Prevention of Murine Atherosclerosis with Bempedoic Acid

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Graduate Program in Biochemistry
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Abstract

Bempedoic acid (BA) is a novel LDL cholesterol-lowering compound. Preclinical studies revealed that BA inhibits hepatic cholesterol and fatty acid synthesis through inhibition of ATP-citrate lyase and activation of AMP-kinase. In the current study we tested the ability of BA to prevent diet-induced metabolic dysregulation, inflammation and atherosclerosis in Ldlr−/− mice fed a high-fat high-cholesterol diet (HFHC). BA supplementation to the HFHC diet at 3, 10 or 30 mg/kg/d significantly attenuated diet-induced hypercholesterolemia, hypertriglyceridemia, hyperglycemia, hyperinsulinemia, fatty liver and obesity over the 12-week study compared to HFHC alone. Livers of BA-treated mice displayed decreased fatty acid synthesis, and increased β-oxidation, AMP-kinase activation, and peroxisome proliferation. BA reduced hepatic and aortic inflammatory gene expression and MAPK signaling, aortic esterified cholesterol, and atherosclerotic lesion development. This demonstrates that BA effectively improves hepatic lipid metabolism and reduces plasma and tissue lipids, markers of inflammation, and atherosclerosis in a mouse model of metabolic dysregulation.

Keywords

LDL, cholesterol, dyslipidemia, atherosclerosis, inflammation, metabolic syndrome, NAFLD, Bempedoic Acid
Co-Authorship Statement

The co-author contributions of this thesis are described below.

Expertise and input for the conceptualization, design, and protocols of this study were provided by Dr. Murray W. Huff, Dr. Roger S. Newton, Dr. Sergey Filippov, and Stephen L. Pinkosky. Dr. Murray Huff also edited this thesis. Dr. J. Geoffrey Pickering provided access to the Molecular Pathology core facility at Robarts for histological analysis. Dr. Robert Gros provided access to the Comprehensive Lab Animal Monitoring System for metabolic cage experiments. Dr. Robert A. Hegele provided access to the London Regional Genomic Centre for gene expression analysis and the AKTA purifier and Superose 6 column for FPLC analysis. Dr. Maria Drangova and Dr. David W Holdsworth provided access to the Lotus Ultra micro-CT scanner for body composition analysis. Brian G. Sutherland, Dawn E. Telford, Jane Y. Edwards, Amy C. Burke, Julia M. St. John, and Andrew Q. Phu assisted with mouse husbandry, metabolic experiments and tissue harvesting. Brian G. Sutherland also assisted with lipid analysis and histological processing of tissues. Jane Y. Edwards also assisted with aortic gene expression analysis. Cindy G. Sawyez assisted with plasma lipid and ELISA analyses. Dr. Hao Yin, Dr. Zengxuan Nong, and Caroline O’Neil assisted with histological staining and quantitative analysis of tissues. Dr. Joseph Umoh, Dr. Maria Drangova, Joy Dunmore-Buyze, and Brian G. Sutherland performed micro-CT scanning. Dr. Joseph Umoh also performed processing and quantitative analyses of micro-CT scans.
There are several individuals I wish to acknowledge for what I have been able to learn from them and for making this project a much more rewarding endeavor.

First, I would like to thank my supervisor, Dr. Murray Huff, for providing me with the opportunity to carry out this work. His investment of time and resources into me made the success of this project possible and were key in my own learning and development as a researcher. I would also like to thank the members of my advisory committee, Dr. Geoffrey Pickering and Dr. Robert Gros, for their guidance and feedback. Thank you to all the members of the Huff Lab who contributed to this project: Brian Sutherland, Cindy Sawyez, Dawn Telford, Jane Edwards, Amy Burke, Andrew Phu, and Julia St. John. Although not involved with this project, I would also like to thank Kyle Seigel, also from the Huff Lab. It was a pleasure have the opportunity to mentor an undergraduate student. I enjoyed getting to work on the flavonoid study together, it’s a shame we couldn’t see it through to completion.

To the many members of the fourth floor Vascular Biology group, I am hugely grateful for opportunities I had to meet and interact with you all. This includes the Gros, Hegele, and Pickering Labs whose collegiality and innovative spirits has been a pleasure to witness. I would especially like to thank Dr. Hao Yin, Sali Farhan, and John Robinson whose enthusiasm and willingness to exchange ideas were very encouraging to see. To Sharon Leung, Sina Ghoreishi, John-Michael Arpino, and Mouhamed Dakroub thank you for the enjoyable discussions.

