</td>
<td>0.9</td>
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<td>16:1 n-7</td>
<td>0.85 ± .05</td>
<td>0.89 ± .07</td>
<td>0.7</td>
<td>401 ± 121</td>
<td>294 ± 52</td>
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<tr>
<td>18:0</td>
<td>4.9 ± .5</td>
<td>4.2 ± .26</td>
<td>0.24</td>
<td>1951 ± 522</td>
<td>1504 ± 304</td>
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<tr>
<td>18:1 n-9</td>
<td>49 ± 1.4</td>
<td>48 ± 2.2</td>
<td>0.8</td>
<td>25743 ± 7618</td>
<td>18251 ± 3839</td>
<td>0.4</td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>24 ± 1.1</td>
<td>24 ± 1.5</td>
<td>0.91</td>
<td>13239 ± 3933</td>
<td>8645 ± 1645</td>
<td>0.31</td>
</tr>
<tr>
<td>18:3 n-3</td>
<td>1.3 ± .053</td>
<td>1.4 ± .08</td>
<td>0.77</td>
<td>632 ± 181</td>
<td>482 ± 98</td>
<td>0.5</td>
</tr>
<tr>
<td>20:3 n-6</td>
<td>0.72 ± .03</td>
<td>0.63 ± .006</td>
<td>0.8</td>
<td>21 ± 6.2</td>
<td>22 ± 4.1</td>
<td>0.9</td>
</tr>
<tr>
<td>20:4 n-6</td>
<td>0.29 ± .1</td>
<td>0.21 ± .022</td>
<td>0.43</td>
<td>67 ± 14</td>
<td>72 ± 15</td>
<td>0.84</td>
</tr>
<tr>
<td>20:5 n-3</td>
<td>ND</td>
<td>ND</td>
<td>--</td>
<td>ND</td>
<td>ND</td>
<td>--</td>
</tr>
<tr>
<td>22:6 n-3</td>
<td>ND</td>
<td>ND</td>
<td>--</td>
<td>ND</td>
<td>ND</td>
<td>--</td>
</tr>
<tr>
<td>SFA</td>
<td>22 ± 2.2</td>
<td>22 ± 1.4</td>
<td>0.98</td>
<td>8739 ± 2339</td>
<td>7556 ± 1183</td>
<td>0.65</td>
</tr>
<tr>
<td>MUFA</td>
<td>50 ± 1.3</td>
<td>50 ± 2.1</td>
<td>0.78</td>
<td>26432 ± 7815</td>
<td>18782 ± 3935</td>
<td>0.38</td>
</tr>
<tr>
<td>PUFA</td>
<td>26 ± 1</td>
<td>27 ± 1.6</td>
<td>0.92</td>
<td>14211 ± 4198</td>
<td>9450 ± 1795</td>
<td>0.32</td>
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<tr>
<td>HUFA</td>
<td>2.1 ± .2</td>
<td>2.1 ± .12</td>
<td>0.94</td>
<td>867 ± 237</td>
<td>725 ± 136</td>
<td>0.6</td>
</tr>
<tr>
<td>n-3</td>
<td>1.5 ± .07</td>
<td>1.5 ± .08</td>
<td>0.96</td>
<td>672 ± 191</td>
<td>521 ± 105</td>
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</tr>
<tr>
<td>n-6</td>
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<td>25 ± 1.6</td>
<td>0.93</td>
<td>13539 ± 4010</td>
<td>8917 ± 1693</td>
<td>0.31</td>
</tr>
<tr>
<td>n-9</td>
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<td>49 ± 2.2</td>
<td>0.77</td>
<td>26014 ± 7695</td>
<td>18450 ± 3881</td>
<td>0.38</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>18 ± 1.4</td>
<td>17 ± .88</td>
<td>0.86</td>
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<td>--</td>
<td>--</td>
</tr>
<tr>
<td>16:0/16:1 ratio</td>
<td>16 ± 1.5</td>
<td>17 ± .9</td>
<td>0.5</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>18:0/18:1 ratio</td>
<td>.1 ± .01</td>
<td>.09 ± .01</td>
<td>0.4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>20:5/18:3 ratio</td>
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<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>20:4/18:2 ratio</td>
<td>.01 ± .004</td>
<td>.009 ± .001</td>
<td>0.41</td>
<td>--</td>
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Figure 3.2: One-way ANOVA of major SFA and MUFA species between H-Chol, LCD, and HCD groups
Figure 3.3: One-way ANOVA of liver n3, n6, and n9 classes between H-Chol, LCD, and HCD groups
Figure 3.4: One-way ANOVA of major n3 and n6 PUFA species between H-Chol, LCD, and HCD
Figure 3.5: One-way ANOVA of n6 to n3 ratio in liver phospholipids and triglycerides between H-Chol, LCD, and HCD groups
STUDY 3: Discussion
This is the first study to our knowledge that has evaluated the fatty acid composition of liver PL and triglyceride in guinea pigs under different dietary schemes. The study, in conjunction with the pathological evidence obtained from previous work (see study 1 and 2) might help in our understanding of NAFLD pathogenesis and how it occurs in guinea pigs, an animal which has yet to gain appreciable interest as a model for NAFLD.

