THE EFFECT OF A KETOGENIC DIET ON HEPATIC CHOLESTEROL METABOLISM

ARIS KHEIRANDISH

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

GRADUATE PROGRAM IN KINESIOLOGY AND HEALTH SCIENCE
YORK UNIVERSITY
TORONTO, ONTARIO
August 2024

Abstract

The ketogenic diet (KD), known for its high-fat, low-carbohydrate composition, has gained popularity for weight loss and metabolic health benefits. Despite these advantages, there are concerns that the diet's high saturated fat content might elevate cholesterol levels and cardiovascular disease (CVD) risk. This study investigates the KD's impact on the molecular mechanisms of cholesterol metabolism in the liver, focusing on cholesterol synthesis markers such as 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) and sterol regulatory element-binding protein-2 (SREBP-2), as well as cholesterol uptake markers including proprotein convertase subtilisin/kexin type 9 (PCSK9) and LDL receptors (LDLr). For that, male Wistar rats (n = 6 per group) were fed for 16 weeks one of the following diets: standard chow (SC, 60% carbohydrates, 13% fat, 27% protein), high-fat sucrose-enriched (HFS, 20% carbohydrates, 60% fat, 20% protein), and ketogenic diet (KD, 0% carbohydrates, 80% fat, 20% protein). Liver tissue was extracted and analyzed for gene expression using real-time PCR and protein content using western blotting. Blood samples were collected to measure circulating cholesterol levels. We found that neither plasma cholesterol levels nor HMG-CoA reductase and SREBP-2 levels in the liver differed among the dietary interventions. However, the KD significantly reduced liver PCSK9 content and expression in comparison other diets, suggesting that the KD enhanced clearance of circulating cholesterol by the liver. To test whether there was a higher amount of LDLr on the membrane compared to the cytoplasm, the ratio of LDLr distribution between these compartments was measured. Importantly, there was an upward trend in the levels of LDLr on the membrane. In conclusion, the KD altered key steps that regulate hepatic cholesterol metabolism and prevented plasma cholesterol levels from increasing, despite its elevated saturated fat content.

Acknowledgments

I would like to express my deepest gratitude to my supervisor, Dr. Rolando Ceddia, for his unwavering guidance, support, and encouragement throughout this journey. Your invaluable expertise illuminated every step of my research endeavors, as you consistently engaged with each experiment and provided the necessary insights to steer me in the right direction. Thank you for your steadfast presence and for always ensuring that I maintained the appropriate pace whenever it was required. Dr. Ceddia, your dedication to excellence and your profound insights have inspired me to strive for the highest standards in my work.

A heartfelt thank you to Daniel for his kindness, patience, and being an exceptional friend and colleague. Daniel, you have imparted invaluable life and work lessons, always with an open ear and a passion for science. I would also like to thank David for helping me with my experiments and has always been there for me. Earnest thanks to Shailee and Mateja for their support and encouragements throughout this journey.

Lastly, to my parents, words cannot express how much I am thankful for your unconditional love, unwavering support, and endless encouragement. Your belief in me has been a constant source of motivation, and I am profoundly grateful for all the sacrifices you have made to help me reach this milestone.

Table of Contents

List of Abbreviations

3HB	3-beta-hydroxybutyrate
AcAc	Acetoacetate
AHA	American Heart Association
ASCVD	Atherosclerotic Cardiovascular Disease
bHLH-Zip	Basic Helix-Loop-Helix-Leucine Zipper
BMI	Body mass index
ChREBP	carbohydrate regulatory element-binding protein
CK	Creatine Kinase
CoA	Coenzyme A
CVD	Cardiovascular disease
DAG	Dietary Approaches to Stop Hypertension
DAG	Diacylglycerol
DASH	Dietary Approaches to Stop Hypertension
DNL	De novo lipogenesis
ER	Endoplasmic reticulum
FH	Familial Hypercholesterolemia
HDL-C	High density lipoprotein cholesterol
HFS	High fat-sucrose
HMG-CoA	3-hydroxy-3-methyl glutaryl coenzyme A
HMG-CoA reductase	3-hydroxy-3-methyl glutaryl coenzyme A reductase
IDL	Intermediate-density lipoprotein
IHD	ischemic heart disease
KD	Ketogenic diet
lbLDL	large buyout LDL subfraction
LCD	Low carbohydrate diet
LDL-C	Low density lipoprotein cholesterol
LDLr	Low density lipoprotein receptor
LIRKO	Liver Insulin Receptor Knockout
LMHR	Lean mass hyper responders
LP(a)	Lipoprotein (a)
LPL	Lipoprotein lipase
NAFLD	Non-alcoholic fatty liver disease
NEFAs	Non-Esterified Fatty Acids
NSERC	Natural Sciences and Engineering Research Council of Canada
PCSK9	Proprotein convertase subtilisin/kexin type 9
PURE	Prospective Urban Rural Epidemiology
S1P	Site-1 Protease

SBP	systolic blood pressure
SC	Standard Chow
SCAP	Sterol Regulatory Element-Binding Protein Cleavage-Activating Protein
sdLDL	Small dense lipoprotein
sdLDL	small, dense LDL
SER	Smooth endoplasmic reticulum
SREBP	Sterol receptor binding protein
SREBP-2	Sterol regulatory element-binding protein 2
SREBP1c	Sterol regulatory element-binding protein 1c
TG	Triglyceride
VLCD	very low carbohydrate diet
VLDL	Very low-density lipoprotein

List of figures

List of tables

1. Introduction

Cholesterol is a lipid molecule that plays a pivotal role in various biological processes, such as production of certain hormones, including sex hormones, vitamin D, and bile[1,2]. Because cholesterol is predominantly lipophilic, it is incorporated into lipoproteins that allow lipid molecules to circulate in the bloodstream and be transported to cells and tissues[3]. Various types of lipoproteins travel through the blood, each serving distinct functions including high-density lipoproteins (HDL) and low-density lipoproteins (LDL)[4]. However, excessive amounts of cholesterol found in LDL-C can cause health problems such as atherosclerosis and heart disease[5,6]. This concern arises from "the diet heart hypothesis", which proposes that high amounts of saturated fat increase cholesterolemia and lead to CVD[7]. To avoid this issue, pharmacological interventions have been developed to manage liver cholesterol biosynthesis. One of the main agents used is Statins[8]. Despite the potential of statins to lower blood cholesterol, negative side effects such as muscle cramps, increased risk of diabetes, myopathy, and rhabdomyolysis have been described in subjects taking these cholesterol lowering drugs[9,10]. Thus, alternative safe and effective approaches to reduce circulating cholesterol to treat or prevent dyslipidemia associated with metabolic disorders are needed. In this context, the KD has emerged as a dietary intervention to treat or prevent metabolic disorders (*e.g.*, dyslipidemia) associated with obesity and type II diabetes[6,11]. The typical KD generally provides ~80% of total calories from fats, 15% from proteins, and 5% from carbohydrates[12]. Several studies have reported controversial results regarding the effect of the KD on cholesterol metabolism, with some studies reporting circulating cholesterol levels being elevated[13], whereas in others cholesterol levels are either reduced[14] or remain unaltered[15]. A study conducted by Walton et al. extended over a 90-day period, focusing on 11 women diagnosed with type II diabetes who adhered to a KD (<30 g of carbohydrate per

day) reported positive lipid profile changes, such as an increase in HDL cholesterol, a significant decrease in triglycerides (TG), and a noteworthy decrease in the triglyceride-to-HDL ratio, whereas LDL levels did not exhibit significant changes[16] . Other study showed a notable decrease in cholesterol and LDL levels in the KD group, along with an increase in HDL (U/L) when compared to the Mediterranean group (55% carbohydrates, 25% fat and 20% protein). These findings suggest that the KD may have a more favorable impact on lipid profiles compared to the Mediterranean diet[17]. Interestingly, some reports have indicated that the most significant rises in LDL-C levels tend to happen in patients who have a lower BMI and an average LDL-C level before starting the diet[18]. Currently, little is known about how the KD affects the molecular mechanisms that regulate cholesterol metabolism at the cellular level. This is particularly relevant in the liver, an organ that produces large amounts of cholesterol daily. Thus, this research was designed to investigate how the KD regulates crucial molecular steps that regulate endogenous cholesterol production in the liver.

2. Literature Review

2.1. Cholesterol

Cholesterol consists of 27 carbons, possesses a distinctive structure comprising a hydrocarbon tail, a sterol nucleus consisting of four hydrocarbon rings, and a hydroxyl group[19] (Figure 1). Both the hydrocarbon tail and the central ring lack polarity, rendering them immiscible with water[20,21]. Due to their insolubility in water, cholesterol transport in conjunction with proteins[4]. Cholesterol plays a crucial role in the cell membrane, contributing to its structural integrity and regulating its fluidity[22]. It serves as a precursor in the synthesis of vitamin D, steroid

hormones (such as cortisol, aldosterone, and adrenal androgens), and sex hormones (including testosterone, estrogens, and progesterone)[1,2,23]. Additionally, cholesterol is a key component of bile acids, which are essential for digestion and the absorption of fat-soluble vitamins A, D, E, and K[24,25].

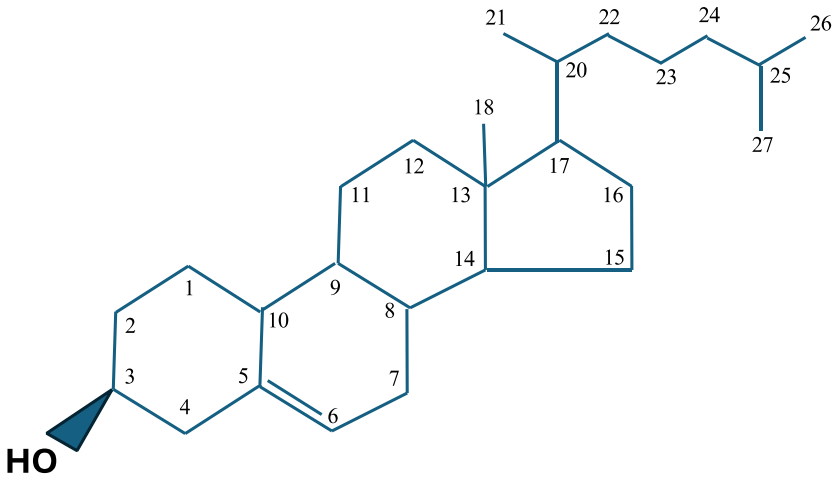

Figure 1. Structure of Cholesterol ($C_{27}H_{46}O$)

2.1.2. Cholesterol transport

Lipoproteins serve the purpose of transporting cholesterol[4]. They comprise intricate structures with a central core containing cholesterol esters and TG[4]. Lipoproteins are encircled by free cholesterol, phospholipids, and apolipoproteins[4]. These apolipoproteins play crucial roles in the formation and function of lipoproteins. Lipoproteins can be categorized into seven classes based on factors such as size, lipid composition, and apolipoprotein content. These classes include chylomicrons, chylomicron remnants, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), LDL, high-density lipoproteins (HDL), and lipoprotein (a)[4,26]. **Chylomicrons** are large TG-rich particles generated in the small intestine. They play a pivotal role in ferrying dietary TG and cholesterol to peripheral tissues and the liver[4,27]. Chylomicrons contain

a spectrum of apolipoproteins, including A-I, A-II, A-IV, A-V, B-48, C-II, C-III, and E [28]. Among these, Apo B-48 serves as the core structural protein with each chylomicron carrying a single Apo B-48[29]. The size of chylomicrons is contingent upon the quantity of fat consumed[30]. The typical chylomicron particle is made up of more than 75% triglycerides and approximately 2% protein [28]. Like other lipoproteins, chylomicrons also transport esterified cholesterol and phospholipids [28]. As chylomicrons donate their cargo to peripheral tissues through its interaction with lipoprotein lipase (LPL), **chylomicron remnants** are formed[30,31]. These remnants exhibit reduced TG and heightened cholesterol contents compared to chylomicrons[30,32,33]. TG are also synthesized endogenously, mostly by the liver. These endogenously produced TG are packed into **VLDLs** in the liver and contain the apolipoproteins B-100, C-I, C-II, C-III, and E[34,35]. They feature Apo B-100 as the central structural protein and a single Apo B-100 molecule is present in every VLDL particle [34]. Much like chylomicrons, the size of VLDL particles varies depending on the TG content within them[34]. Elevated TG production in the liver results in the secretion of larger VLDL particles[34]. Nonetheless, VLDL particles are comparatively smaller than chylomicrons[32]. The typical VLDL particle contains TG (50–70%) and 10% protein[36].VLDL particles also donate their TG content to peripheral tissues in a similar fashion as chylomicrons operate[34] . As the TG content of VLDL particles reduce through lipoprotein lipase (LPL-mediated) TG hydrolysis, cholesterol-rich **IDL** particles are formed. Upon losing their apoE content, **LDL** particles carrying the bulk of circulating cholesterol are formed [34]. This lipoprotein is characterized by the presence of a single Apo B-100 per LDL particle[37] . LDL encompasses a range of particles distinguished by size and density[38] . Notably, an abundance of small dense LDL particles is observed in conjunction with hypertriglyceridemia, low HDL levels, obesity, type II diabetes as well as inflammatory conditions[39,40]. These small dense LDL particles are considered more pro-atherogenic than their

larger counterparts for several reasons[39]. They more easily penetrate into arterial walls and display enhanced binding to intra-arterial proteoglycans, thereby becoming trapped within the arterial wall. Furthermore, small dense LDL particles are more susceptible to oxidation, potentially increasing their uptake by macrophages[39,41]. The liver also produces **HDL** particles[42], which in their nascent form are the smallest of all lipoproteins[43]. HDL contain approximately 35–50% phospholipids, 5–10% sphingolipids, 30–40% cholesterol esters, 5–10% free cholesterol, and 5–12% triglycerides[43]. HDL particles are also associated with various apolipoproteins, including A-I, A-II, A-IV, C-I, C-II, C-III, and E. Apo A-I serves as the core structural protein, with each HDL particle potentially containing multiple Apo A-I molecules[44]. These particles play a pivotal role in reverse cholesterol transport, facilitating the transfer of cholesterol from peripheral tissues to the liver, which represents a potential mechanism underlying the anti-atherogenic properties of HDL[45,46]. Lipoprotein(a), or Lp(a), is a particle that resembles LDL-C [26]. It is composed of a core containing TGs and cholesteryl esters, enveloped by a membrane of phospholipids and free cholesterol. The distinctive feature of Lp(a) lies in its protein components, which include apolipoprotein B-100 (ApoB) and apolipoprotein(a) (apo(a)). The apo(a) is covalently linked to ApoB via a disulfide bond, imparting a unique structure to Lp(a) [26]. The synthesis of Lp(a) is predominantly controlled by the LPA gene, which encodes for multiple kringle IV (KIV) repeats in apo(a). The number of these KIV repeats varies among individuals, leading to different isoforms of apo(a) and contributing to the heterogeneity of Lp(a) particles[26] . Lp(a) is synthesized in the liver, where it assembles either on the hepatocyte surface or within the space of Disse. After synthesis, Lp(a) circulates in the bloodstream, where it interacts with various cellular components and undergoes modifications that affect its function and potential to cause disease [26]. Elevated levels of Lp(a) are linked to the development of various CVDs through several intricate

mechanisms such as calcification. It transports oxidized phospholipids to sites of vascular injury and aortic valve leaflets. This leads to endothelial dysfunction, lipid deposition, and calcification of the vessel walls and valve leaflets[26].

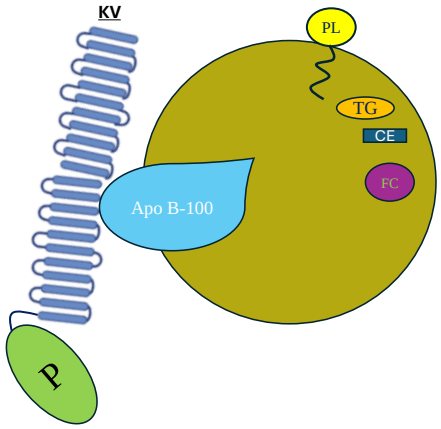

Figure 2. Lp(a) is a lipoprotein particle that contains apoB-100, which serves as its core protein component. This particle also includes various lipid components such as cholesterol esters (CE), free cholesterol (FC), phospholipids (PL), and triglycerides (TG). The unique structural feature of Lp(a) is its covalent attachment to apo(a) through a disulfide bond. Apo(a) itself contains multiple kringle IV domains, which contribute to its distinct properties and functions.

2.1.3. Sources of Cholesterol

Dietary cholesterol

Sterols are an essential component of cell membranes, produced by both animals and plants. In animals, the primary sterol is cholesterol[47] . Dietary cholesterol is mainly found in animal-based foods, primarily in egg yolk, shrimp, beef, pork, poultry, cheese, and butter [48] . In contrast, plants do not contain cholesterol but have similar compounds called phytosterols, such as sitosterol, campesterol, and stigmasterol[49]. Phytosterols contribute to the structure and stability of plant cell membranes and differ from cholesterol in the side chain attached at the C-17 position[50]. Unlike cholesterol, phytosterols are poorly absorbed, with an absorption rate of about 2-5%, compared to 60% for cholesterol[49,51]. Cholesterol has poor solubility in the gut's watery

environment and depends on bile micelles for digestion and absorption [52]. These micelles, which contain bile acids, free cholesterol, phospholipids, and TGs, facilitate the transport of cholesterol across the intestinal lumen-enterocyte interface[52]. The Niemann-Pick C1-like 1 protein (NPC1L1) serves as a sterol transporter. It enables the movement of cholesterol and plant sterols from the intestinal lumen into the intestinal cells[53]. Once inside the intestinal cells, cholesterol is converted into cholesteryl esters by acyl-CoA cholesterol acyltransferase (ACAT) [54]. Cholesteryl esters are then packaged into chylomicrons along with dietary TG for transport initially through the lymphatic system and then into the bloodstream (Figure 3)[54,55]. On average, the diet provides about 400 mg of cholesterol daily, whereas the liver produces approximately 1 gram daily [53] .