Another group worthy of very special acknowledgement is the lab of Dr. Khosrow Adeli, without whom I can certainly say I would not be where I am today. During my brief time working there I had the privilege of being mentored by Dr. Mark Dekker, while also working alongside Chris Baker, Mark Naples, Rianna Zhang, Wei Qui, Sara Farr, and Marsel Lino.

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excited to watch bempedoic acid continue through clinical development and am honored that I was able to play a part (however small) in this endeavor.

I wish to express my sincere gratitude to my family and friends who supported me over the past few years. Most notably I wish to thank my Mom and Dad for their unending love and support.

Above all, I thank God.

*Soli Deo Gloria*, glory to God alone.
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<tr>
<td>ABCA1</td>
<td>ATP-binding cassette transporter A1</td>
</tr>
<tr>
<td>ABCG5</td>
<td>ATP-binding cassette transporter G5</td>
</tr>
<tr>
<td>ACAT</td>
<td>acyl-CoA: cholesterol acyl transferase</td>
</tr>
<tr>
<td>ACC</td>
<td>acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACL</td>
<td>ATP-citrate lyase</td>
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<tr>
<td>ACO</td>
<td>fatty acyl-CoA oxidase</td>
</tr>
<tr>
<td>ACS</td>
<td>acyl-CoA synthetase</td>
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<tr>
<td>ADP</td>
<td>adenosine 5’-diphosphate</td>
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<td>AKT</td>
<td>protein kinase B</td>
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<td>ALT</td>
<td>alanine transaminase</td>
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<td>AMP</td>
<td>adenosine 5’-monophosphate</td>
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<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>ANCOVA</td>
<td>analysis of covariance</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>AP1</td>
<td>activating protein 1</td>
</tr>
<tr>
<td>Apo</td>
<td>apolipoprotein</td>
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<tr>
<td>ApoB100</td>
<td>apolipoprotein B100</td>
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<tr>
<td>AST</td>
<td>aspartate transaminase</td>
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<td>ATGL</td>
<td>adipose triglyceride lipase</td>
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BA  bempedoic acid (ETC-1002)
cAMP  cyclic-AMP
CC3  cleaved caspase 3
CD  cluster of differentiation
CE  cholesteryl ester
CETP  cholesterol ester transfer protein
CoA  coenzyme A
CPT1α  carnitine palmitoyltransferase 1-alpha
CRCT  cAMP response element binding protein-regulated transcription coactivator
CRP  C-reactive protein
CVD  cardiovascular disease
DAG  diacylglycerol
DAMP  damage-associated molecular pattern molecule
DGAT  diacylglycerol acyltransferase
DNA  deoxyribonucleic acid
ER  endoplasmic reticulum
ERK1/2  extracellular related kinase 1/2
FAS  fatty acid synthase
FC  free cholesterol
FOXO1  forkhead box-O1
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<td>FPLC</td>
<td>fast-performance liquid chromatography</td>
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<tr>
<td>G6P</td>
<td>glucose 6-phosphatase</td>
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<td>GS</td>
<td>glycogen synthase</td>
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<td>GSK3</td>
<td>glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GTT</td>
<td>glucose tolerance test</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
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<tr>
<td>HFHC</td>
<td>high-fat, high-cholesterol</td>
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<td>HMG-CoA</td>
<td>3-hydroxy-3-methyl-glutaryl-CoA</td>
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<td>HMGCR</td>
<td>HMG-CoA reductase</td>
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<td>HMGCS</td>
<td>HMG-CoA synthase</td>
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<tr>
<td>hsCRP</td>
<td>high sensitivity C-reactive protein</td>
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<td>HSL</td>
<td>hormone sensitive lipase</td>
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<td>HTGL</td>
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<td>IDL</td>
<td>intermediate-density lipoprotein</td>
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<td>IHC</td>
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<td>IKK</td>
<td>inhibitor of IκBα kinase</td>
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<td>IL-1β</td>
<td>interleukin 1-beta</td>
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<td>IL-6</td>
<td>interleukin 6</td>
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<td>INSIG</td>
<td>insulin signaling gene</td>