Many aspects of hepatic lipid metabolism are affected by cholesterol status. For instance, increased dietary cholesterol is known to exert tremendous influence on stearoyl-CoA desaturase 1 (SCD-1), the enzyme responsible for the conversion of saturated fatty acids (SFA) to monounsaturated fatty acids (MUFA) (21). MUFA, primarily palmitoleate (16:1) or oleate (18:1), are the preferred fatty acid substrates for the synthesis of triglycerides, PL, as well as cholesterol esters, and a disruption of SCD-1 activity, such as that which occurs in the SCD-1 null mouse (22) results in decreased synthesis of these molecules. Here we observed that guinea pigs fed the H-Chol had a shift in the ratio of SFA and MUFA in both PL and triglyceride fractions. Specifically, both fractions were shown to contain a greater percentage of MUFA and this occurred at the expense of SFA. Oleate was shown to be responsible for the increased MUFA of both fractions, thus implicating SCD-1 as a likely mediator. The reason for enhanced SCD-1 activity under H-Chol presumably stems from an increased cellular need to buffer incoming free cholesterol through esterification, a process mediated by acyl-CoA cholesterol acyltransferase (ACAT) (23). By forming the preferred palmitoleate or oleate, cells then use ACAT to attach these fatty acids to the cholesterol molecule,
rendering it much more hydrophobic and at the same time eliminating any potential cytotoxic or membrane effects (24). Free cholesterol resides in close proximity to PLs within membranes. It is important not to underestimate cholesterol's ability to influence membrane function, as has recently been shown by Andrew Brown’s group (25). In this study an enantiomer of cholesterol was utilized in several cell experiments and shown to be as effective as natural cholesterol in eliciting homeostatic responses. This was attributed to the specific membrane effects that cholesterol is responsible for and argues that cholesterol might play a much more profound role in modulating membranes than previously thought.

One of the most interesting findings was that H-Chol triggered a significant depletion of PL arachidonic acid. Because there was no difference between H-Chol and L-Chol in terms of PL linoleic acid levels (the precursor to arachidonic acid), the reduction cannot be attributed to a dietary deficiency of linoleate. To us this indicates that the arachidonic acid contained in membrane PL of H-Chol livers underwent phospholipase-mediated release and was able to engage in the production of proinflammatory prostaglandins, thromboxanes, or leukotrienes, though we do not have evidence to directly support these claims at this time. It may also be the case that the decrease of PL arachidonic acid reflects an increased utilization of this lipid in H-Chol livers. Whatever the underlying mechanism(s), it was significant that this alteration has also been observed in humans with varying stages of NAFLD (18). Specifically, Puri et al (18) found that in humans with NASH, there was a significant reduction in arachidonic
acid content of free fatty acids, triglycerides, and importantly in PC. This again occurred despite no differences in linoleic acid levels between control and NASH patients.