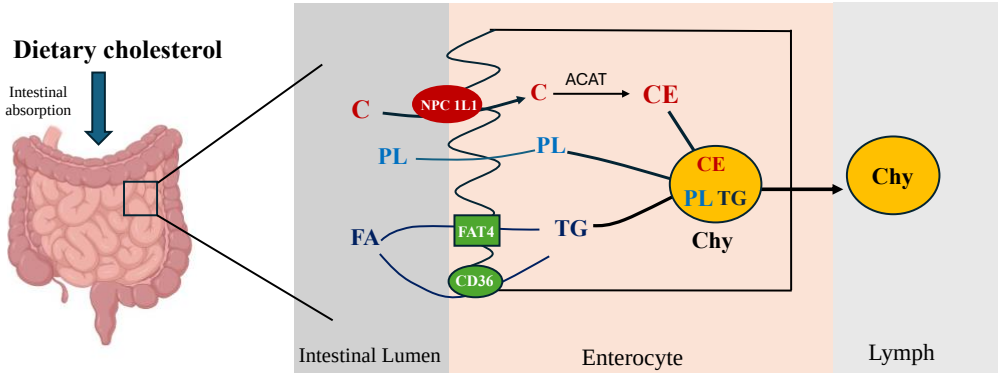

Figure 3. Intestinal absorption of dietary cholesterol. Dietary cholesterol (C) enters the intestinal cells via NPC1L1 and is converted to cholesteryl esters by ACAT. Fatty acids (FA) are brought into the cells by specific proteins like FATP4 and CD36. These components are then packaged into chylomicrons, which are transported to the lymphatic system for distribution throughout the body. Chy = chylomicron, PL= phospholipid.

2.1.4. Endogenous cholesterol production

Although all cells have the capacity for cholesterol synthesis, the liver is the primary site for this process[56]. Endogenous cholesterol synthesis involves enzymes located in both the cytoplasm and the smooth endoplasmic reticulum (SER). The initial step of cholesterol biosynthesis involves the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA.

Subsequently, a cytosolic enzyme named HMG-CoA synthase, adds a third molecule of acetyl-CoA to acetoacetyl-CoA, producing a six-carbon compound known as 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA)[3]. The regulatory enzyme HMG-CoA reductase, located in the SER, then catalyzes the reduction of HMG-CoA to mevalonate (MVA)[3]. The latter is a key rate-limiting step in endogenous cholesterol synthesis[3]. Through a series of reactions (Figure 4), MVA is converted into 3-isopentenyl pyrophosphate, then to farnesyl pyrophosphate, squalene, and lanosterol. In the ER, 19 additional enzymatic steps convert lanosterol into cholesterol, with the final step being catalyzed by 7-dehydrocholesterol reductase, which converts 7-dehydrocholesterol to cholesterol[3,57,58].

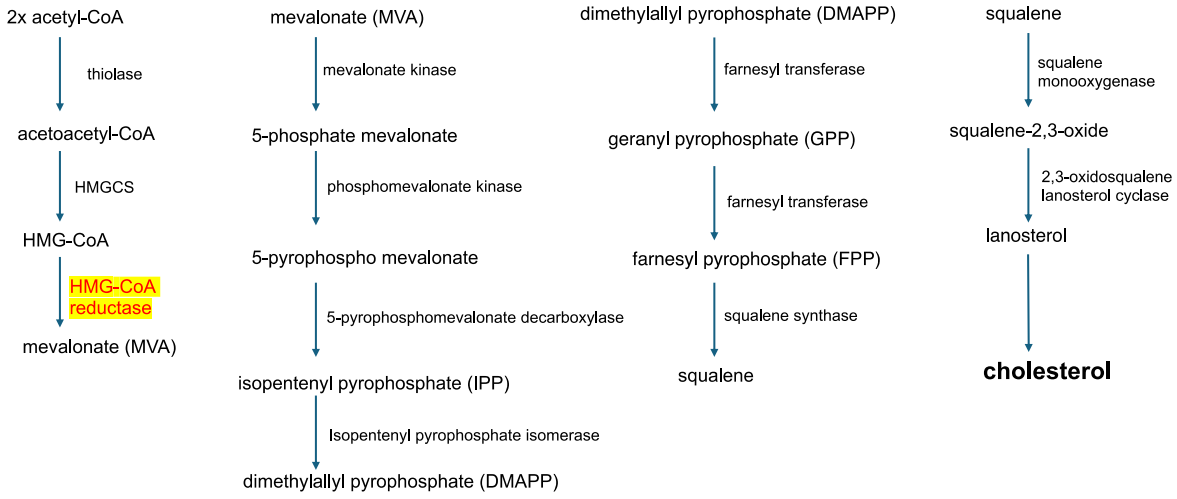

Figure 4. The pathway for endogenous cholesterol biosynthesis.

Regulation of HMG-CoA reductase activity

As previously mentioned, endogenous cholesterol synthesis is regulated by the activity of HMG-CoA reductase[59]. When cholesterol levels are high, it triggers a negative feedback mechanism that reduces the activity of this rate limiting enzyme[59]. This regulation was first highlighted in the late 1970s when researchers studied a molecule called ML-236B, which acts as

a competitive inhibitor of HMG-CoA reductase in rat liver [59,60]. When administered orally to rats, ML-236B acutely lowered plasma cholesterol levels[60]. Its structure was found to be identical to compactin, a drug independently isolated from Penicillium brevicompactum by Brown et al[60]. Then researchers used compactin to study how the enzyme HMG-CoA reductase is regulated in human cells. They found that when cells were treated with compactin, the production of mevalonate was suppressed[59,60]. HMG-CoA reductase is an ER-localized glycoprotein, and its activity is up-regulated at the transcriptional level by SREBP-2 when intracellular cholesterol levels are low[61].

SREBP-2 Activation, and Regulation

SREBPs, which include SREBP1a, SREBP1c, and SREBP-2, are basic-helix-loop-helix leucine zipper (bHLH-Zip) transcription factors that control the synthesis and cellular uptake of cholesterol and fatty acids, two essential components of cell membranes[62]. However, it is SREBP-2 that regulates the expression of HMG-CoA reductase, LDLr, and PCSK9, which are major determinants of endogenous synthesis and uptake of cholesterol by the liver [63,64]. In its inactive form, SREBP-2 is a 125kDa molecule bound to the endoplasmic reticulum (ER) through its NH_2 terminal domain[65–67]. There are two crucial sequential steps required for SREBP-2 to become active: 1) transportation of SREBP-2 from the ER to the Golgi apparatus and 2) cleavage of SREBP-2 in the Golgi. When cellular cholesterol levels decrease in the ER plasma membrane, SREBP-cleavage activating protein (SCAP) binds to the COOH-terminal of SREBP-2. SCAP functions as both a sterol sensor and an escort for SREBPs. The decrease in cholesterol induces a conformational change in SCAP, which then allows it to bind to COPII-coated vesicle (Coat Protein Complex II) [62,67,68]. This vesicle is what allows the transportation of SREBP-SCAP

complex from the ER to the Golgi apparatus. Once in the Golgi apparatus, SREBP-2 is cleaved by Site 1 protease (S1P) and S2P proteases to release the NH_2-terminal of SREBP-2 (nSREBP-2). This allows SREBP-2 to be active and enter the nucleus where it binds to a specific DNA sequence termed sterol response element (SREs), ultimately inducing gene transcription[62,67,68](Figure 5).

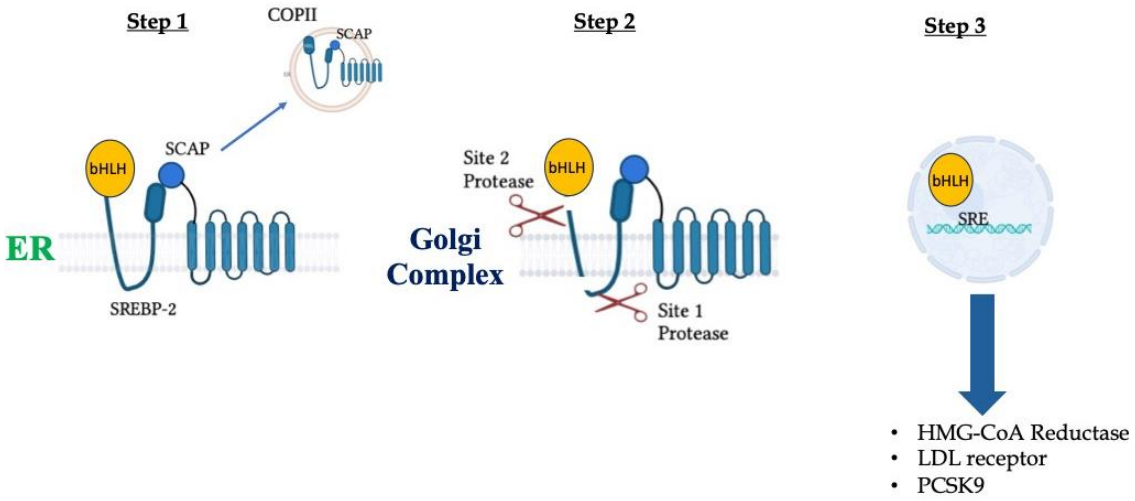

Figure 5. Sterol-mediated proteolytic release of SREBP-2 from the membranes involving SCAP. The latter serves as both a sterol sensor and an escort for SREBP-2. In conditions where cells experience cholesterol depletion, SCAP facilitates the transportation of SREBP-2 from the ER to the Golgi apparatus. Within the Golgi, two proteases, S1P and S2P, act consecutively to release the NH_2-terminal bHLH-Zip domain from the Golgi membrane. Subsequently, the bHLH-Zip domain translocates into the nucleus and binds to a SRE located in the enhancer/promoter region of target genes. This binding event triggers the activation of genes that are involve cholesterol synthesis.

Conversely, when intracellular cholesterol levels rise, SCAP undergoes a conformational change that promotes the binding of SCAP-SREBP complex to Insig (Insulin-induced gene). Insig is a resident protein of the ER, and as a result, the Insig/SCAP/SREBP-2 complex remains localized to the ER membrane[68]. This localization effectively impedes the transport of SREBP-2 to the Golgi apparatus, preventing its subsequent cleavage and nuclear translocation[68]. This leads to a decrease

in transcription of the target genes, resulting in a decline in cholesterol synthesis and uptake[69](Figure 6).

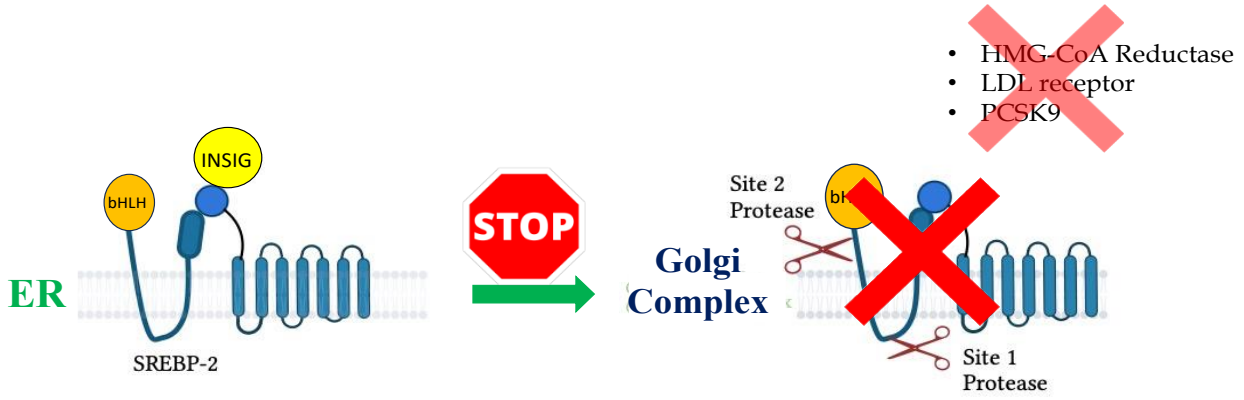

Figure 6. Insig-mediated regulation of SCAP/SREBP transport: When cholesterol accumulates in ER membranes, it binds to SCAP, inducing a change in its structure and leading the SCAP-SREBP complex to associate with either Insig-1 or Insig-2, both of which are closely related polytopic membrane proteins. Once the SCAP-SREBP complex binds to Insig, it becomes incapable of being incorporated into COPII-coated vesicles. Consequently, the SREBP retained in the ER remains unprocessed, resulting in a decrease in the transcription of target genes. This leads to reduced cholesterol synthesis and uptake.

2.1.5. The LDLr and Cholesterol Uptake

The LDLr is a member of a group of cell-surface glycoproteins synthesized in the ER[70]. The LDLr undergoes glycosylation in the ER producing a precursor of the LDLr. This precursor then undergoes additional glycosylation in the Golgi complex, where it transforms into an active LDLr with an observed molecular weight of 160 kDa[71]. The LDLr is subsequently directed towards specialized areas on the cell membrane. The LDLr is responsible for the clearance of LDL particles rich in cholesterol (LDL-C) from the bloodstream. Upon binding of LDL to its receptor on the cell membrane, the entire complex undergoes internalization via coated vesicles through the process of endocytosis[72]. Following internalization, these vesicles merge to form endosomes [72]. Within the endosomal environment, the drop in pH triggers the release of the LDL particle from the LDLr,

allowing the latter to recycle to the cell surface for another round of endocytosis (Figure 7) [73]. It is recognized that cholesterol regulates the expression of the LDLr through a negative feedback mechanism mediated by SREBP-2 [73].

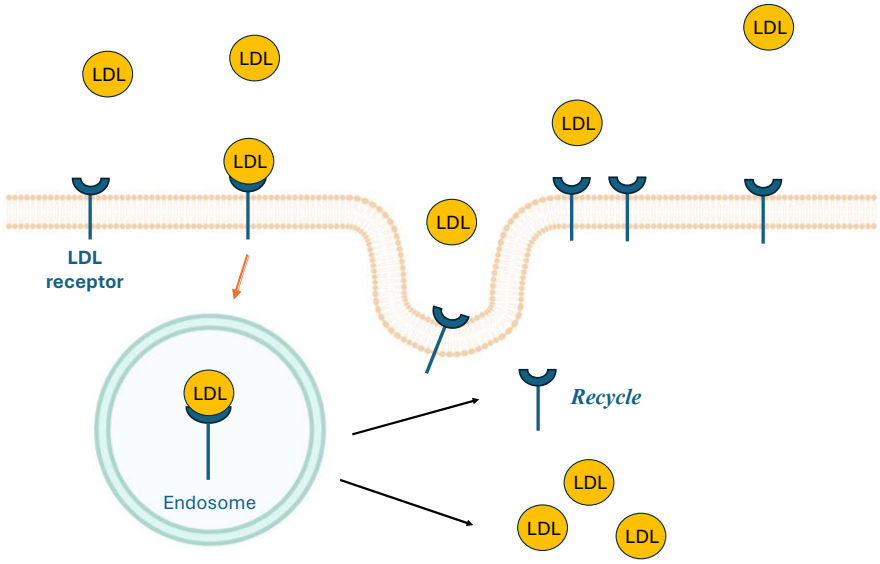

Figure 7. Diagram depicting cholesterol uptake through LDL binding to its receptor. Reductions in intracellular cholesterol levels activate SREBP-2. This activation prompts the translocation of SREBP-2 to the nucleus, where it initiates the expression of cholesterol regulatory genes such as LDLr. The LDLr present on the cell surface captures circulating LDL and facilitates the transport of the cholesterol from plasma to hepatocytes for degradation. Subsequently, the receptor undergoes recycling back to the cell surface.

PCSK9

PCSK9 belongs to the proprotein convertase family of secretory serine endoproteases. It is primarily synthesized and secreted by the liver, although lower levels of PCSK9 expression have also been detected in the intestine, kidney, and brain[74]. PCSK9 plays a pivotal role as a regulator of blood cholesterol uptake by the liver [75,76]. PCSK9 acts by binding to LDLr located on the plasma membrane of liver cells[77]. Following this binding event, the PCSK9-LDLr complex is internalized by the cell via clathrin-coated vesicles[77,78]. Once inside the cell, the PCSK9-LDLr complex is

transported to lysosomes, where it undergoes degradation [77,78]. This degradation process results in the reduction of the number of functional LDLr on the cell surface. With fewer LDLr available for LDL particle binding and internalization, there is a consequent increase in plasma LDL-C levels (Figure 8) [77]. PCSK9 levels in the bloodstream are positively associated with factors such as diabetes mellitus, obesity, and atherosclerosis [79,80]. Multiple studies in adults have demonstrated an association between type II diabetes and elevated PCSK9 levels [81]. Research on cells and mice further indicates that insulin stimulates PCSK9 production, implying that hyperinsulinemia seen in obesity and type II diabetes may increase PCSK9 levels[81]. In addition, preclinical studies have found that overexpression of PCSK9 promotes atherosclerosis, whereas reducing PCSK9 levels provides protection against it[80]. Inhibiting PCSK9 pharmacologically has been shown to significantly reduce cardiovascular risk[80].