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IR  insulin receptor
IRS  insulin receptor substrate
ITT  insulin tolerance test
IκBα  inhibitor of NF-κB-alpha
JNK  c-Jun N-terminal kinase
LCAT  lecithin: cholesterol acyl transferase
LDL  low-density lipoprotein
LDL-C  LDL cholesterol
LDLR  LDL receptor
Ldlr/-  LDLR knockout mice
LPL  lipoprotein lipase
LPS  lipopolysaccharide
M1  classically activated macrophage
M2  alternatively activated macrophage
MAP2K  mitogen-activated protein kinase kinase
MAP3K  mitogen-activated protein kinase kinase kinase
MAPK  mitogen-activated protein kinase
mg/kg  mg per kg body weight per day
MMP  matrix metalloproteinase
MRI  magnetic resonance imaging
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<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
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<td>mTORC1</td>
<td>mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>MTP</td>
<td>microsomal triglyceride transfer protein</td>
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<tr>
<td>NAFLD</td>
<td>non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NASH</td>
<td>non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>Nf-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<td>non-HDL-C</td>
<td>non-HDL-cholesterol</td>
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<td>NPC1L1</td>
<td>Neimann-Pick C1-like protein 1</td>
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<td>OCT</td>
<td>Optimum Cutting Temperature</td>
</tr>
<tr>
<td>ORO</td>
<td>Oil red-O</td>
</tr>
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<td>oxLDL</td>
<td>oxidized LDL</td>
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<td>PAMP</td>
<td>pathogen-associated molecular pattern molecule</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCSK9</td>
<td>proprotein convertase subtilisin/kexin 9</td>
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<tr>
<td>PEPCK</td>
<td>phosphoenolpyruvate carboxykinase</td>
</tr>
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<td>PGC1α</td>
<td>PPAR-gamma coactivator 1-alpha</td>
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<td>phosphoinositide-dependent kinase 1</td>
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<td>protein kinase C</td>
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<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
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<td>Description</td>
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<tr>
<td>PPRE</td>
<td>PPAR response element</td>
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<td>PRAS40</td>
<td>proline-rich AKT substrate of 40 kDa</td>
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<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
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<tr>
<td>qRT-PCR</td>
<td>quantitative real-time reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>RAPTOR</td>
<td>regulatory associated protein of mTOR</td>
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<td>RCT</td>
<td>reverse cholesterol transport</td>
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<td>RER</td>
<td>respiratory exchange ratio</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>SAA</td>
<td>serum amyloid A</td>
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<tr>
<td>SCAP</td>
<td>SREBP cleavage-activating protein</td>
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<td>SEM</td>
<td>standard error of the mean</td>
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<td>SH2</td>
<td>Src homology 2</td>
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<td>SMA</td>
<td>smooth muscle alpha-actin</td>
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<td>smooth muscle cell</td>
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<td>SRB1</td>
<td>scavenger receptor B1</td>
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<td>sterol regulatory element-binding protein</td>
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<td>total cholesterol</td>
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<td>TRL</td>
<td>triglyceride-rich lipoprotein</td>
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<td>TSC2</td>
<td>tubular sclerosis complex 2</td>
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<td>ULK</td>
<td>unc 51-like kinase.</td>
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<td>UPR</td>
<td>unfolded protein response</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VCO₂</td>
<td>volume of carbon dioxide produced</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low-density lipoprotein</td>
</tr>
<tr>
<td>VO₂</td>
<td>volume of oxygen consumed</td>
</tr>
<tr>
<td>μCT</td>
<td>micro-computed tomography</td>
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Chapter 1