Guinea pigs fed the LCD or HCD had almost identical changes in all parameters measured in this study, which was unexpected given the different metabolic changes each diet elicits. The only difference that reached statistical significance was an increase in the amount of palmitate within PLs of guinea pigs fed the HCD. The mechanistic importance of this observation is unclear. Because these animals were coming off of a six week dietary cholesterol challenge prior to these diets, we compared the responses of LCD and HCD to the H-Chol group. The results indicate that in both PL and triglycerides there were statistically significant increases in MUFA along with concomitant decreases in PUFA. An increase in oleate in both fractions was shown to be responsible for the elevated MUFA content of LCD and HCD guinea pigs. We should point out that triglycerides contained MUFA as the most predominant fatty acid specie, while PL was primarily composed of SFA and PUFA. When we evaluated the percent composition of total omega 6, the LCD and HCD groups had significantly less omega 6 in both PL and triglyceride fractions as compared to H-Chol. This reduction in omega 6 proved to be due to a significant reduction in PL and triglyceride content of linoleic acid (18:2n6) (figure 3.4 a,b).

As mentioned previously, cholesterol is found situated between PLs in membranes with its free, polar hydroxyl group on the 3rd carbon facing the aqueous exterior and its fused steroid nucleus and methylated side chain buried deep within the lipid bilayer. In mammalian cells the plasma membrane contains the highest
concentration of cholesterol (about 17% total lipid) whereas organelles such as the mitochondria tend to have much lower levels (3%). The ER is home to all of the pivotal transmembrane proteins that are responsible for governing cholesterol homeostasis, yet maintains a very tight level of membrane cholesterol at a low range (3-5%). Brown and Goldstein recently demonstrated that the ER is remarkably sensitive to changes in membrane cholesterol concentration such that when the ER content exceeds 5% cholesterol (on a molar basis), homeostatic responses are initiated which effectively shut down pathways of cholesterol biosynthesis (26). So, in situations where cholesterol is relatively abundant such as during a dietary cholesterol challenge, it is reasonable to consider the potential for increased membrane accumulation, which may impact membrane lipid organization. We believe this occurs in the guinea pigs fed LCD or HCD as they were shown to be markedly depleted in total phospholipid content as compared to H-Chol ($p = 0.001$). Furthermore this reduction in PL content likely contributes to the advanced pathological changes in these groups as plasma ALT and AST were shown to be negatively correlated with total hepatic PL content ($r = -.7$, $p = 0.0004$ and $r = -.8$, $p = 0.0002$ for ALT and AST respectively). Though we did not identify the specific PL species in this analysis, it seems logical that this reduction of PL might be PC, as it is the most abundant PL in liver cells. The fact that PC has also been seen to be reduced as humans transition from steatosis to NASH (18) lends credence to our findings. It may also be that PE is also reduced, thereby altering the PC to PE membrane ratio, which has been shown to be reduced in mice and humans with NAFLD (17). Maintenance of a proper PC to PE ratio is also critical for recovery from surgically-induced liver loss, such
as partial hepatectomy (PHx) (27). We should point out that in those guinea pigs with more advanced injury, PL content was not always reduced, indicating the involvement of other as of yet unknown factors. However the most severe pathological injury (biliary hyperplasia) occurred in the HCD guinea pig which did in fact display the lowest level of hepatic PL content.