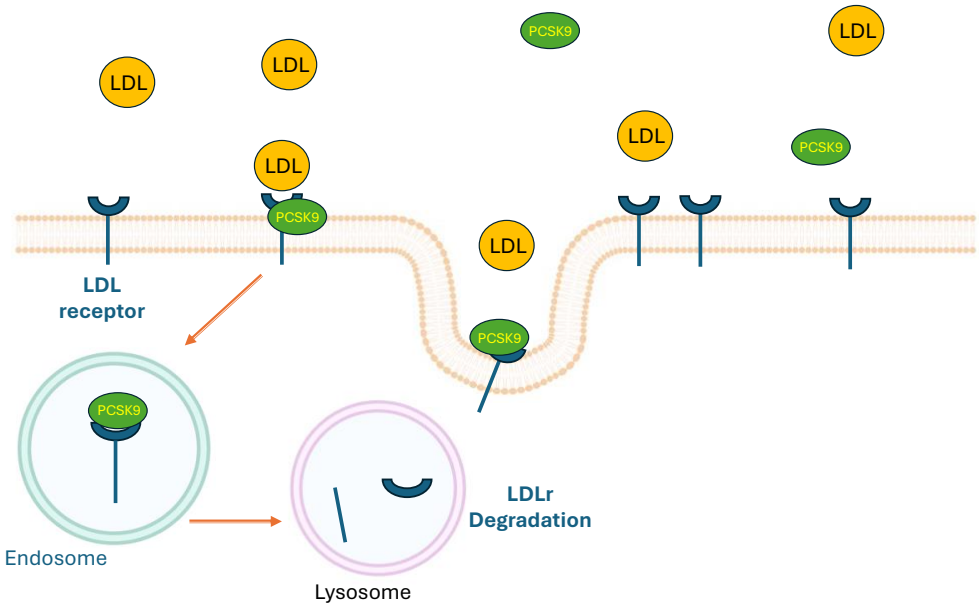

Figure 8. Mechanism of PCSK9-Mediated LDLr degradation. Circulating PCSK9 binds the LDLr leading to internalization of the PCSK9-LDLr complex via endocytosis. This ultimately causes degradation of LDLr within lysosomes.

Familial Hypercholesterolemia (FH)

Carl Müller first showed that high levels of cholesterol and the onset of heart disease at a young age tend to run in families as dominant genetic traits [3]. FH was further characterized by Avedis Khachadurian, who described two clinical forms of FH [3]. Mutations can be either homozygous or heterozygous. Homozygotes carry the same mutation in both alleles, while compound heterozygotes have different mutations in each allele[82,83]. Homozygous individuals exhibited severe hypercholesterolemia from birth, with plasma cholesterol levels reaching approximately 800 mg/dl and were susceptible to heart attacks as early as 5 years old[3]. Conversely, heterozygous patients presented with cholesterol levels ranging from 300 to 400 mg/dl experienced early-onset heart attacks (between the ages of 35 and 60)[3]. The three main genetic mutations associated with FH are defects in the LDLr, ApoB, and PCSK9, with LDL receptor defects being the most common[84]. In the 1970s, Joseph Goldstein and Michael Brown elucidated the fundamental defect in LDLr function in FH, a discovery that earned them the Nobel Prize in 1985 [3]. Over 1,700 mutations in the LDLr gene have been identified in FH[85]. These include large fragment deletions, small fragment deletions, insertions, point mutations, and splicing mutations[85]. Such genetic alterations result in dysfunctional LDLr proteins, impairing the clearance of LDL particles from the bloodstream [85]. Consequently, elevated serum LDL-C levels lead to early onset atherosclerosis and an increased risk of CVD[85]. Another underlying mutation observed in FH is in the ApoB gene plays a central role in the interaction between LDL and the LDLr, which significantly influences plasma cholesterol levels [86]. Mutations in the ApoB gene are less prevalent compared to genetic defects in the LDLr[87]. Patients with ApoB mutations, often referred to as "familial defective ApoB patients", have a single ApoB gene mutation that reduces ApoB's affinity for the LDLr, resulting in increased circulating LDL-C levels [86]. However, these patients typically

experience less severe elevations in LDL-C compared to those with LDLr gene defects[88]. As the third gene associated with FH, PCSK9 has been discovered to decrease the liver's uptake of LDL-C by enhancing the degradation of LDLr in endosomes and lysosomes[89]. Therefore, pharmacological agents that inhibit PCSK9 are now available for managing FH [90].

Cardiovascular disease (CVD)

CVD primarily originates from atherosclerosis, an arterial condition leading to myocardial infarction, stroke, and various other complications[91]. This disease predominantly affects the subendothelial space of medium and large arteries, especially where blood flow is disturbed or bifurcates[92]. It has been shown that risk factors such as hyperlipidemia, obesity and inflammation significantly contribute to the onset and progression of atherosclerotic cardiovascular disease (ASCVD) [92]. Research also highlights that elevated levels of LDL-C are a key factor in the development of atherosclerosis [92]. Therefore, pharmaceutical agents such as statins are available to lower LDL-C levels [92]. Statins are designed to reduce LDL levels by inhibiting the activity of HMG-CoA reductase[93] . Beyond merely competing with the enzyme's usual substrate, statins also change the enzyme's shape upon binding to its active site. This structural alteration prevents HMG-CoA reductase from becoming functional[94], which results in a decrease in cholesterol synthesis and content within the liver[93]. Even when patients reach the recommended LDL-C targets through statin treatment, they still face a considerable risk of ASCVD[92]. Moreover, the use of statins is associated with myotoxicity, which can manifest as myopathy, myalgia, myositis, or rhabdomyolysis[95]. In clinical trials, statin toxicity is often characterized by myalgia or muscle weakness with creatine kinase (CK) levels exceeding 10 times the normal upper limit[95]. Rhabdomyolysis is a medical condition characterized by the rapid breakdown of damaged or injured skeletal muscle[96]. This disruption of muscle integrity results in the release of intracellular

components, including myoglobin into the bloodstream and extracellular space[96].The condition can lead to severe complications such as acute renal failure, disseminated intravascular coagulation, and even death [95]. Alternatively, dietary approaches are used to reduce cholesterol levels. In the 1950s, Ancel Keys proposed the idea that increased intake of saturated fat raises cholesterol levels, thereby increasing the risk of CVD [97]. Based on this hypothesis, later the American Heart Association (AHA) and Dietary Guidelines for Americans (DGA) recommended dietary guidelines to reduce fat intake, particularly saturated fat, and promoting diets rich in fruits, vegetables and whole grains to lower cholesterol levels and reduce the risk of CVD [97,98]. Contrary to the traditional view, some studies have questioned the direct relationship between dietary cholesterol and CVD risk[99]. The Prospective Urban Rural Epidemiology (PURE) study, the largest epidemiological cohort study ever conducted, provides evidence that challenges the traditional diet-heart hypothesis[98,99]. The PURE study tracked individuals aged 35–70 years from 2003 to 2013 across 18 countries, with a median follow-up period of 7.4 years. The study's findings revealed that saturated fat intake was not linked to an increased risk of myocardial infarction or CVD mortality. Instead, higher saturated fat consumption was significantly associated with lower total mortality and a reduced risk of stroke [98,99]. Recent studies highlight that the size and distribution of LDL-C particles are crucial for understanding their role in CVD. Research indicates that smaller, dense LDL particles are significantly more atherogenic compared to larger, buoyant LDL particles, thus posing a greater risk for CVD [100].

2.2.1. Ketogenic Diet (KD)

The KD is characterized by its emphasis on a high-fat and low-carbohydrate dietary pattern, where carbohydrate intake is restricted to 50g per day[101]. It produces ketone bodies namely acetoacetate (AcAc), 3-beta-hydroxybutyrate (3HB) and acetone in a process known as ketogenesis[102]. By limiting the intake of dietary carbohydrates, insulin secretion is maintained at low levels[14], allowing for enhanced lipolysis and release of non-esterified fatty acids (NEFAs) by the white adipose tissue[103]. In the liver, NEFAs undergo beta-oxidation and generate acetyl-CoA, the substrate for ketogenesis. Through this process, the KD leads to the induction of a state known as nutritional ketosis aimed at raising and sustaining blood ketone levels between 0.5 to 3 mg/dL[14,103]. This dietary approach shifts the body's energy source from glucose to ketone bodies derived from fat metabolism, promoting fat as the primary fuel[6]. Diet and lifestyle modifications are recommended as the first-line treatment for adults with hypertension, prediabetes, type II diabetes, and obesity, as these conditions significantly increase the risk of severe health complications, including stroke, myocardial infarction, and premature death[104]. The Dietary Approaches to Stop Hypertension (DASH) diet, endorsed by the American Heart Association for managing blood pressure, emphasizes fruits, vegetables, whole grains, and low-fat dairy while restricting saturated fats, total fats, and sodium intake [104]. Another effective dietary option is the very low-carbohydrate diet (VLCD), also known as the KD[104]. In a randomized clinical trial comparing the DASH and VLCD, researchers found that the VLC diet led to greater improvements in systolic blood pressure (SBP), glycemic control, and weight over a 4-month period in these adults[104]. However, LCDs like the KD raise concerns about potential adverse effects on serum lipid levels, particularly increasing LDL-C and CVD. In this context, a study involving 83 obese patients with a body mass index (BMI) over 35 kg/m², who also had high glucose and cholesterol levels,

followed a specific KD regimen that included 30 g of carbohydrates, protein at 1 g/kg body weight, 20% saturated fat, and 80% polyunsaturated and monounsaturated fats [105]. The findings from this study revealed a significant increase in HDL-C and a decrease in LDL-C after in the KD group. Additionally, there were significant reductions in both TG and blood glucose levels after 24 weeks[105]. Also, a study conducted by Walton et al. extended over a 90-day period, focusing on 11 women diagnosed with type II diabetes who adhered to a KD (<30 g of carbohydrate per day). This study reported positive lipid profile changes, such as an increase in HDL-C, a significant decrease in TGs, and a noteworthy decrease in the TG-to-HDL ratio, whereas LDL levels did not exhibit significant changes[6] . As previously mentioned, the size of LDL particles is crucial in assessing the risk of CVD[100]. Studies have shown that small, dense LDL (sdLDL, diameter <25.5 nm) are more susceptible to oxidation. Oxidized LDL leads to foam cell formation in macrophages and the progression of atherosclerotic lesions [38,39,41,106]. However, a study involving men who were free of ischemic heart disease (IHD) at baseline found that large buoyant LDL subfraction (lbLDL, diameter ≥25.5 nm) were not associated with an increased risk of IHD [107]. Instead, the cardiovascular risk was primarily linked to the accumulation of small-dense LDL (sdLDL) particles[107]. Additionally, individuals with increased concentrations of lbLDL were characterized by lower plasma TG and insulin levels, lower BMI, and higher plasma HDL-C concentrations [107]. These findings challenge traditional views and underscore the need to understand individual variability in response to diets. This is particularly relevant in a subgroup of individuals named lean mass hyper-responders (LMHR) that exhibits unique lipid responses to the KD.

LMHR

Typically, patients with the LMHR phenotype have a lean physique and are characterized by TG levels of ≤ 70 mg/dL, HDL-C levels of ≥ 80 mg/dL, and LDL-C levels of ≥ 200 mg/dL[108].

A potential mechanistic hypothesis for this relationship, known as the "lipid energy model," suggests that carbohydrate restriction in lean individuals increases the reliance on fat as a metabolic substrate [18]. In this state, the liver increases VLDL secretion, which is matched by enhanced breakdown of VLDL triglycerides (VLDL-TG) in the peripheral tissues, a process known as "VLDL turnover," facilitated by LPL[109]. LPL releases (NEFAs) from TGs within VLDL, providing energy for storage or use by tissues like peripheral tissues[109,110] . This process increases both HDL-C and LDL-C and reduce TG levels in circulation [109]. For instance, a case study evaluated a 38-year-old male with a normal BMI and no familial history of CVD. Historically, his LDL-C levels had remained within the range of 100-140 mg/dL. However, subsequent to initiating a KD rich in saturated fats, his LDL-C levels dramatically increased to 496 mg/dL[18]. Despite these markedly elevated LDL-C levels, computed tomography (CT) imaging showed no evidence of coronary artery calcification, which is commonly recognized as a significant indicator of atherosclerosis [18,111] .

sdLDL particles are closely linked to high TGs and low HDL-C levels[100], which together characterize an atherogenic lipoprotein phenotype that is significant in the development of atherosclerosis[100]. Therefore, it is important to examine the subfractions of LDL under the KD. To address this, a study investigated the effects of a six-week KD on fasting and post-meal serum biomarkers in 20 normolipidemic men of normal weight[112]. Twelve participants transitioned from their usual diet (17% protein, 47% carbohydrate, and 32% fat) to a KD (30% protein, 8% carbohydrate, and 61% fat)[112]. Prior to and following the intervention, levels of fasting blood lipids, LDL particle size, oxidized LDL, as well as post-meal TG and insulin responses to a high-fat meal were assessed. The KD led to significant increases in both mean and peak LDL particle diameter, indicating a shift towards larger LDL particles [112]. Also, results revealed notable

reductions in fasting serum TG (by 33%), post-meal lipemia following a high-fat meal (by 29%), and fasting serum insulin concentrations (by 34%) following adherence to the KD[112]. Levels of fasting serum total and LDL-C, as well as oxidized LDL, remained unaffected, and there was a tendency for HDL-C to increase with the KD (by 11.5%; p=0.066) [112]. Although the aforementioned studies identified differences in LDL-C levels under KD, the molecular details underlying the diet's impact on critical mechanisms that regulate endogenous cholesterol production by the liver are still largely unknown.

The effects of the KD on liver lipid metabolism

Hepatic de novo lipogenesis (DNL) is a crucial biosynthetic process in the liver, that includes both synthesis of fatty acids and the subsequent production of TG[113,114]. This pathway is part of the broader metabolic activities in the liver, primarily fueled by substrates from glycolysis and carbohydrate metabolism[114]. Consequently, diets rich in carbohydrates can significantly enhance the DNL pathway by providing an abundant supply of substrates, thereby increasing DNL rates[114]. The latter is regulated by two key transcription factors: sterol regulatory element-binding protein 1c (SREBP1c) activated by insulin, and carbohydrate regulatory element-binding protein (ChREBP), which is stimulated by carbohydrates [115]. Activation of SREBP1c and ChREBP triggers the expression of key lipogenic genes involved in lipogenesis [115,116]. This process elucidates the link between a high-carbohydrate diet and the overproduction of TG, potentially leading to the development of non-alcoholic fatty liver disease (NAFLD) [115,117]. Insulin resistance associated with obesity is a major contributor to the pathogenesis of NAFLD and tends to worsen as the disease progresses[115,117]. Recently, KD has gained recognition as an effective non-pharmaceutical approach for managing NAFLD and other metabolic disorders in obese

individuals[115,118]. KD reduces insulin levels and initiates a metabolic shift that significantly boosts fatty acid oxidation and ketone production[115,118]. The study by Jani et al. on male Wistar rats, which involved a KD composed of 0% carbohydrates, 80% fat, and 25% protein, demonstrated that despite the high fat content, the KD not only exerts anti-steatogenic and insulin-sensitizing effects on the liver but also lowers the levels of diacylglycerol (DAG)[115]. Additionally, the expression of SREBP1c and ChREBP, remained unchanged in rats on the KD. However, rats fed a HFS diet, which led to hepatic steatosis and insulin resistance, had significantly higher mRNA levels of ChREBP in the liver compared to those on the KD[115]. Given these findings on lipid metabolism, it is crucial to explore how the KD influence cholesterol metabolism in the liver, particularly in terms of regulatory mechanisms and overall hepatic health. While SREBP1c is primarily involved in the regulation of fatty acid synthesis[62], SREBP-2 primarily targets genes involved in cholesterol biosynthesis and uptake [62–64]. Observing the effects of KD on SREBP1c may imply similar impacts on SREBP-2 activities. In a study involving mice with a liver insulin receptor knockout (LIRKO), a reduction in the expression of SREBP-2 and cholesterol synthesis was observed[119].

DNL plays a crucial role in liver steatosis and is closely tied to VLDL production [120]. The connection between DNL and VLDL production lies in the fact that the fatty acids produced via DNL are incorporated into TG, which are a major component of VLDL particles [34,113,114]. Given that Jani's research observed a reduction in DNL activity and DAG levels with the KD [115], it is likely that this could impact VLDL and LDL levels, potentially modulating their production and influencing overall cholesterol metabolism in the liver. In line with this, an obese mouse model was fed a KD for a duration of 7 weeks. A significant decrease in cholesterol levels was observed in the VLDL fraction of the KD-fed obese mice[121]. The KD was effective in improving hyperglycemia, thereby preventing the development of steatosis[121].

Rationale

Despite the rising popularity and therapeutic promise of the KD [15], concerns persist regarding its high saturated fat content and the potential impact on cholesterol levels and CVD risk [7,97]. Given the liver's central role in cholesterol metabolism[122], we aimed to assess the impact of the KD on molecular mechanisms involved in hepatic cholesterol metabolism. This understanding could contribute to the development of more effective dietary guidelines and therapeutic strategies for managing cholesterol levels and reducing CVD risk.

Objectives

Specific objectives:

1. To determine the effects of obesogenic and ketogenic diets on circulating levels of total cholesterol, LDL-C/VLDL and HDL-C.
2. To examine the effects of KD, HFS and SC on SREBP-2 and HMG-CoA reductase.
3. To assess the effects of obesogenic and ketogenic diets on the key molecular determinants of cholesterol uptake LDLr and PCSK9 in the liver.

Hypotheses

I hypothesize that:

1. The elevated saturated fat content of the KD will increase circulating levels of LDL-C.
2. Abundant dietary intake of saturated fat will supress the expression of HMG-CoA Reductase and SREBP-2 in the liver ultimately limiting endogenous cholesterol synthesis.
3. The increase in cholesterol levels under the KD will impair cholesterol uptake by increasing PCSK9 and reduce LDLr levels in the liver.