1 Review of thesis topics

1.1 General Introduction

Cardiovascular disease (CVD) continues to be a leading cause of death in developed countries, exacerbated in part by the increasing prevalence of Type 2 Diabetes and the metabolic syndrome (1, 2). The rates of CVD mortality have been declining over the previous decades due to significant advancements in our understanding of its pathophysiology and therapies for risk management (3, 4). Underlying the progression of CVD is atherosclerosis, a chronic inflammatory condition, which can develop silently for decades and not be identified until after a major cardiovascular event has occurred, such as myocardial infarction, stroke, or peripheral artery disease (1, 5).

1.2 Cardiovascular Disease and Dyslipidemia

It has been known for some time now that cholesterol plays a major role in the development of CVD and over the past century there have been huge advancements in our understanding of this relationship (4). However, in addition to its role in CVD progression it is important for many other bodily functions, both as a structural and signaling molecule. Cholesterol is required for the synthesis of steroid hormones, bile acid, fat soluble vitamins and myelin sheath while also serving as a major structural component of cell membranes (6, 7).

Cholesterol traveling within the circulation is packaged into small micelle particles known as lipoproteins (further characterized in section 1.2.2). Low- and high-density lipoproteins (LDL and HDL, respectively) are two of the classes of lipoproteins by which cholesterol is transported within the circulation. Plasma LDL cholesterol (LDL-C) or non-HDL cholesterol (HDL-C) have been established for some time as major risk factors for CVD (8, 9). For example, evidence from randomized clinical trials examining statin efficacy have demonstrated that for every 1 mmol/L lowering in LDL-C, there was a 22% reduction in major vascular events and a 10% reduction in all-cause mortality (10). An
LDL-C concentration >5.0 mmol/L would be considered severe dyslipidemia likely due to a genetic disorder and warrant pharmacological treatment (11, 12). HDL-C has long been thought to be cardioprotective but more recently, multiple therapies that pharmacologically raise HDL have so far failed to demonstrate therapeutic benefit (13). Clinicians will typically measure plasma LDL-C and HDL-C concentrations both to estimate CVD risk and to gauge efficacy of treatment.

Dyslipidemia consists of elevated plasma LDL-C (hypercholesterolemia) and TG (hypertriglyceridemia) and low HDL-C and thus increases the risk of developing CVD. It can either be of genetic origin or secondary to other factors such as age, obesity, diabetes, and metabolic syndrome (14, 15). This lipid profile often seen in diabetics is referred to as diabetic dyslipidemia which, at least in part explains why patients with diabetes are at a much greater risk for developing CVD (16, 17).

The high incidence of CVD is linked to, and likely driven by the increasing prevalence of so-called “diseases of affluence” such as insulin resistance (leading to Type 2 Diabetes), obesity, and hepatic steatosis, all of which play a role in the development of atherogenic dyslipidemia and CVD (18–20). This cluster of risk factors, along with high blood pressure, have become known as the metabolic syndrome (15, 21). The patient population represented by this clustering of cardiometabolic abnormalities has been increasing in prevalence for some time, and will likely continue to do so thereby representing a significant challenge when it comes to managing the worldwide CVD burden (22).

Altogether, there is a growing need for therapies that will further lower LDL (or non-HDL) cholesterol while also treating other risk factors associated with the metabolic syndrome. The standard of care used clinically has improved tremendously over the years and there are several experimental therapies with significant therapeutic potential in late stage clinical development (reviewed in section 1.7). However, in order to counter the increasing CVD prevalence, there is a need for a better understanding of the molecular pathways that govern disease progression in order to identify novel therapeutic targets.
1.2.1 Atherogenesis

Vessels that are most susceptible to atherosclerosis are the medium and large sized arteries. The structure of a normal artery is shown in Figure 1-1. On the most basic level, it consists of an outermost, collagen-rich layer (adventitia), followed by an elastic middle layer (media), then a thin innermost layer (intima) facing the vessel lumen and exposed to continuous blood flow (Figure 1-1). Additionally, the medial layer is lined externally and internally by the external and internal elastic membranes, respectively (23). Each layer is defined by their cell types and macromolecule extracellular matrix composition. The intima is a thin layer of endothelial cells surrounded by a subendothelial space comprised of extracellular matrix proteins such as collagen and elastin. Humans and other larger mammals also contain smooth muscle cells (SMCs) within the intima in contrast to model organisms such as rodents, which do not (23, 24). The internal elastic membrane marks the start of the media. This thick elastin-rich layer made up of many SMCs and extends until the external elastic membrane (25). Lastly, the adventitial layer contains a heterogeneous combination of cells including vascular progenitor cells, fibroblasts and resident arterial macrophages. High collagen content within the adventitia provides tensile strength to the arteries (25–27).