There are several outstanding questions that emerge from this study. Where exactly in the liver do these changes to fatty acid composition occur? Triglycerides are stored in the hydrophobic cores of lipid droplets within the cytoplasm, however PLs are scattered throughout several organelle membranes. It would be extremely interesting to determine if H-Chol induces these changes we observed to a similar extent in places like the ER, mitochondria, or golgi. Which species of PL are involved is another question of significance. Because PC is the most abundant PL, we speculate that this might be where the bulk of these changes to fatty acid composition occur. With regard to the LCD and HCD groups an important question is whether the reduction in PL content is due to either depleted PC, PE, or a combination of both. Given the large surface area that the plasma membrane has, it may be that most of the depletion occurs here, although it may also be that organelles like the ER are experiencing a shortage of PL. To what extent does cholesterol accumulate in membranes? We did not measure membrane cholesterol content, but determining whether feeding a H-Chol diet results in membrane accumulation is an avenue that should be explored. It is quite possible that overloading guinea pig livers with cholesterol results in increased membrane accretion, which may oversaturate the complexing ability of cholesterol with surrounding PL. Answers to these
pressing questions will hopefully begin to tease apart the function of PL metabolism in NAFLD. Research of this kind will also likely begin to shift the attention of researchers towards the importance of organelle lipid composition in disease progression. Scientific evidence from such experiments is eagerly awaited.

References:


CONCLUDING REMARKS

We have come to the conclusion of the present investigation and should reflect upon some of the major findings. The overarching hypothesis of this dissertation centered around the pathophysiological impact of excess dietary cholesterol on hepatic...
function and in particular, how this dietary scheme has the potential to promote non-alcoholic fatty liver disease (NAFLD) in guinea pigs, an underappreciated animal model in metabolic disease research. Indeed, study 1 demonstrated that feeding a cholesterol-enriched diet caused guinea pigs to develop fatty liver and several pathological features observed in the more advanced stage of the disease spectrum. Several questions arise.

Is cholesterol solely responsible for the transition of steatosis to non-alcoholic steatohepatitis in those affected guinea pigs? How is it that some guinea pigs in the cholesterol-enriched group developed steatosis and others displayed more inflammatory pathological features? Is there an underlying genetic component that makes some guinea pigs more sensitive to cholesterol-induced disease? Answers will only come from more in-depth studies that employ a potent mixture of genetics, biochemical, and pathological experiments.

Another hypothesis that drove the dissertation was that manipulating dietary macronutrients may be a potential therapeutic modality to improve histological features of NAFLD. Quite unexpectedly, the results from study 2 suggest this may not be the case as guinea pigs fed either a low-carbohydrate, high fat diet or a high-carbohydrate, high-fat diet developed more severe hepatic dysfunction and pathological features of injury. This could imply that macronutrient manipulation may be unable to rescue guinea pigs following cholesterol-induced injury. An interesting experiment that should be done in the future would be to subject guinea pigs to the dietary intervention and then place them on a high-cholesterol diet. It may be that one diet is more hepatoprotective, a consequence of the metabolic effects of specific macronutrients.
The third study provided us with some more in-depth answers when it comes to the lipidomic changes that a cholesterol challenge elicits. The results largely reaffirmed what was previously published, specifically that high cholesterol promotes an increase in the content of unsaturated fatty acids, both in triglycerides as well as phospholipids. What was particularly rewarding was the observation that arachidonic acid was significantly depleted in phospholipids following a cholesterol challenge, a finding that has been repeatedly shown in studies conducted in humans. An equally interesting observation was that both low and high carbohydrate diets were profoundly depleted of membrane phospholipids compared to the high cholesterol group. What species of phospholipid are depleted, and perhaps equally important, what organelles do these changes occur in the liver? Future experiments should begin to take a more nuanced approach to understand alterations in fatty acid metabolism and see how this relates to the pathological changes in the liver. This may indeed represent a new frontier in the study of NAFLD, lipid metabolism, and dietary interventions. No doubt it is an exciting time to be a researcher in this field. Hopefully this work along with others will result in a truly exciting situation, that is, potent therapeutic modalities to finally gain control over a disease that has, and continues to have such an overwhelming impact on much of the world today.