Statement of Labour

The work in this study was performed by Aris Kheirandish, including homogenization of liver tissues, RNA extraction, real-time PCR analysis, and protein assays. Aris was also responsible for conducting all western blots, data analysis, interpretation of results for serum lipid markers, preparation of figures, and thesis writing. Dr. Shailee Jani and Dr. Daniel Da Eira, former PhD students in Dr. Rolando Ceddia's laboratory, carried out the in vivo animal work (animal feeding, blood collection, and tissue extraction), which was essential to this research. Dr. Da Eira also provided mentorship and experimental guidance. Mr. David De Caprio assisted in running protein assays. Dr. Rolando Ceddia was the principal investigator for this research study. He was responsible for designing the study, overseeing its implementation, securing funding, and providing intellectual guidance throughout the project. Dr. Ceddia also contributed significantly to the interpretation of results and provided critical feedback during the thesis writing process, ensuring the scientific rigor and integrity of the research.

Manuscript

The ketogenic diet lowers liver PCSK9 and prevents blood cholesterol fluctuation in male rats

Aris Kheirandish, Daniel Da Eira, Shailee Jani, David De Caprio, Rolando B. Ceddia

Muscle Health Research Center, School of Kinesiology and Health Science, York University, Toronto, Ontario, M3P 1J3, Canada

Running head: Ketogenic diet and hepatic cholesterol metabolism

To whom correspondence should be addressed:
Prof. Rolando B. Ceddia
Muscle Health Research Centre, School of Kinesiology and Health Science
York University, 4700 Keele St., North York, Ontario, M3J 13P, Canada.
Tel.: 416-736-2100 (Ext. 77204); Fax: 416-736-5774
E-mail: roceddia@yorku.ca

Abstract

The ketogenic diet (KD), known for its high-fat, low-carbohydrate composition, has gained popularity for weight loss and metabolic health benefits. Despite these advantages, there are concerns that the diet's high saturated fat content might elevate cholesterol levels and cardiovascular disease (CVD) risk. This study investigates the KD's impact on the molecular mechanisms of cholesterol metabolism in the liver, focusing on cholesterol synthesis markers such as 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) and sterol regulatory element-binding protein-2 (SREBP-2), as well as cholesterol uptake markers including proprotein convertase subtilisin/kexin type 9 (PCSK9) and LDL receptors (LDLr). For that, male Wistar rats were assigned to three dietary groups: standard chow (SC, 60% carbohydrates, 13% fat, 27% protein), high-fat sucrose-enriched (HFS, 20% carbohydrates, 60% fat, 20% protein), and ketogenic diet (KD, 0% carbohydrates, 80% fat, 20% protein). Liver tissue was extracted and analyzed for gene expression using real-time PCR and protein content using western blotting. Blood samples were collected to measure circulating cholesterol levels. We found that neither plasma cholesterol levels nor HMG-CoA reductase and SREBP-2 levels in the liver differed among the dietary interventions. However, the KD significantly reduced liver PCSK9 content and expression in comparison to other diets, suggesting that the KD enhanced clearance of circulating cholesterol by the liver. To test whether there was a higher amount of LDLr on the membrane compared to the cytoplasm, the ratio of LDLr distribution between these compartments was measured. Although a higher availability of LDLr on the membrane was found under KD, it did not reach statistical significance. In conclusion, the KD altered key steps that regulate hepatic cholesterol metabolism and prevented plasma cholesterol levels from increasing, despite its elevated saturated fat content.

Introduction

Cholesterol is a lipid molecule essential for various biological processes, including the production of sex hormones, vitamin D, and bile acids [1,2]. This critical lipid can be obtained either through dietary sources or synthesized endogenously via a complex pathway in the liver [3,48,123]. The latter primarily takes place in the endoplasmic reticulum of hepatocytes (ER)[123], where the enzyme HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate, a precursor in cholesterol[122]. However, excessive amounts of cholesterol found in low density-lipoprotein cholesterol (LDL-C) have raised concerns due to their association with health issues such as atherosclerosis and heart disease [5,6]. To manage this risk, pharmacological interventions have been developed to regulate cholesterol biosynthesis in the liver. Statins, one of the main agents used [8], inhibit HMG-CoA reductase to suppress endogenous cholesterol production[93,124]. Despite their effectiveness, statins are associated with side effects, including myopathy [125]. A study on skeletal muscle-specific HMG-CoA reductase knockout mice revealed that the lack of HMG-CoA reductase enzyme activity led to severe myopathy and necrosis in muscle tissue, it surprisingly did not affect plasma cholesterol levels[125]. Alternatively, dietary strategies are employed as safe and effective approaches to lower cholesterol levels. In the 1950s, Ancel Keys proposed that increased intake of saturated fat raises cholesterol levels, thereby increasing the risk of CVD [97]. Building on this hypothesis, guidelines have recommended reducing fat consumption, particularly saturated fats, and promoting diets rich in fruits, vegetables, and whole grains to lower cholesterol levels and reduce CVD risk [97,98]. Contrary to this approach, the KD has garnered significant attention for its potential health benefits [6,126]. This diet is characterized by high fat, low carbohydrate (maximum 50g/day), and moderate protein intake [101]. Studies indicate that the KD reduces risk factors for CVD such as obesity, type II diabetes, and dyslipidemia [6,11]. Yet, there are still concerns regarding

its impact on LDL-C levels [127]. Several studies have reported controversial results regarding the effect of the KD on cholesterol metabolism, with some studies reporting cholesterol levels being elevated[13], whereas in others cholesterol levels are either reduced[14] or remain unaltered[15] . To better understand this discrepancy, it is important to examine the molecular mechanisms involved in cholesterol synthesis, particularly in the liver, which produces the majority of daily cholesterol. The endogenous cholesterol production, occurring in the ER of hepatocytes, involves a complex series of over 30 enzymatic reactions[3,123]. Among these, the enzyme HMG-CoA reductase plays a pivotal role in cholesterol synthesis[3,61,77]. The latter is an ER-localized glycoprotein that functions as the rate-limiting enzyme for cholesterol biosynthesis[61]. The activity of HMG-CoA reductase directly influences the amount of cholesterol synthesized in the liver. When sufficient cholesterol accumulates, the activity of HMG-CoA reductase is suppressed through a negative feedback mechanism[3,59]. Conversely, low cholesterol levels trigger the activation of the transcription factor SREBP-2, which then enhances the activity of HMG-CoA reductase to increase cholesterol synthesis. Therefore, SREBP-2 is a crucial regulator of cholesterol synthesis in the liver [62–64]. Beyond its role in cholesterol synthesis, SREBP-2 also regulates key markers involved in cholesterol uptake, such as LDLr and PCSK9, underscoring its significant impact on cholesterol management [62–64]. The LDLr is responsible for the clearance of LDL-C from the bloodstream [73]. However, when there is high amount of hepatic cholesterol, a protein known as PCSK9 primarily synthesized and secreted by the liver, binds to LDLr located on the plasma membrane of hepatocytes[77,128]. Following this binding event, the PCSK9-LDLr complex is internalized and degraded by lysosomes [77,78]. Therefore, with fewer LDLr available for LDL particle binding and internalization, there is a consequent increase in plasma LDL-C levels [77]. Currently, most studies on the KD have not addressed its specific metabolic effects on hepatic cholesterol metabolism. To

further understand these mechanisms, we conducted an experiment where male Wistar rats were fed three different diets for 16 weeks: SC, HFS, and KD.

Material and methods

Reagents – FA-free bovine serum albumin (BSA), Protease (cOmplete Ultra Tablets) and phosphatase (PhosSTOP) inhibitors obtained from Roche Diagnostics GmbH (Mannheim, Germany). Cholesterol Assay Kit - HDL and LDL/VLDL was purchased from Abcam (CAT# ab65390). The Subcellular Protein Fractionation Kit for Tissues (CAT#87790) was purchased from Thermofisher Scientific. **Antibodies** – The following antibodies were used in this study: β-actin (CAT#4967S) and NA/K-ATPase: (CAT#3010S) were purchased from Cell signalling. Proprotein convertase subtilisin/kexin type 9 (PCSK9, CAT#AB31762), LDL receptor (LDLr, CAT #AB30532), Sterol regulatory element-binding protein 2 (SREBP-2, CAT #AB30682) were purchased from Abcam. HMG-CoA reductase (CAT #ARC0496), was purchased from Invitrogen.

Animals and diet – Male albino rats of the Wistar strain, weighing approximately 250g, were housed at a temperature of 22°C with a 12/12-hour light/dark cycle. These rats were divided into three groups: one group received a standard laboratory chow diet consisting of 27% protein, 13% fat, and 60% carbohydrates (Lab Diet Cat #5012), a high-fat-sucrose (HFS) diet consisting of 20% protein, 60% fat, and 20% carbohydrates (Research Diets, Cat #D12492), and a ketogenic diet (KD) consisting of 20.0% protein, 80.0% fat, and 0.0% carbohydrates (Research Diets Cat #D03022101). At the end of the 16-week feeding period, the animals were anesthetized, and tissues were collected for use in experiments. In addition, blood samples were collected from all fed animals between 15:00 and 16:00h through saphenous vein bleeding. Plasma Beta-hydroxybutyrate levels for each diet are as follow: SC: 0.15 ± 0.01, HFS :0.15 ± 0.02 and KD

0.29 ± 0.02 mM. The animal procedures outlined in this proposal received specific approval from the Committee on the Ethics of Animal Experiments at York University (York University Animal Care Committee, YUACC), with permit number 2016-5. These procedures were carried out strictly in accordance with the guidelines set forth by YUACC. All surgical procedures were conducted under ketamine/xylazine anesthesia, and every effort was made to minimize any discomfort or suffering experienced by the animals.

Quantitative PCR analysis – Liver samples were placed in liquid nitrogen and stored at -80°C until RNA isolation. Afterward, RNA extraction was performed using TRIzolTM (ThermoFisher Scientific, Waltham, MA, USA), and the RNA was dissolved in sterile water. Complementary DNA (cDNA) synthesized from 2 µg of RNA following the manufacturer's instructions, utilizing the ABM EasyScriptTM Reverse Transcriptase cDNA Synthesis kit (Diamed, Mississauga, ON, Canada). For real-time PCR, specific primers were previously designed using PrimerQuest software (IDT), based on probe sequences accessible in the Affymetrix database (NetAffx™ Analysis Centre, http://www.affymetrix.com/analysis) for each target gene. The samples were ran in duplicates on a 96-well plate, with each 20 µl reaction containing 4 µl of cDNA, 0.4 µl of primer, 10 µl of Brightgreen 2x qPCR Master mix (Diamed, Mississauga, ON, Canada), and 5.6 µl of RNA-free water. The qPCR analysis was conducted using a Bio-Rad CFX96 Real Time PCR Detection System (Bio-Rad, Mississauga, ON, Canada) with the following amplification conditions: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 65°C for 1 minute, with a gradual temperature increment from 65°C to 95°C by 0.5°C steps. All gene expressions were normalized to the housekeeping gene β-actin or TBP, and the relative variations in gene expression between different treatment groups determined using the ΔΔCt method[129]. The results presented as fold changes

relative to the control (Con) group. The primers for PCSK9, LDLr, SREBP-2 and HMG-CoA Reductase are listed in Table 1.

Subcellular fractionation of liver tissue – For the separation of cytoplasmic and membrane fractions, a protein fractionation kit from Thermo Fisher was used. Briefly, 200 mg liver tissue homogenized using cytoplasmic extraction buffer. The homogenate was then transferred to a Pierce Tissue Strainer. Following centrifugation of the strainer, the supernatant, containing the cytoplasmic extract, was transferred into a new tube. Ice-cold membrane extraction buffer was added to the remaining pellet, which was then subjected to another round of centrifugation. The resulting supernatant containing the membrane extract was collected into a separate tube and kept on ice. For immediate use, both fractions were maintained on ice for downstream applications and analysis. For long-term storage, the fractions were stored at -80°C.

Western blotting – Liver samples were extracted, snap frozen in liquid nitrogen, and the tissues preserved in a freezer set at -80 degrees Celsius for future utilization. Tissues were homogenized with 350 µl of lysis buffer containing 135 mM NaCl, 1 mM $MgCl_2$, 2.7 mM KCl, 20 mM Tris, 1% Triton X-100, 10% glycerol, protease (cOmplete Ultra Tablets) and phosphatase (PhosSTOP) inhibitors obtained from Roche Diagnostics GmbH (Mannheim, Germany). Then, the homogenized samples were centrifuged for 10 minutes at 4°C, resulting in the separation of the supernatant, which then were transferred to a separate microtube to measure protein concentration using Bradford assay. The samples were diluted 1:1 in 2× Laemmli then heated at 95°C for 5 min and subject to SDS-PAGE. Twenty-five µg of protein from each liver sample was loaded on to gels and subjected to SDS-PAGE, transferred to PVDF membrane. The samples were probed with antibodies at a ratio of 1:1000, and β-actin serve as the loading control. Densitometry analysis was performed using the Scion Image software.

Statistical Analysis – Statistical analyses were conducted by using One-way ANOVAs with Bonferroni post-hoc tests using the GraphPad Prism Version 9.4.0 statistical analysis program. Statistical significance was set at $p<0.05$. Bars represent mean ± SD.

Results

Effect of the KD on HDL, LDL+VLDL, and total cholesterol levels in the plasma – Across the SC, HFS, and KD groups, cholesterol levels, including HDL (Fig. 9A), LDL (Fig. 9B), and total cholesterol (Fig. 9C), showed no significant variation.

Effects of the KD on SREBP-2 and HMG-CoA reductase expression and protein content – Gene expression analysis revealed that SREBP-2 expression increased by 1.7-fold in the KD group compared to the SC group (Fig. 10A). However, no significant difference in SREBP-2 gene expression was observed between the HFS and KD groups (Fig. 10 A). Despite differences in SREBP-2 gene expression, the protein content of this transcription factor did not differ among the three dietary interventions (Fig. 10B). Similarly, HMG-CoA reductase expression was significantly reduced by 50% in KD compared to SC (Fig. 10C), whereas the protein content remained unchanged among the three dietary groups (Fig.10D).

Effect of the KD on PCSK9 and LDLr gene expression and protein content – After observing no significant changes in proteins involved in cholesterol synthesis, it became important to examine the molecular machinery involved in the cholesterol uptake pathway. Interestingly, content and gene expression of PCSK9 reduced by 50% under KD, but not in HFS fed rats in comparison to SC (Fig. 11A, B respectively). Despite the significant reduction in PCSK9 under the KD, protein

as well as the mRNA levels of total LDLr remained unchanged (Fig. 11 C, D). Similarly, no changes were observed in HFS-fed rats compared to the SC group.

Cellular localization of LDLr in the liver and its ratio between membrane and cytoplasm – Although there was a slight decrease in cytoplasmic LDLr levels in both the HFS and KD groups (Fig. 13A), this reduction did not reach statistical significance. Similarly, a trend towards increased LDLr levels on the cell membrane was observed, but this change was not significant (Fig. 13B). Given the dynamic nature of LDLr, which continuously recycles between the cell membrane and cytoplasm, we assessed its distribution ratio between these two compartments. While no changes were observed in the HFS group, there was a tendency for higher LDLr levels on the membrane in the KD group, though this increase did not reach statistical significance (Fig. 13C).

Discussion

The main finding of this research was that even though the content of saturated fat in the KD was much higher than the SC and the typical obesogenic HFS diet, none of the circulating lipids (cholesterol, LDL-C, VLDL, and HDL) differed among the groups. However, the molecular mechanisms involved in cholesterol metabolism in the liver were significantly affected by diet composition. This was evident in the diets that were rich in saturated fat such as the HFS and the KD, suggesting that the diet-specific effects on SREBP-2, HMG-CoA reductase, LDLr, and PCSK9 allowed the liver to manage cholesterol metabolism in a way that plasma levels of this lipid remained unchanged among the different dietary interventions. Thus, at least in rats, the main concern that the high content of saturated fat in a KD could be atherogenic is not supported by our data.

Because HMG-CoA reductase has a crucial role in endogenous cholesterol synthesis in the liver [59], we expected that a diet rich in saturated fat such as the HFS and the KD would increase cholesterol levels in the liver. This, in turn, would suppress the activity of HMG-CoA reductase in hepatocytes based on demonstrations that the expression of this enzyme would be downregulated as intracellular cholesterol levels rise [59] . In this context, we indeed found that the gene expression of HMG-CoA reductase was reduced in the KD, although the protein content was not significantly altered in liver samples from all dietary interventions, regardless of their saturated fat content. Importantly, although the KD was devoid of carbohydrate, it contained the highest amount of saturated fat. This was also accompanied by no alteration in levels of HDL, LDL/VLDL, and total cholesterol in rats under the KD. This is at odds with the tenets of the diet-heart hypothesis stating that saturated fat intake increases cholesterol levels, LDL-C in particular, and ultimately elevates the risk of CVD [130]. These tenets have served as the basis to support dietary guidelines that advise reducing saturated fat intake[130]. Contrary to previous assumptions [99], research has shown that saturated fat intake does not correlate with an increased risk of myocardial infarction or CVD mortality [98,99]. In fact, it appears that it is the size of LDL particles that plays a critical role in elevating CVD risk [131]. In this context, it has been reported that small, dense LDL (sdLDL) lipoproteins are more prone to oxidation, which renders these particles unable to bind LDLr and make macrophages to engulf them more avidly and turn into foam cells[39,41,106]. This latter event seems to be critical for the development of atherosclerotic lesions[39] . Conversely, findings from a study on men initially free from ischemic heart disease (IHD) reveal that large buoyant LDL (lbLDL) particles are not linked to increased IHD risk. Instead, cardiovascular risk was more closely associated with the presence of sdLDL particles [107]. Interestingly, in studies reporting KD-induced elevation of LDL levels have attributed this effect to a higher presence of lbLDL than the

ldLDL particles in the circulation [112], which may exert a protective effect against atherogenesis. Notably, in this study we did not measure LDL particle size. Thus, it is possible that even though no significant differences existed between HFS and KD with respect to total plasma LDL levels, these dietary interventions could have affected the size distribution of these particles. Additionally, evidence suggests that higher intakes of saturated fatty acids, which are less prone to oxidation, are associated with an increase in LDL particle size compared to diets lower in saturated fat [132,133].