The initiation of atherogenesis begins when apolipoprotein (apo) B-containing lipoproteins circulating in the blood enter the arterial intima (Figure 1-2A). This occurs predominantly in arteries experiencing oscillatory, non-uniform, and lower velocity flow (28). While in the subendothelial space lipoproteins will interact with the extracellular matrix leading to their retention and modification. Oxidative modification by reactive oxygen species (ROS) or lipoxygenases of lipids and LDL (oxLDL) elicits immune responses and complex interactions with and between the endothelial cells, SMCs, and immune cells (29, 30). Endothelial cells will express adhesion molecules and secrete chemokines resulting in the recruitment of leukocytes (mostly monocytes) to the area of lipid deposition. Within the arterial intima, monocytes will differentiate into macrophage and phagocytose (engulf) accumulated oxLDL particles, generating lipid-loaded foam cells (31) (Figure 1-2A). Lipid deposition within the intima, referred to as fatty streaks, accumulates over time resulting in the thickening of the intimal space forming a growing
atherosclerotic plaque (Figure 1-2B). Plaque development continues with the infiltration and proliferation of SMCs from the media and monocytes from the circulation (Figure 1-2C). Cellular apoptosis and the accumulation of debris and cholesterol crystals lead to the formation of a necrotic core. SMCs secrete collagen to generate a fibrous cap overlaying the growing plaque and conferring stability (31–33) (Figure 1-2D). Several factors can lead to the destabilization, erosion and potential rupture of the plaque fibrous cap, including a growing necrotic core, collagen degradation by enzymes such as matrix metalloproteases (MMPs), along with plaque neovascularization, microcalcification and remodeling (32–36). Plaque rupture exposes the highly thrombotic necrotic core to the blood (Figure 1-2E). Clinically, this can lead to the sudden onset of major vascular events as the thrombi can occlude the lumen or break off from the plaque and occlude distal arteries in locations such as the heart or brain, presenting clinically as a myocardial infarct or stroke, respectively (5, 29, 36).
A blood vessel consists of three main layers: the outermost, collagen-rich layer (adventitia), the elastic middle layer (media), and the thin, innermost layer (intima) facing the vessel lumen. Each of these layers are separated by elastic membranes and are further characterized by unique cell types (21-25).
Figure 1-2 Atherogenesis

The development of atherosclerosis occurs over several decades, beginning early in life when lipoproteins circulating in the blood enter the arterial intima. Once here, monocytes will differentiate into macrophage and phagocytose accumulated oxidized LDL particles, generating foam cells (A). Continued lipid deposition within the intima generates fatty streaks, resulting in the thickening of the intimal space forming a growing atherosclerotic plaque (B). Plaque development continues with the infiltration and proliferation of smooth muscle cells from the media which begin to secrete collagen to stabilize the growing plaque (C). Cellular apoptosis and the accumulation of debris and cholesterol crystals lead to the formation of a necrotic core (C). Continued secretion of collagen generates a fibrous cap overlaying the growing plaque and conferring stability, while the necrotic core can continue to grow (D). Thinning of the fibrous cap causes destabilization of the plaque and increases its susceptibility to rupture. Plaque rupture exposes the highly thrombotic necrotic core to the blood (E). Thrombi formation can occlude blood vessels and rapidly lead to serious clinical events (26-34).
1.2.2 Lipoproteins

In addition to cholesterol, lipoproteins contain triacylglycerol, phospholipids and proteins, the ratios of which determine the density and thus classification of the lipoprotein. They are spherical particles with an outer hydrophilic monolayer of free cholesterol, phospholipids, and apolipoproteins surrounding a hydrophobic core of triacylglycerol (TG) and cholesteryl ester (CE). Lipoprotein solubility allows for the transportation of insoluble hydrophobic lipids within the circulation throughout the body (37).

In addition to different lipid composition, lipoprotein classes also differ in terms of size, density, and the types of apolipoproteins with which they associate. The largest, lowest density class of lipoproteins are chylomicrons which originate from the intestine and contain predominantly dietary TG with a small amount of CE derived from the diet or reabsorbed biliary cholesterol. Very low-density lipoproteins (VLDL) are synthesized by the liver and its core contains primarily TG plus a greater amount of CE content than chylomicrons. Hydrolysis of chylomicron and VLDL TG result in smaller, denser particles referred to as chylomicron remnants and intermediate-density lipoproteins (IDL), respectively. Collectively, chylomicrons and their remnants, IDL, and VLDL all contain apoB and are referred to as triglyceride-rich lipoproteins (TRLs). Further removal of IDL-TG by hepatic lipase leads to the formation of LDL, which contains a relatively high CE content. Lastly, high-density lipoproteins (HDL) also contains a high CE content but serve a unique function known as reverse cholesterol transport, which delivers cholesterol from peripheral tissues back to the liver for potential excretion as bile (37, 38). Further characterization of the classes of lipoproteins in terms of composition, origin, associated apolipoproteins, and function are described in Table 1-1 and their metabolism is summarized in section 1.3.
Table 1-1 Lipoprotein classification