Transcriptional regulation of HMG-CoA reductase is primarily influenced by the activity of SREBP-2[61]. The latter undergoes several steps in order to be activated[62,67,68]. In its quiescent state, SREBP-2 is located in ER. To initiate the activation process of SREBP-2, SCAP serves as chaperone to facilitate the travel of this transcription factor to the Golgi[62,67,68]. Subsequently, SREBP-2 is cleaved and undergoes translocation to the nucleus where it promotes the expression of genes such as HMG-CoA reductase, PCSK9, and LDLr [62,67,68]. In this study, neither LDLr nor PCSK9 protein levels significantly differed among the dietary interventions, although we found that SREBP-2 gene expression was higher in livers from KD- than SC- and HFS-fed rats. This apparent discrepancy could be due to differences in post-translational modifications that led to unaltered levels of SREBP-2 in the tissue. Moreover, because we did not assess the intracellular localization of SREBP-2, we cannot discard different levels of activity of this transcription factor under dietary conditions that affect the abundance of cellular cholesterol.

PCSK9 is an important protein involved in regulating cholesterol levels [77]. It binds to LDLr on liver cells, leading to the degradation of these receptors [77]. This reduces the liver's ability to remove LDL cholesterol from the blood, thereby increasing circulating LDL cholesterol levels[77]. While PCSK9 is primarily regulated by SREBP-2, the observed reduction in PCSK9 under the KD suggests broader metabolic changes that align with existing literature. In fact, it has been reported

that PCSK9 induction is often associated with increased hepatocellular triglyceride content and hepatic lipogenesis[134]. Insulin is likely the most important hormonal factor influencing lipogenesis[135], particularly with respect to the process by which the body converts excess carbohydrates into fatty acids and stores them as fat[136]. Supporting this, a study demonstrated that PCSK9 mRNA levels decreased by 73% in mice after 24 hours of fasting, resulting in a twofold reduction in protein levels[137]. However, when the mice were refed a high-carbohydrate diet, PCSK9 expression was restored[137]. This significant decrease in PCSK9 during fasting may reflect lower insulin levels and a shift of metabolism away from lipogenesis and towards fat utilization. In line with this, the drastic reduction in carbohydrate intake under a low-carbohydrate, high-fat diet leads to a significant metabolic shift[138]. This shift is underlined by activation of adipose tissue lipolysis and β-oxidation in liver and skeletal muscles, resulting in an overall increased reliance on fat for fuel as opposed to glucose[138]. Indeed, as reported by Jani et al., the KD induced a metabolic shift that prioritized fatty acid oxidation and reduced lipogenesis in hepatocytes[115]. The latter can also support the significant reduction in PCSK9 observed in our study. Research on the impact of diet interventions, particularly the KD, on markers involved in cholesterol metabolism is still relatively scarce. In our study, where we implemented a diet high in fat and devoid of carbohydrates, we observed a notable reduction in PCSK9 levels. This finding contrasts with another study conducted on male Wistar rats that were fed a western diet (40% fat, 35% sucrose (17.5% fructose)) for 2 and 6 weeks [139]. The study reported significant decreases in the expression of key liver cholesterol metabolism markers, specifically LDLr, PCSK9, and SREBP-2. The reductions in PCSK9 and LDLr were attributed to the lowered expression of SREBP-2 and ultimately increased cholesterol levels in the circulation under the western diet[139]. In this study, a significant reduction in PCSK9 levels was observed, despite no changes in the cholesterol

synthesis pathway. This suggests that the liver may be increasing its uptake of cholesterol from the bloodstream under the KD. However, even with this notable reduction in PCSK9, there were no significant changes in total LDLr levels across dietary interventions. This seems at odds with the role PCSK9 in down-regulating LDLr availability at the cell membrane [77]. However, it could be that a higher rate of LDLr recycling between cytoplasm and membrane could be taking place under KD conditions [72]. To further investigate this possibility, we performed subcellular fractionation to determine the ratio of LDLr between the cell membrane (memb) and the cytoplasm (cyto). We found that the LDLr memb/cyto ratio was higher under the KD, although not statistically significant. Thus, it appears that the KD enhances liver ability to clear circulating LDL-C. The lack of statistical significance could be due to the limited number of animals that underpowered the results and the inherent variability in the data. Thus, further studies are required to address these issues.

To conclude, despite concerns regarding high saturated fat content of the KD causing dyslipidemia and increasing CVD risk, we observed no significant change in LDL/VLDL and HDL plasma levels. This outcome aligns with the unchanged hepatic levels of cholesterol synthesis markers like SREBP-2 and HMG-CoA reductase. Furthermore, the KD did not alter total LDLr levels in the liver, despite causing a significant reduction in PCSK9 levels in this organ. However, we found a trend towards higher rates of LDLr memb/cyto recycling in the liver of KD-fed rats, which suggests that this dietary intervention enhances the ability of hepatocytes to uptake cholesterol. Therefore, even though the saturated fat content of the KD was much higher than the SC and the typical obesogenic HFS diet, in our rodent model the liver managed cholesterol metabolism in a way that plasma levels of this lipid remained unchanged among the different dietary interventions.

Figures and table

Table 1: *Primer sequences used for qPCR analysis.*

Gene	Forward	Reverse
PCSK9	CCTCATAGGCCTGGAGTTTATTC	CAGTGTTAAGGATCCGGCTATAC
LDLr	CACATCTGCAAGGACCTCAA	GCTCCTGACACTCGTCAATATC
SREBP-2	AGGTCTAGGGATGGGTGAAA	GTGGGAAGGAACAGGACAATTA
HMG-CoA reductase	GTAACTGGAGAGTGCAGAGAAAG	CAGTTTGTAGGCTGGGATGT
β-actin	GTGGAAAAGATGACCCAGATC	CACCGCCTGGATGGCTACGT
Tbp	TACAGGTGGCAGCATGAAGTGACA	AACCAACAATCACCAGCAGCAGTG

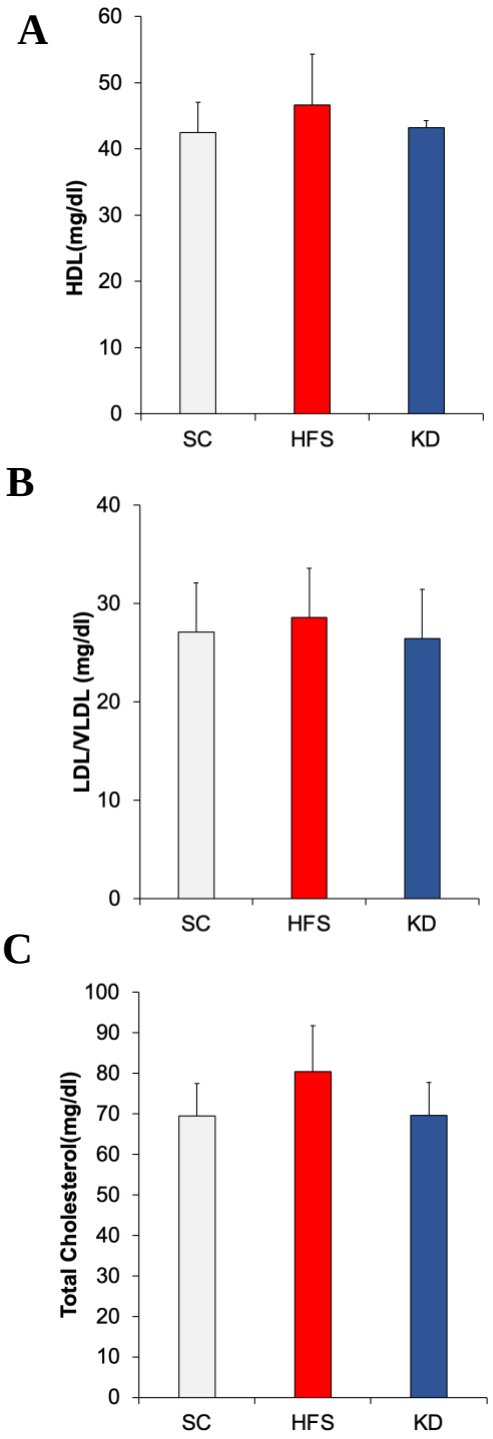

Figure 9. The effects of SC, HFS and KD on HDL (A), LDL/VLDL (B) and total cholesterol (C). There was no significant difference among three different dietary interventions. One-way ANOVAs were used to compare groups. n=5-6 rats. Bars represent mean + SD.

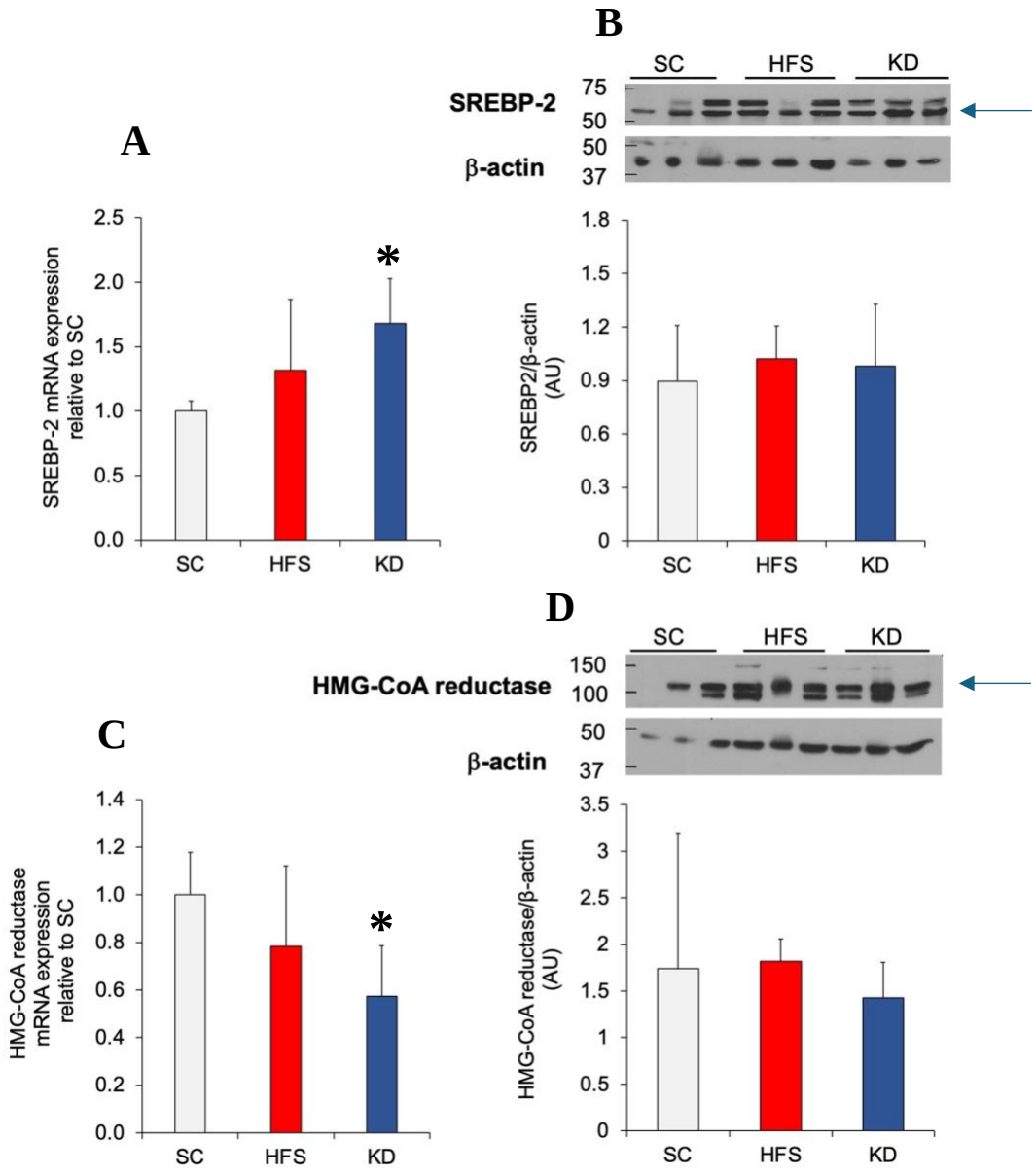

Figure 10. KD increased SREBP-2 (A) and reduced HMG-CoA reductase (C) mRNA expression. However, this was accompanied by no alteration in protein content levels in both SREBP-2 and HMG-CoA reductase (B and D respectively). The HFS diet maintained unaltered liver mRNA expressions and protein levels of SREBP-2 (A and B) and HMG-CoA reductase (C and D). *p<0.05 vs. SC. One way ANOVA was used to compare groups. n=5-6 rats. Bars represent mean ± SD. A.U., arbitrary units.

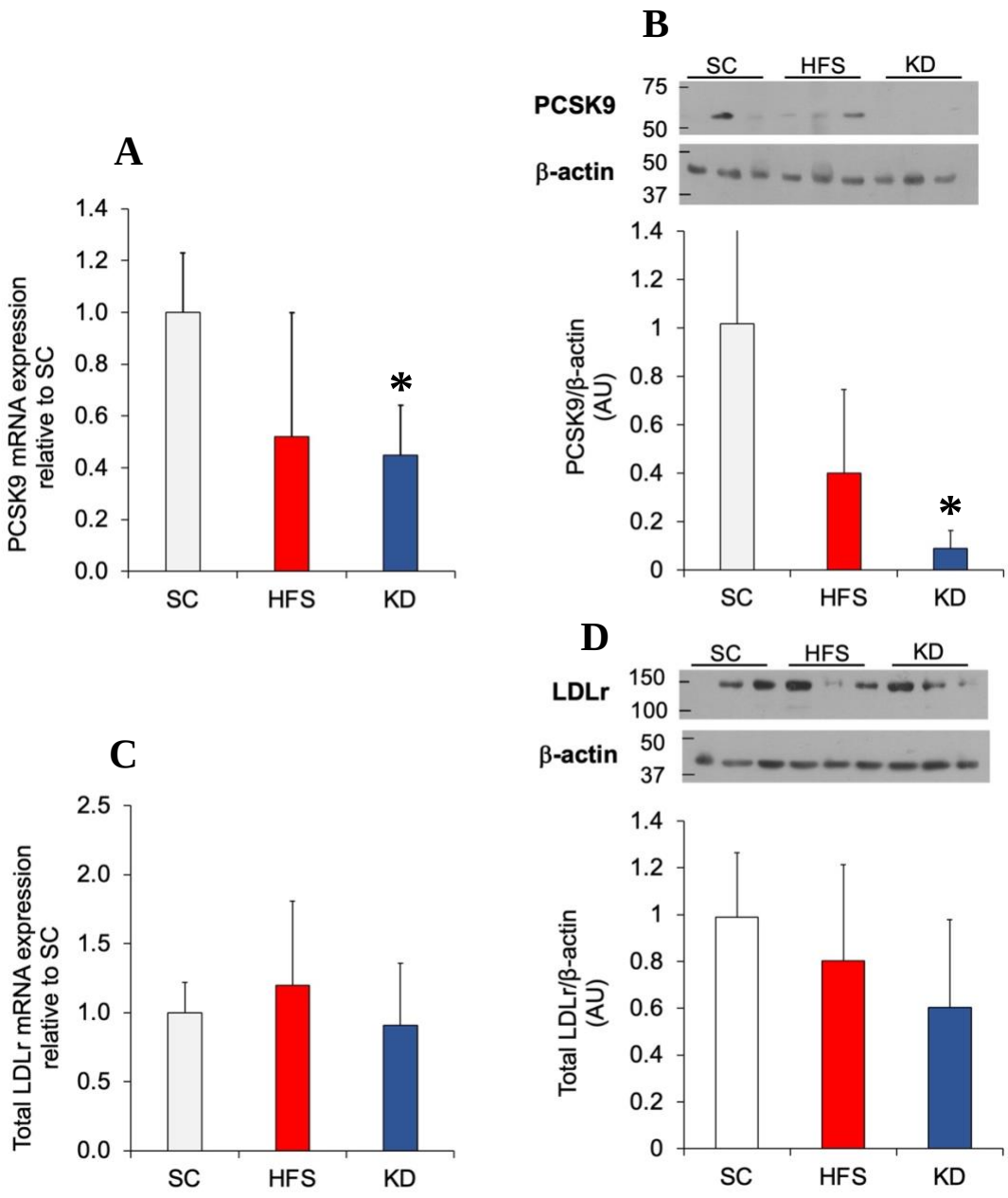

Figure 11. The mRNA and protein content levels of PCSK9 under HFS did not change compared to SC (A and B respectively). However, both mRNA expression (A) and content (B) of PCSK9 downregulated by 50% with KD in comparison to SC and HFS. Contrary to reduction in PCSK9 levels under the KD, total LDLr levels remained unaltered irrespective of the diet (C, D). *$p<0.05$ vs. SC. One way ANOVA was used to compare groups. n=5-6 rats. Bars represent mean ± SD. A.U., arbitrary units.

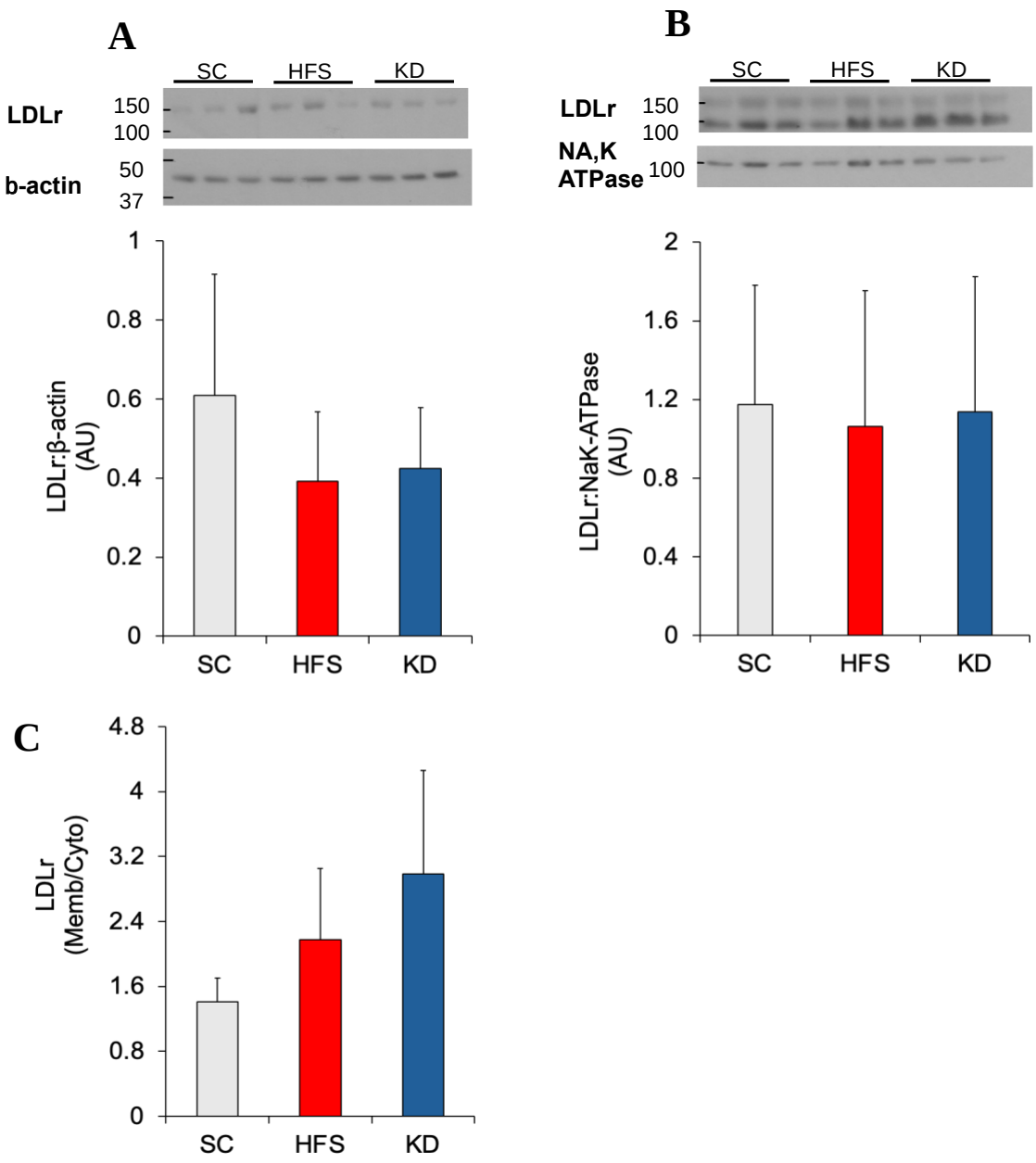

Figure 12. The levels of LDLr in cytoplasm (A) and membrane (B) remained the same among the groups. Although the ratio of distribution in membrane increased 40% under the KD (C), it was not statistically significant. Representative blots and densitometric analyses of levels in cytoplasmic and membrane cellular fractions were corrected by loading controls β-actin and Na, K-ATPase, respectively. AU = arbitrary units. Mean ± SD. One-way ANOVA was used to compare groups. n=5-6 rats.

Limitations of the study

In this study, we aimed to identify key mechanisms involved in hepatic cholesterol metabolism influenced by HFS and KD, focusing on how these diets regulate crucial markers involved in cholesterol synthesis and uptake by the liver. Although we observed no significant changes in SREBP-2 and HMG-CoA reductase protein levels, we did detect significant alterations in their gene expression under the KD. However, a limitation of our study is that we did not measure proteins involved in the activation of SREBP-2 before it enters the nucleus, such as Insig1, which binds to SREBP-2 in the endoplasmic reticulum in response to high cholesterol levels, and SCAP, which chaperones SREBP-2 to the Golgi complex. Including these measurements would have provided a more comprehensive understanding of the regulatory processes affecting cholesterol metabolism in response to dietary interventions.

Our study measured both total LDLr levels and their distribution between the membrane and cytoplasm. While we observed that the ketogenic diet increased LDLr levels on the membrane compared to the cytoplasm, a larger sample size could have minimized variability and enhanced the statistical significance of these findings.

Future directions

Our study provides evidence by demonstrating that saturated fat intake did not affect LDL-C levels. This finding challenges the conventional understanding that high saturated fat intake directly increases LDL-C, a marker often linked to cardiovascular risk. Given the complexity of cholesterol metabolism, it is recognized that LDL-C levels alone do not fully capture cardiovascular risk. Instead, the size and density of LDL particles may play a more significant role,

with smaller, denser LDL particles being more atherogenic. Considering this, it is essential to assess LDL-C particle size and distribution under the KD. Such an investigation could help clarify whether the unchanged LDL-C levels observed in our study are accompanied by shifts in LDL particle size or type. Moreover, future research should focus on exploring the effects of KD on hepatic cholesterol metabolism in female Wistar rats. It is crucial to investigate whether the findings observed in male rats translate to females. Specifically, future studies should assess markers involved in both cholesterol synthesis and uptake, such as SREBP-2, PCSK9, and LDLr in female rats.

Another potential direction for future research would be to examine PCSK9 levels in the blood, which we were unable to measure in the current study due to the large blood volume required by the available assay kits. By assessing circulating PCSK9, future studies could determine whether the reductions in hepatic PCSK9 observed in our study are mirrored in the bloodstream, offering a more comprehensive understanding of how KD influences cholesterol metabolism. These proposed future directions carry significant implications for understanding hepatic cholesterol metabolism and the broader impacts of KD.

References

1. Cortes VA, Busso D, Maiz A, Arteaga A, Nervi F, Rigotti A. Physiological and pathological implications of cholesterol. *Front Biosci (Landmark Ed)*. 2014;19(3):416-428. doi:10.2741/4216
2. Chiang JYL, Ferrell JM, Wu Y, Boehme S. Bile Acid and Cholesterol Metabolism in Atherosclerotic Cardiovascular Disease and Therapy. *Cardiol Plus*. 2020;5(4):159-170.
3. Duan Y, Gong K, Xu S, Zhang F, Meng X, Han J. Regulation of cholesterol homeostasis in health and diseases: from mechanisms to targeted therapeutics. *Signal Transduct Target Ther*. 2022;7(1):265. doi:10.1038/s41392-022-01125-5
4. Das P, Ingole N. Lipoproteins and Their Effects on the Cardiovascular System. *Cureus*. Published online November 15, 2023. doi:10.7759/cureus.48865
5. Soliman GA. Dietary Cholesterol and the Lack of Evidence in Cardiovascular Disease. *Nutrients*. 2018;10(6). doi:10.3390/nu10060780
6. Dowis K, Banga S. The Potential Health Benefits of the Ketogenic Diet: A Narrative Review. *Nutrients*. 2021;13(5). doi:10.3390/nu13051654
7. Teicholz N. A short history of saturated fat: the making and unmaking of a scientific consensus. *Curr Opin Endocrinol Diabetes Obes*. 2023;30(1):65-71. doi:10.1097/MED.0000000000000791
8. Alsheikh-Ali AA, Maddukuri P V, Han H, Karas RH. Effect of the magnitude of lipid lowering on risk of elevated liver enzymes, rhabdomyolysis, and cancer: insights from large randomized statin trials. *J Am Coll Cardiol*. 2007;50(5):409-418. doi:10.1016/j.jacc.2007.02.073
9. Di Stasi SL, MacLeod TD, Winters JD, Binder-Macleod SA. Effects of statins on skeletal muscle: a perspective for physical therapists. *Phys Ther*. 2010;90(10):1530-1542. doi:10.2522/ptj.20090251
10. Tomaszewski M, Stępień KM, Tomaszewska J, Czuczwar SJ. Statin-induced myopathies. *Pharmacol Rep*. 2011;63(4):859-866. doi:10.1016/s1734-1140(11)70601-6
11. Salas Noain J, Minupuri A, Kulkarni A, Zheng S. Significant Impact of the Ketogenic Diet on Low-Density Lipoprotein Cholesterol Levels. *Cureus*. 2020;12(7):e9418. doi:10.7759/cureus.9418
12. Harvey KL, Holcomb LE, Kolwicz SC. Ketogenic Diets and Exercise Performance. *Nutrients*. 2019;11(10). doi:10.3390/nu11102296
13. Schmidt T, Harmon DM, Kludtke E, Mickow A, Simha V, Kopecky S. Dramatic elevation of LDL cholesterol from ketogenic-dieting: A Case Series. *Am J Prev Cardiol*. 2023;14:100495. doi:10.1016/j.ajpc.2023.100495
14. Gershuni VM, Yan SL, Medici V. Nutritional Ketosis for Weight Management and Reversal of Metabolic Syndrome. *Curr Nutr Rep*. 2018;7(3):97-106. doi:10.1007/s13668-018-0235-0
15. Goldberg IJ, Ibrahim N, Bredefeld C, et al. Ketogenic diets, not for everyone. *J Clin Lipidol*. 2021;15(1):61-67. doi:10.1016/j.jacl.2020.10.005
16. Walton CM, Perry K, Hart RH, Berry SL, Bikman BT. Improvement in Glycemic and Lipid Profiles in Type 2 Diabetics with a 90-Day Ketogenic Diet. *J Diabetes Res*. 2019;2019:8681959. doi:10.1155/2019/8681959
17. Masood I, Noreen S, Raza K, Khalid W, Rahim MA, Mohamedahmed KA. Effect of ketogenic diet and hypocaloric Mediterranean diet on metabolic and endocrine parameter

in women suffering from Polycystic Ovary Syndrome. *Int J Food Prop*. 2023;26(2):3187-3196. doi:10.1080/10942912.2023.2275528
18. Naveh N, Avidan Y, Zafrir B. Extreme Hypercholesterolemia Following a Ketogenic Diet: Exaggerated Response to an Increasingly Popular Diet. *Cureus*. 2023;15(8):e43683. doi:10.7759/cureus.43683
19. Doole FT, Kumarage T, Ashkar R, Brown MF. Cholesterol Stiffening of Lipid Membranes. *J Membr Biol*. 2022;255(4-5):385-405. doi:10.1007/s00232-022-00263-9
20. Subczynski WK, Pasenkiewicz-Gierula M, Widomska J, Mainali L, Raguz M. High Cholesterol/Low Cholesterol: Effects in Biological Membranes: A Review. *Cell Biochem Biophys*. 2017;75(3-4):369-385. doi:10.1007/s12013-017-0792-7
21. Miller WL, Bose HS. Early steps in steroidogenesis: intracellular cholesterol trafficking. *J Lipid Res*. 2011;52(12):2111-2135. doi:10.1194/jlr.R016675
22. Zhang J, Li Q, Wu Y, et al. Cholesterol content in cell membrane maintains surface levels of ErbB2 and confers a therapeutic vulnerability in ErbB2-positive breast cancer. *Cell Commun Signal*. 2019;17(1):15. doi:10.1186/s12964-019-0328-4
23. Chiang JYL, Ferrell JM, Wu Y, Boehme S. Bile Acid and Cholesterol Metabolism in Atherosclerotic Cardiovascular Disease and Therapy. *Cardiol Plus*. 2020;5(4):159-170.
24. Li T, Chiang JYL. Regulation of bile acid and cholesterol metabolism by PPARs. *PPAR Res*. 2009;2009:501739. doi:10.1155/2009/501739
25. Schmidt DR, Holmstrom SR, Fon Tacer K, Bookout AL, Kliewer SA, Mangelsdorf DJ. Regulation of Bile Acid Synthesis by Fat-soluble Vitamins A and D. *Journal of Biological Chemistry*. 2010;285(19):14486-14494. doi:10.1074/jbc.M110.116004
26. Duarte Lau F, Giugliano RP. Lipoprotein(a) and its Significance in Cardiovascular Disease: A Review. *JAMA Cardiol*. 2022;7(7):760-769. doi:10.1001/jamacardio.2022.0987
27. Gugliucci A. The chylomicron saga: time to focus on postprandial metabolism. *Front Endocrinol (Lausanne)*. 2023;14:1322869. doi:10.3389/fendo.2023.1322869
28. Gugliucci A. The chylomicron saga: time to focus on postprandial metabolism. *Front Endocrinol (Lausanne)*. 2023;14:1322869. doi:10.3389/fendo.2023.1322869
29. Behbodikhah J, Ahmed S, Elyasi A, et al. Apolipoprotein B and Cardiovascular Disease: Biomarker and Potential Therapeutic Target. *Metabolites*. 2021;11(10). doi:10.3390/metabo11100690
30. Julve J, Martín-Campos JM, Escolà-Gil JC, Blanco-Vaca F. Chylomicrons: Advances in biology, pathology, laboratory testing, and therapeutics. *Clin Chim Acta*. 2016;455:134-148. doi:10.1016/j.cca.2016.02.004
31. Giammanco A, Cefalù AB, Noto D, Averna MR. The pathophysiology of intestinal lipoprotein production. *Front Physiol*. 2015;6:61. doi:10.3389/fphys.2015.00061
32. Chait A, Ginsberg HN, Vaisar T, Heinecke JW, Goldberg IJ, Bornfeldt KE. Remnants of the Triglyceride-Rich Lipoproteins, Diabetes, and Cardiovascular Disease. *Diabetes*. 2020;69(4):508-516. doi:10.2337/dbi19-0007
33. Krauss RM, King SM. Remnant lipoprotein particles and cardiovascular disease risk. *Best Pract Res Clin Endocrinol Metab*. 2023;37(3):101682. doi:10.1016/j.beem.2022.101682
34. Huang JK, Lee HC. Emerging Evidence of Pathological Roles of Very-Low-Density Lipoprotein (VLDL). *Int J Mol Sci*. 2022;23(8). doi:10.3390/ijms23084300
35. Zhang D, Wei Y, Huang Q, et al. Important Hormones Regulating Lipid Metabolism. *Molecules*. 2022;27(20). doi:10.3390/molecules27207052

36. Lee HC, Akhmedov A, Chen CH. Spotlight on very-low-density lipoprotein as a driver of cardiometabolic disorders: Implications for disease progression and mechanistic insights. *Front Cardiovasc Med*. 2022;9:993633. doi:10.3389/fcvm.2022.993633
37. Martin SS, Qasim AN, Mehta NN, et al. Apolipoprotein B but not LDL cholesterol is associated with coronary artery calcification in type 2 diabetic whites. *Diabetes*. 2009;58(8):1887-1892. doi:10.2337/db08-1794
38. Ivanova EA, Myasoedova VA, Melnichenko AA, Grechko A V, Orekhov AN. Small Dense Low-Density Lipoprotein as Biomarker for Atherosclerotic Diseases. *Oxid Med Cell Longev*. 2017;2017:1273042. doi:10.1155/2017/1273042
39. Vekic J, Zeljkovic A, Cicero AFG, et al. Atherosclerosis Development and Progression: The Role of Atherogenic Small, Dense LDL. *Medicina (Kaunas)*. 2022;58(2). doi:10.3390/medicina58020299
40. Cho Y, Lee SG, Jee SH, Kim JH. Hypertriglyceridemia is a major factor associated with elevated levels of small dense LDL cholesterol in patients with metabolic syndrome. *Ann Lab Med*. 2015;35(6):586-594. doi:10.3343/alm.2015.35.6.586
41. Kotani K, Tsuzaki K, Sakane N, Taniguchi N. The Correlation Between Small Dense LDL and Reactive Oxygen Metabolites in a Physical Activity Intervention in Hyperlipidemic Subjects. *J Clin Med Res*. 2012;4(3):161-166. doi:10.4021/jocmr870w
42. Habib A, Mihas AA, Abou-Assi SG, et al. High-density lipoprotein cholesterol as an indicator of liver function and prognosis in noncholestatic cirrhotics. *Clin Gastroenterol Hepatol*. 2005;3(3):286-291. doi:10.1016/s1542-3565(04)00622-6
43. Kontush A, Lhomme M, Chapman MJ. Unraveling the complexities of the HDL lipidome. *J Lipid Res*. 2013;54(11):2950-2963. doi:10.1194/jlr.R036095
44. Kontush A, Lindahl M, Lhomme M, Calabresi L, Chapman MJ, Davidson WS. Structure of HDL: particle subclasses and molecular components. *Handb Exp Pharmacol*. 2015;224:3-51. doi:10.1007/978-3-319-09665-0_1
45. Ouimet M, Barrett TJ, Fisher EA. HDL and Reverse Cholesterol Transport. *Circ Res*. 2019;124(10):1505-1518. doi:10.1161/CIRCRESAHA.119.312617
46. Kontush A, Chapman MJ. Antiatherogenic small, dense HDL--guardian angel of the arterial wall? *Nat Clin Pract Cardiovasc Med*. 2006;3(3):144-153. doi:10.1038/ncpcardio0500
47. Law M. Plant sterol and stanol margarines and health. *BMJ*. 2000;320(7238):861-864. doi:10.1136/bmj.320.7238.861
48. Soliman GA. Dietary Cholesterol and the Lack of Evidence in Cardiovascular Disease. *Nutrients*. 2018;10(6). doi:10.3390/nu10060780
49. Poli A, Marangoni F, Corsini A, et al. Phytosterols, Cholesterol Control, and Cardiovascular Disease. *Nutrients*. 2021;13(8). doi:10.3390/nu13082810
50. Jones PJH, AbuMweis SS. Phytosterols as functional food ingredients: linkages to cardiovascular disease and cancer. *Curr Opin Clin Nutr Metab Care*. 2009;12(2):147-151. doi:10.1097/mco.0b013e328326770f
51. Berger A, Jones PJH, Abumweis SS. Plant sterols: factors affecting their efficacy and safety as functional food ingredients. *Lipids Health Dis*. 2004;3:5. doi:10.1186/1476-511X-3-5
52. Cohn JS, Kamili A, Wat E, Chung RWS, Tandy S. Dietary phospholipids and intestinal cholesterol absorption. *Nutrients*. 2010;2(2):116-127. doi:10.3390/nu2020116

53. Iqbal J, Hussain MM. Intestinal lipid absorption. *Am J Physiol Endocrinol Metab*. 2009;296(6):E1183-94. doi:10.1152/ajpendo.90899.2008
54. Park SW. Intestinal and hepatic niemann-pick c1-like 1. *Diabetes Metab J*. 2013;37(4):240-248. doi:10.4093/dmj.2013.37.4.240
55. Alves-Bezerra M, Cohen DE. Triglyceride Metabolism in the Liver. *Compr Physiol*. 2017;8(1):1-8. doi:10.1002/cphy.c170012
56. Trapani L. Regulation and deregulation of cholesterol homeostasis: The liver as a metabolic "power station." *World J Hepatol*. 2012;4(6):184. doi:10.4254/wjh.v4.i6.184
57. Rybarczyk A, Formanowicz D, Radom M, Formanowicz P. Cholesterol Metabolism Pathways Disturbances in Atherosclerosis—Analyses Using Stochastic Petri Net-Based Model. *Applied Sciences*. 2023;13(10):6149. doi:10.3390/app13106149
58. Pirahanchi Y, Sinawe H, Dimri M. *Biochemistry, LDL Cholesterol*.; 2023.
59. DeBose-Boyd RA. Feedback regulation of cholesterol synthesis: sterol-accelerated ubiquitination and degradation of HMG CoA reductase. *Cell Res*. 2008;18(6):609-621. doi:10.1038/cr.2008.61
60. Brown MS, Faust JR, Goldstein JL, Kaneko I, Endo A. Induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in human fibroblasts incubated with compactin (ML-236B), a competitive inhibitor of the reductase. *J Biol Chem*. 1978;253(4):1121-1128.
61. Luo J, Yang H, Song BL. Mechanisms and regulation of cholesterol homeostasis. *Nat Rev Mol Cell Biol*. 2020;21(4):225-245. doi:10.1038/s41580-019-0190-7
62. Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *Journal of Clinical Investigation*. 2002;109(9):1125-1131. doi:10.1172/JCI15593
63. Madison BB. Srebp2: A master regulator of sterol and fatty acid synthesis. *J Lipid Res*. 2016;57(3):333-335. doi:10.1194/jlr.C066712
64. Burke AC, Dron JS, Hegele RA, Huff MW. PCSK9: Regulation and Target for Drug Development for Dyslipidemia. *Annu Rev Pharmacol Toxicol*. 2017;57:223-244. doi:10.1146/annurev-pharmtox-010716-104944
65. Shimano H, Shimomura I, Hammer RE, et al. Elevated levels of SREBP-2 and cholesterol synthesis in livers of mice homozygous for a targeted disruption of the SREBP-1 gene. *J Clin Invest*. 1997;100(8):2115-2124. doi:10.1172/JCI119746
66. Xue L, Qi H, Zhang H, et al. Targeting SREBP-2-Regulated Mevalonate Metabolism for Cancer Therapy. *Front Oncol*. 2020;10:1510. doi:10.3389/fonc.2020.01510
67. DeBose-Boyd RA, Ye J. SREBPs in Lipid Metabolism, Insulin Signaling, and Beyond. *Trends Biochem Sci*. 2018;43(5):358-368. doi:10.1016/j.tibs.2018.01.005
68. Espenshade PJ. Cholesterol Synthesis and Regulation. In: *Encyclopedia of Biological Chemistry*. Elsevier; 2013:516-520. doi:10.1016/B978-0-12-378630-2.00070-0
69. Radhakrishnan A, Goldstein JL, McDonald JG, Brown MS. Switch-like control of SREBP-2 transport triggered by small changes in ER cholesterol: a delicate balance. *Cell Metab*. 2008;8(6):512-521. doi:10.1016/j.cmet.2008.10.008
70. Gent J, Braakman I. Low-density lipoprotein receptor structure and folding. *Cell Mol Life Sci*. 2004;61(19-20):2461-2470. doi:10.1007/s00018-004-4090-3
71. Matsui M, Sakurai F, Elbashir S, Foster DJ, Manoharan M, Corey DR. Activation of LDL receptor expression by small RNAs complementary to a noncoding transcript that overlaps

the LDLR promoter. *Chem Biol*. 2010;17(12):1344-1355. doi:10.1016/j.chembiol.2010.10.009
72. Afonso MS, Machado RM, Lavrador MS, Quintao ECR, Moore KJ, Lottenberg AM. Molecular Pathways Underlying Cholesterol Homeostasis. *Nutrients*. 2018;10(6). doi:10.3390/nu10060760
73. Zhang Y, Ma KL, Ruan XZ, Liu BC. Dysregulation of the Low-Density Lipoprotein Receptor Pathway Is Involved in Lipid Disorder-Mediated Organ Injury. *Int J Biol Sci*. 2016;12(5):569-579. doi:10.7150/ijbs.14027
74. Lagace TA. PCSK9 and LDLR degradation: regulatory mechanisms in circulation and in cells. *Curr Opin Lipidol*. 2014;25(5):387-393. doi:10.1097/MOL.0000000000000114
75. Xia XD, Peng ZS, Gu HM, Wang M, Wang GQ, Zhang DW. Regulation of PCSK9 Expression and Function: Mechanisms and Therapeutic Implications. *Front Cardiovasc Med*. 2021;8:764038. doi:10.3389/fcvm.2021.764038
76. Spolitu S, Dai W, Zadroga JA, Ozcan L. Proprotein convertase subtilisin/kexin type 9 and lipid metabolism. *Curr Opin Lipidol*. 2019;30(3):186-191. doi:10.1097/MOL.0000000000000601
77. Xiao X, Luo Y, Peng D. Updated Understanding of the Crosstalk Between Glucose/Insulin and Cholesterol Metabolism. *Front Cardiovasc Med*. 2022;9:879355. doi:10.3389/fcvm.2022.879355
78. Seidah NG, Prat A. The Multifaceted Biology of PCSK9. *Endocr Rev*. 2022;43(3):558-582. doi:10.1210/endrev/bnab035
79. Guo Y, Yan B, Tai S, Zhou S, Zheng XL. PCSK9: Associated with cardiac diseases and their risk factors? *Arch Biochem Biophys*. 2021;704:108717. doi:10.1016/j.abb.2020.108717
80. Ruscica M, Macchi C, Giuliani A, et al. Circulating PCSK9 as a prognostic biomarker of cardiovascular events in individuals with type 2 diabetes: evidence from a 16.8-year follow-up study. *Cardiovasc Diabetol*. 2023;22(1):222. doi:10.1186/s12933-023-01948-8
81. Levenson AE, Shah AS, Khoury PR, et al. Obesity and type 2 diabetes are associated with elevated PCSK9 levels in young women. *Pediatr Diabetes*. 2017;18(8):755-760. doi:10.1111/pedi.12490
82. Prem P, Muneshwar KN, Agrawal S, Jaiswal A. The Impact of Increased Homozygosity on Human Fertility: A Comprehensive Review. *Cureus*. 2023;15(11):e49000. doi:10.7759/cureus.49000
83. Cuchel M, Bruckert E, Ginsberg HN, et al. Homozygous familial hypercholesterolaemia: new insights and guidance for clinicians to improve detection and clinical management. A position paper from the Consensus Panel on Familial Hypercholesterolaemia of the European Atherosclerosis Society. *Eur Heart J*. 2014;35(32):2146-2157. doi:10.1093/eurheartj/ehu274
84. Abifadel M, Boileau C. Genetic and molecular architecture of familial hypercholesterolemia. *J Intern Med*. 2023;293(2):144-165. doi:10.1111/joim.13577
85. Wang X, Jiang L, Sun LY, et al. Genetically confirmed familial hypercholesterolemia in outpatients with hypercholesterolemia. *J Geriatr Cardiol*. 2018;15(6):434-440. doi:10.11909/j.issn.1671-5411.2018.06.006
86. Dhingra S, Bansal MP. Hypercholesterolemia and apolipoprotein B expression: regulation by selenium status. *Lipids Health Dis*. 2005;4:28. doi:10.1186/1476-511X-4-28

87. Lv X, Wang C, Liu L, et al. Screening and verifying the mutations in the LDLR and APOB genes in a Chinese family with familial hypercholesterolemia. *Lipids Health Dis*. 2023;22(1):175. doi:10.1186/s12944-023-01935-8
88. Pejic RN. Familial hypercholesterolemia. *Ochsner journal*. 2014;14(4):669-672.
89. Guo Q, Feng X, Zhou Y. PCSK9 Variants in Familial Hypercholesterolemia: A Comprehensive Synopsis. *Front Genet*. 2020;11:1020. doi:10.3389/fgene.2020.01020
90. Lambert CT, Sandesara P, Isiadinso I, et al. Current Treatment of Familial Hypercholesterolaemia. *Eur Cardiol*. 2014;9(2):76-81. doi:10.15420/ecr.2014.9.2.76
91. Qiao YN, Zou YL, Guo SD. Low-density lipoprotein particles in atherosclerosis. *Front Physiol*. 2022;13:931931. doi:10.3389/fphys.2022.931931
92. Qiao YN, Zou YL, Guo SD. Low-density lipoprotein particles in atherosclerosis. *Front Physiol*. 2022;13:931931. doi:10.3389/fphys.2022.931931
93. Istvan E. Statin inhibition of HMG-CoA reductase: a 3-dimensional view. *Atheroscler Suppl*. 2003;4(1):3-8. doi:10.1016/s1567-5688(03)00003-5
94. Stancu C, Sima A. Statins: mechanism of action and effects. *J Cell Mol Med*. 2001;5(4):378-387. doi:10.1111/j.1582-4934.2001.tb00172.x
95. Tomaszewski M, Stępień KM, Tomaszewska J, Czuczwar SJ. Statin-induced myopathies. *Pharmacol Rep*. 2011;63(4):859-866. doi:10.1016/s1734-1140(11)70601-6
96. Torres PA, Helmstetter JA, Kaye AM, Kaye AD. Rhabdomyolysis: pathogenesis, diagnosis, and treatment. *Ochsner journal*. 2015;15(1):58-69.
97. Newport MT, Dayrit FM. The Lipid-Heart Hypothesis and the Keys Equation Defined the Dietary Guidelines but Ignored the Impact of Trans-Fat and High Linoleic Acid Consumption. *Nutrients*. 2024;16(10). doi:10.3390/nu16101447
98. Teicholz N. A short history of saturated fat: the making and unmaking of a scientific consensus. *Curr Opin Endocrinol Diabetes Obes*. 2023;30(1):65-71. doi:10.1097/MED.0000000000000791
99. Dehghan M, Mente A, Zhang X, et al. Associations of fats and carbohydrate intake with cardiovascular disease and mortality in 18 countries from five continents (PURE): a prospective cohort study. *Lancet*. 2017;390(10107):2050-2062. doi:10.1016/S0140-6736(17)32252-3
100. Srisawasdi P, Vanavanan S, Rochanawutanon M, Kruthkul K, Kotani K, Kroll MH. Small-dense LDL/large-buoyant LDL ratio associates with the metabolic syndrome. *Clin Biochem*. 2015;48(7-8):495-502. doi:10.1016/j.clinbiochem.2015.01.011
101. Crosby L, Davis B, Joshi S, et al. Ketogenic Diets and Chronic Disease: Weighing the Benefits Against the Risks. *Front Nutr*. 2021;8:702802. doi:10.3389/fnut.2021.702802
102. Laffel L. Ketone bodies: a review of physiology, pathophysiology and application of monitoring to diabetes. *Diabetes Metab Res Rev*. 1999;15(6):412-426. doi:10.1002/(sici)1520-7560(199911/12)15:6<412::aid-dmrr72>3.0.co;2-8
103. Fletcher JA, Deja S, Satapati S, Fu X, Burgess SC, Browning JD. Impaired ketogenesis and increased acetyl-CoA oxidation promote hyperglycemia in human fatty liver. *JCI Insight*. 2019;5(11). doi:10.1172/jci.insight.127737
104. Saslow LR, Jones LM, Sen A, et al. Comparing Very Low-Carbohydrate vs DASH Diets for Overweight or Obese Adults With Hypertension and Prediabetes or Type 2 Diabetes: A Randomized Trial. *Ann Fam Med*. 2023;21(3):256-263. doi:10.1370/afm.2968
105. Dashti HM, Mathew TC, Hussein T, et al. Long-term effects of a ketogenic diet in obese patients. *Exp Clin Cardiol*. 2004;9(3):200-205.

106. Nagy L, Tontonoz P, Alvarez JG, Chen H, Evans RM. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. *Cell*. 1998;93(2):229-240. doi:10.1016/s0092-8674(00)81574-3
107. St-Pierre AC, Cantin B, Dagenais GR, et al. Low-density lipoprotein subfractions and the long-term risk of ischemic heart disease in men: 13-year follow-up data from the Québec Cardiovascular Study. *Arterioscler Thromb Vasc Biol*. 2005;25(3):553-559. doi:10.1161/01.ATV.0000154144.73236.f4
108. Norwitz NG, Feldman D, Soto-Mota A, Kalayjian T, Ludwig DS. Elevated LDL Cholesterol with a Carbohydrate-Restricted Diet: Evidence for a "Lean Mass Hyper-Responder" Phenotype. *Curr Dev Nutr*. 2022;6(1):nzab144. doi:10.1093/cdn/nzab144
109. Norwitz NG, Soto-Mota A, Kaplan B, et al. The Lipid Energy Model: Reimagining Lipoprotein Function in the Context of Carbohydrate-Restricted Diets. *Metabolites*. 2022;12(5). doi:10.3390/metabo12050460
110. Abumrad NA, Cabodevilla AG, Samovski D, Pietka T, Basu D, Goldberg IJ. Endothelial Cell Receptors in Tissue Lipid Uptake and Metabolism. *Circ Res*. 2021;128(3):433-450. doi:10.1161/CIRCRESAHA.120.318003
111. Ji B, Liu XB. Coronary artery calcification: concepts and clinical applications. *Ann Med Surg (Lond)*. 2024;86(5):2848-2855. doi:10.1097/MS9.0000000000002016
112. Sharman MJ, Kraemer WJ, Love DM, et al. A ketogenic diet favorably affects serum biomarkers for cardiovascular disease in normal-weight men. *J Nutr*. 2002;132(7):1879-1885. doi:10.1093/jn/132.7.1879
113. Kersten S. Mechanisms of nutritional and hormonal regulation of lipogenesis. *EMBO Rep*. 2001;2(4):282-286. doi:10.1093/embo-reports/kve071
114. Sanders FWB, Griffin JL. De novo lipogenesis in the liver in health and disease: more than just a shunting yard for glucose. *Biol Rev Camb Philos Soc*. 2016;91(2):452-468. doi:10.1111/brv.12178
115. Jani S, Da Eira D, Stefanovic M, Ceddia RB. The ketogenic diet prevents steatosis and insulin resistance by reducing lipogenesis, diacylglycerol accumulation and protein kinase C activity in male rat liver. *J Physiol*. 2022;600(18):4137-4151. doi:10.1113/JP283552
116. Kersten S. Mechanisms of nutritional and hormonal regulation of lipogenesis. *EMBO Rep*. 2001;2(4):282-286. doi:10.1093/embo-reports/kve071
117. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med*. 2002;346(16):1221-1231. doi:10.1056/NEJMra011775
118. Watanabe M, Tozzi R, Risi R, et al. Beneficial effects of the ketogenic diet on nonalcoholic fatty liver disease: A comprehensive review of the literature. *Obes Rev*. 2020;21(8):e13024. doi:10.1111/obr.13024
119. Miao J, Haas JT, Manthena P, et al. Hepatic insulin receptor deficiency impairs the SREBP-2 response to feeding and statins. *J Lipid Res*. 2014;55(4):659-667. doi:10.1194/jlr.M043711
120. Heeren J, Scheja L. Metabolic-associated fatty liver disease and lipoprotein metabolism. *Mol Metab*. 2021;50:101238. doi:10.1016/j.molmet.2021.101238
121. Okuda T, Morita N. A very low carbohydrate ketogenic diet prevents the progression of hepatic steatosis caused by hyperglycemia in a juvenile obese mouse model. *Nutr Diabetes*. 2012;2(11):e50. doi:10.1038/nutd.2012.24

122. Nemes K, Åberg F, Gylling H, Isoniemi H. Cholesterol metabolism in cholestatic liver disease and liver transplantation: From molecular mechanisms to clinical implications. *World J Hepatol*. 2016;8(22):924-932. doi:10.4254/wjh.v8.i22.924
123. Brown MS, Goldstein JL. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell*. 1997;89(3):331-340. doi:10.1016/s0092-8674(00)80213-5
124. Selva-O'Callaghan A, Alvarado-Cardenas M, Pinal-Fernández I, et al. Statin-induced myalgia and myositis: an update on pathogenesis and clinical recommendations. *Expert Rev Clin Immunol*. 2018;14(3):215-224. doi:10.1080/1744666X.2018.1440206
125. Osaki Y, Nakagawa Y, Miyahara S, et al. Skeletal muscle-specific HMG-CoA reductase knockout mice exhibit rhabdomyolysis: A model for statin-induced myopathy. *Biochem Biophys Res Commun*. 2015;466(3):536-540. doi:10.1016/j.bbrc.2015.09.065
126. Dyńka D, Kowalcze K, Charuta A, Paziewska A. The Ketogenic Diet and Cardiovascular Diseases. *Nutrients*. 2023;15(15). doi:10.3390/nu15153368
127. Retterstøl K, Svendsen M, Narverud I, Holven KB. Effect of low carbohydrate high fat diet on LDL cholesterol and gene expression in normal-weight, young adults: A randomized controlled study. *Atherosclerosis*. 2018;279:52-61. doi:10.1016/j.atherosclerosis.2018.10.013
128. Raal F, Panz V, Immelman A, Pilcher G. Elevated PCSK9 levels in untreated patients with heterozygous or homozygous familial hypercholesterolemia and the response to high-dose statin therapy. *J Am Heart Assoc*. 2013;2(2):e000028. doi:10.1161/JAHA.112.000028
129. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25(4):402-408. doi:10.1006/meth.2001.1262
130. Astrup A, Teicholz N, Magkos F, et al. Dietary Saturated Fats and Health: Are the U.S. Guidelines Evidence-Based? *Nutrients*. 2021;13(10). doi:10.3390/nu13103305
131. Burén J, Ericsson M, Damasceno NRT, Sjödin A. A Ketogenic Low-Carbohydrate High-Fat Diet Increases LDL Cholesterol in Healthy, Young, Normal-Weight Women: A Randomized Controlled Feeding Trial. *Nutrients*. 2021;13(3). doi:10.3390/nu13030814
132. Vieira SA, McClements DJ, Decker EA. Challenges of Utilizing Healthy Fats in Foods. *Advances in Nutrition*. 2015;6(3):309S-317S. doi:10.3945/an.114.006965
133. Froyen E. The effects of fat consumption on low-density lipoprotein particle size in healthy individuals: a narrative review. *Lipids Health Dis*. 2021;20(1):86. doi:10.1186/s12944-021-01501-0
134. Ruscica M, Ferri N, Macchi C, et al. Liver fat accumulation is associated with circulating PCSK9. *Ann Med*. 2016;48(5):384-391. doi:10.1080/07853890.2016.1188328
135. Kersten S. Mechanisms of nutritional and hormonal regulation of lipogenesis. *EMBO Rep*. 2001;2(4):282-286. doi:10.1093/embo-reports/kve071
136. Song Z, Xiaoli AM, Yang F. Regulation and Metabolic Significance of De Novo Lipogenesis in Adipose Tissues. *Nutrients*. 2018;10(10). doi:10.3390/nu10101383
137. Costet P, Cariou B, Lambert G, et al. Hepatic PCSK9 expression is regulated by nutritional status via insulin and sterol regulatory element-binding protein 1c. *J Biol Chem*. 2006;281(10):6211-6218. doi:10.1074/jbc.M508582200

138. Santamarina AB, Mennitti L V, de Souza EA, et al. A low-carbohydrate diet with different fatty acids' sources in the treatment of obesity: Impact on insulin resistance and adipogenesis. *Clin Nutr*. 2023;42(12):2381-2394. doi:10.1016/j.clnu.2023.09.024
139. St-Amand R, Ngo Sock ÉT, Quinn S, Lavoie JM, St-Pierre DH. Two weeks of western diet disrupts liver molecular markers of cholesterol metabolism in rats. *Lipids Health Dis*. 2020;19(1):192. doi:10.1186/s12944-020-01351-2

Appendix

The Thermo Scientific™ Subcellular Protein Fractionation Kit for Tissues (Cat# 87790)

Tissue Preparation

- Thaw all buffers.
- Place liver tissues in nitrogen.
- Weigh 50-200mg of liver samples.
- Add ice-cold CEB (Cytoplasm Extraction Buffer) containing protease inhibitors to the tissue depend on how much sample is being used. (example for 200µg tissue 2000µl CEB, 1300µl MEB).
- Homogenize the samples with CEB with protease inhibitor.
- Transfer the homogenized tissue into the Pierce Tissue Strainer. Centrifuge at 500 × g for 5 minutes. Quickly transfer the supernatant (cytoplasmic extract) into a clean, pre-chilled tube on ice.
- Add ice-cold MEB (Membrane Extraction Buffer) with protease inhibitors to the pellet.
- Vortex the tube at the highest setting for 5 seconds, then incubate at 4°C for 10 minutes with gentle mixing.
- Centrifuge at 3000 × g for 5 minutes. Carefully transfer the supernatant (membrane extract) to a clean, pre-chilled tube on ice.
- Store the samples at -80.

Cholesterol Assay Kit - HDL and LDL/VLDL (ab65390)

Preparation of Cholesterol Working Standard for Colorimetric Assay:

- To prepare 200 µL of a 0.25 µg/µL Cholesterol Working Standard, dilute 25 µL of the provided Cholesterol Standard (2 µg/µL solution) with 175 µL of Assay Buffer II.

Preparation and Measurement of Total and Free Cholesterol

- Mix 100 µL of the sample with 100 µL of 2X Precipitation Buffer in microcentrifuge tubes.
- Incubate the mixture for 10 minutes at room temperature.
- Centrifuge the sample at 2000 × g (5000 rpm on a bench microcentrifuge) for 10 minutes at room temperature.
- Carefully transfer the supernatant to a new tube. This is the HDL fraction.
- Centrifuge the precipitate again at 2000 × g for 10 minutes at room temperature to remove any remaining HDL.
- Carefully remove any trace amount of supernatant.
- Resuspend the precipitate in 200 µL of PBS. This is the LDL/VLDL fraction.

Total Cholesterol Reaction Mix:

- Prepare 50 µL of Total Cholesterol Reaction Mix for each reaction.

If performing multiple assays, prepare a master mix to ensure consistency across all reactions. For example, if preparing for 10 reactions, mix enough reagents to prepare 500 µL of Total Cholesterol Reaction Mix.

- Mix the samples and incubate them at 37°C for 60 minutes, ensuring they are protected from light.
- Immediately measure the output using a microplate reader, setting it to OD 570 nm for a colorimetric assay or Ex/Em = 535/587 nm for a fluorometric assay.

- Calculate Cholesterol Concentration:

Cholesterol concentration(μg/μL) =(V/A×D) ×DF

A = Amount of cholesterol determined from the standard curve (in μg)
D = Dilution factor of the sample (if any dilution was performed)
V = Volume of the sample used in the reaction (in μL)
DF = Additional dilution factor if the sample was further diluted to fall within the standard curve range

Measuring Gene Expression in liver using PCR

RNA Extraction using TRIzol:

- Sterilize the work area and all consumables.
- Place 50 mg of tissue into a 2 ml tube containing 1 ml of TRIzol.
- Homogenize the samples using a mechanical homogenizer.
- Incubate the samples at room temperature for 5 minutes to allow for the dissociation of the nucleoprotein complex.
- Add 200 μl of chloroform to each tube.
- Vigorously vortex each tube for 15 seconds.
- Incubate the samples at room temperature for 2-3 minutes.
- Centrifuge the samples at 13,000 RPM for 15 minutes at 4°C.
- Extract the aqueous (top) phase carefully, avoiding the other two phases.
- Transfer the aqueous phase into a new, sterile 1.5 ml Eppendorf tube.
- Add 500 μl of 100% isopropanol to the aqueous phase and mix by inverting the tube several times.
- Incubate the samples at room temperature for 10 minutes.
- Centrifuge the samples at 13,000 RPM for 20 minutes at 4°C.

- After centrifugation, a pellet should be visible at the bottom of the tube.
- Discard the supernatant and wash the pellet with 1 ml of 75% ethanol.
- Centrifuge the tubes at 13,000 RPM for 20 minutes at 4°C.
- Repeat steps 7 and 8 to perform a second wash of the pellet.
- Discard the supernatant.
- Air dry the pellet for 5-10 min.
- Resuspend the RNA pellet in 20 µl of sterile ddH2O by gently pipetting up and down multiple times.
- Evaluate the RNA sample's concentration and quality using a Nanodrop spectrophotometer.
- Either proceed to cDNA synthesis or preserve the samples at -80°C until required.

cDNA Synthesis using ABM EasyScript™ Reverse Transcriptase cDNA Synthesis Kit

1. Thaw RNA samples and all reagents on ice.
2. Add 2 µg of each RNA sample to sterile tubes.
3. Adjust the total volume to 12.5 µl with ddH2O.
4. Add 1 µl of random primers and 1 µl of dNTP mix to each sample.
5. Briefly centrifuge the tubes.
6. Incubation Steps:
7. Incubate the samples at 65°C for 5 minutes.
8. Immediately place the samples on ice for 1 minute.
9. Briefly centrifuge the tubes again.
10. Master Mix Preparation:

Prepare a master mix containing per sample:

11. µl of 5X RT buffer, 0.5 µl of RNAse OFF Ribonuclease Inhibitor, 1 µl of OneScript mix.
12. Add 5.5 µl of this master mix to each tube.
13. Mixing and Incubation:
14. Mix the components well and briefly centrifuge to collect the contents.
15. Incubate the samples at 25°C for 10 minutes.
16. Perform cDNA synthesis by incubating the tubes at 42°C for 50 minutes.
17. Stop the reaction by heating the samples at 85°C for 5 minutes.
18. Incubate the samples on ice.
19. Proceed to PCR immediately or store cDNA samples at -20°C.

PCR

1. Thaw cDNA samples.
2. In a 96-well plate, load 10 ng of cDNA per well.

To each sample, add: 0.4 µl of the primer of interest, 5.6 µl of ddH2O, 10 µl of BrightGreen 2x qPCR Master mix.

3. Centrifuge the plate at 2000 RPM for 5 minutes at 4°C.

qPCR Analysis:

4. Perform qPCR analysis using the following amplification conditions:

Initial denaturation: 95°C for 10 minutes. 40 cycles of:

Denaturation: 95°C for 15 seconds

Annealing/extension: 65°C for 1 minute, a 0.5°C stepwise increment from 65°C to 95°C.

5. Normalize the results to the control gene β-actin or TBP.
6. Calculate fold change using the ΔΔCt method.

Western Blotting:

Protein Extraction

1. Tissue Extraction and Storage:

- Immediately freeze extracted tissue in liquid nitrogen.

- Store tissue at -80°C until homogenization.

2. preparation of Tissue and Lysis Buffer:

- Place 50 mg of BAT into 2 ml Eppendorf tubes already containing 200 µl of lysis buffer.

- The lysis buffer consists of:

  - 25 mM Tris-HCl,

  - 25 mM NaCl (pH 7.4),

  - 1 mM MgCl2,

  - 2.7 mM KCl,

  - 1% Triton-X

  - Protease and phosphatase inhibitors

3. Homogenize the tissue sample using a mechanical homogenizer.

4. Sonicate the sample three times for 3 seconds each time at 40% amplitude.

5. Centrifuge the samples at 13,000 RPM at 4°C for 10 minutes.

6. Remove the samples from the centrifuge.

7. Extract the protein lysate from the Eppendorf, avoiding the pellet formed at the bottom of the tube.

8.Transfer the lysate into a separate, clean Eppendorf tube.

9. Extract an aliquot for protein quantification using the Bradford method.

10. Samples can be used immediately for Western Blotting or stored at -80°C for future use.

Lysate Preparation with Laemmli Buffer

- Mix 50 µl of 2-mercaptoethanol with 950 µl of 2X Laemmli buffer.
- Dilute the lysate samples in a 1:1 ratio with Laemmli buffer.
- Boil the lysate/Laemmli mixture at 95°C for 5 minutes.
- Briefly centrifuge the lysate/Laemmli mixture.
- Use the prepared samples for Western blot analysis.

Western Blotting:

Gel Components Preparation

1. 1.5 M Tris-HCl, pH 8.8:

- Dissolve 27.3 g of Tris base in 80 ml of deionized water.
- Adjust the pH to 8.8 with 6N HCl.
- Bring the total volume to 150 ml with deionized water.

2. 0.5 M Tris-HCl, pH 6.8:

- Dissolve 6 g of Tris base in 60 ml of deionized water.
- Adjust the pH to 6.8 with 6N HCl.
- Bring the total volume to 100 ml with deionized water.

3. 10X Electrode (Running) Buffer, pH 8.3 (1 L):

- Dissolve 30.3 g of Tris base, 144 g of glycine, and 10 g of SDS in deionized water.
- Bring the total volume to 1 L with deionized water.

4. 10% Ammonium Persulfate (APS):

- Dissolve 100 mg of ammonium persulfate in 1 ml of deionized water.
- Prepare APS fresh each time before use.

Western Blotting: Gel Formulations

Resolving gel:

% Gel	ddH2O (ml)		30% Acrylamide/Bis (ml)		Gel buffer (ml) pH 8.8		10% (w/v) SDS (ml)	
	2 Gels	4 Gels	2 Gels	4 Gels	2 Gels	4 Gels	2 Gels	4 Gels
7%	7	8.61	17.23	3.97	7.93	4.25	8.5	0.17
8%	8.05	16.1	4.53	9.06	4.25	8.5	0.17	0.34
10%	6.91	13.82	5.67	11.34	4.25	8.5	0.17	0.34
12%	5.78	11.56	6.8	13.6	4.25	8.5	0.17	0.34

Stacking gel:

ddH2O (ml)		30% Acrylamide/Bis (ml)		Gel buffer (ml) pH 6.8		10% (w/v) SDS (ml)	
2 Gels	4 Gels	2 Gels	4 Gels	2 Gels	4 Gels	2 Gels	4 Gels
3.94	7.88	0.87	1.73	1.63	3.25	0.065	0.13

1. Resolving Gel

- Begin by preparing the monomer solution with the specified reagents. Add 100 µl of 10% APS and 10 µl of TEMED last, mixing well before pouring into the gel plates. Similarly, for the stacking gel, mix other components first, then add 50 µl of 10% APS and 5 µl of TEMED. Pour this on top of the resolving gel and insert well combs gently. Allow the gels to set for 15-20 minutes.

2. Loading and Running the Gel:

- Once gels are set, place the plates in running clamps and position them in the running apparatus. Fill the apparatus with 1X Running Buffer, remove the well combs, and load 25 µg of protein into each well. Run the gel at 110V for 2 hours.

Transfer Buffers

10X Transfer Buffer

- 25 mM Tris base
- 192 mM Glycine
- Dissolve in distilled water to a total volume of 1 L

1X Transfer Buffer (1 L)

- 700 ml of distilled water
- 200 ml of methanol
- 100 ml of 10X transfer buffer

PVDF Membrane Activation

1. Place pre-cut PVDF membranes in a container.
2. Submerge the membranes in 100% methanol for 30 seconds.
3. Rinse the membranes quickly with distilled water, then submerge them for 2 minutes in distilled water.
4. Discard the water and pour 1X Transfer Buffer over the membranes.
5. Let the membranes equilibrate for 10 minutes.

Wet Transfer Protocol

1. Prepare a dish with transfer cassettes, fiber pads, and pre-cut filter paper.
2. Add enough 1X Transfer buffer to cover the bottom and soak the filter paper and fiber pads.
3. Assemble the cassette: add a fiber pad, followed by two pre-soaked filter papers.
4. Remove a gel-containing plate from the running apparatus and transfer the gel onto the filter paper in the cassette.

5. Place a membrane on top of the gel, remove any bubbles, and add filter papers and fiber pads as described.
6. Clamp the cassette shut, place it in the transfer apparatus, and close the apparatus.
7. Incubate at 4°C and set the transfer to 120V for 2 hours.

Probing the Membrane

Buffers:

10X Wash Buffer (1 L)

- 60.57 g of Tris base
- 87.66 g of NaCl

1X Wash Buffer (1 L)

- Dilute the 10X buffer with distilled water
- Add 500 µl of Tween
- Add 500 µl of NP40

Blocking Buffer (3% BSA)

- Dissolve 1.5 g of crude BSA in 50 ml of 1X Wash buffer

Blocking the Membrane

1. After transfer, prepare containers with 8 ml of blocking buffer.
2. Place the membrane in a container and shake for 1 hour.

Incubation with Primary Antibody

1. Dilute the primary antibody in blocking buffer as needed.
2. Discard the blocking buffer and add the primary antibody preparation.
3. Incubate the membrane for 2 hours at room temperature with gentle shaking.
4. Wash the membrane with 1X wash buffer 5 times for 10 minutes each.

Incubation with Secondary Antibody

1. Dilute the secondary antibody in blocking buffer.
2. Discard the wash buffer and add the secondary antibody.
3. Incubate for 1 hour at room temperature with gentle shaking.
4. Wash the membrane with 1X wash buffer 5 times for 10 minutes each.

Developing the Membrane

1. Discard the secondary antibody and let excess liquid drain.
2. Add Luminata Western HRP Chemiluminescent Substrate and shake gently for 2 minutes.
3. Discard the substrate and place the membranes in film development cassettes.
4. In a dark room, expose film to the membranes for various times to obtain different exposures.
5. Develop the film in Developer Fluid until bands appear, wash in water, fix in Fixer Fluid, and rinse off residual fixer with water.

Primary antibodies dilutions and loading attempts

PCSK9: 1:2000 – 50µg loaded on the gel

LDLr: 1:1000 – 50µg loaded on the gel

HMG-CoA reductase: 1:500 – 60µg loaded on the gel

SREBP-2: 1:200 – 60µg loaded on the gel

Body Weight (BW):

HFS-fed animals gained significantly more weight than the **SC** and **KD-fed animals** at several time points:

- **Week 8:**
  - HFS group: 409.77 ± 14.6 g
  - SC group: 370.21 ± 14.3 g
  - KD group: 374.24 ± 6.6 g
- **Week 12:**
  - HFS group: 452.3 ± 15.7 g
  - SC group: 400.47 ± 15 g
  - KD group: 404.69 ± 6.5 g
- **Week 16:**
  - HFS group: 470.81 ± 17.1 g
  - SC group: 421.29 ± 15.7 g
  - KD group: 425.54 ± 4.7 g

- Statistically significant weight gain was observed in the HFS group at weeks 8, 12, and 16 when compared to the SC and KD groups.