<table>
<thead>
<tr>
<th>Lipoprotein Class</th>
<th>Composition (% mass)</th>
<th>Source of Production</th>
<th>Associated Apolipoproteins</th>
<th>Primary Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons</td>
<td>84% TG, 8% PL, 6% CE, 2% protein</td>
<td>Intestine</td>
<td>AI, AII, AIV, AV, B48, CI, CII, CIII, E</td>
<td>Delivery of dietary TG</td>
</tr>
<tr>
<td>Very Low Density Lipoproteins (VLDL)</td>
<td>55% TG, 20% PL, 15% CE, 9% protein</td>
<td>Liver</td>
<td>AV, B100, C1, CII, CIII, E</td>
<td>Delivery of endogenous TG</td>
</tr>
<tr>
<td>Intermediate Density Lipoproteins (IDL)</td>
<td>29% TG, 26% PL, 34% CE, 11% protein</td>
<td>VLDL catabolism</td>
<td>B100, CI, CII, CIII, E</td>
<td>Delivery of endogenous TG and CE</td>
</tr>
<tr>
<td>Low Density Lipoproteins (LDL)</td>
<td>8% TG, 22% PL, 50% CE, 20% protein</td>
<td>VLDL/IDL catabolism</td>
<td>B100</td>
<td>Delivery of cholesterol</td>
</tr>
<tr>
<td>High Density Lipoproteins (HDL)</td>
<td>8% TG, 30% PL, 29% CE, 33% protein</td>
<td>Liver and intestine secreted apoAl</td>
<td>AI, AII, AIV, AV, CI, CII, CIII, E</td>
<td>Reverse cholesterol transport from periphery to liver</td>
</tr>
</tbody>
</table>

1.3  Lipoprotein Metabolism

Lipoproteins can either be formed from exogenous dietary lipids in intestinal cells (enterocytes), or from de novo synthesis in liver cells (hepatocytes).

1.3.1  Enteric lipoprotein production from exogenous lipids

Dietary lipids are readily absorbed by small intestine enterocytes for packaging into chylomicrons for transport throughout the body. In the gut, dietary TG is hydrolyzed by pancreatic lipases to free fatty acids and monoacylglycerol while CE is hydrolyzed to a free cholesterol and free fatty acid (39). Following entry into the enterocyte via active and passive transporters, these lipids are re-esterified into TG and CE to be packaged into chylomicrons. Microsomal triglyceride transport protein (MTP) facilitates chylomicron formation by loading lipids onto the scaffolding protein apoB48. The fully-formed, TG-rich chylomicron is secreted from the enterocyte into the lymphatics, eventually entering the bloodstream. Within the lymphatics and circulation, chylomicrons become enriched with apolipoproteins E and C. Now the fully-formed chylomicron particle contains apolipoproteins B48, CI, CII, CIII, and E and will circulate to peripheral tissues for further metabolism (section 1.3.3) (39–42).

1.3.2  Hepatic lipoprotein production

The liver acts as a central mediator of lipid homeostasis by controlling the production of lipoproteins to be secreted for use by the rest of the body. This involves the formation of apoB100 containing particles known as VLDL. TG for export are synthesized from free fatty acids derived from either chylomicrons, adipose tissue TG, or from de novo synthesis (lipogenesis). Cholesterol to be exported can be chylomicron-derived from the exogenous pathway but the majority of cholesterol in the body is synthesized de novo by the liver (reviewed in section 1.4).

Similar to apoB48 and chylomicrons, apoB100 serves as the scaffold for hepatic VLDL synthesis. Newly synthesized apoB100 peptides are rapidly degraded if there is low TG availability. When TG is available, apoB100 is co-translationally lipidated with TG, CE,
and phospholipids by MTP in the endoplasmic reticulum (ER) lumen. The newly assembled VLDL particle can now be secreted from the hepatocyte. Constant synthesis and degradation of apoB peptides in enterocytes and hepatocytes allows for the rapid export of lipoprotein particles when there is a sudden influx of intracellular TG (43).

HDL production occurs in the circulation after the hepatic synthesis and secretion of apoAI. Free apoAI binds to ATP-binding cassette (ABC)-A1 (ABCA1) on the surface of peripheral tissues which facilitates the loading of phospholipid and free cholesterol. This generates a discoidal particle known as pre-β HDL, which can undergo further lipidation by ABCG1 and cholesterol esterification by lethicin-cholesterol acyltransferase (LCAT) generating more mature, spherical HDL particles (44, 45).

1.3.3 Metabolism of circulating lipoproteins

As chylomicrons and VLDL travel through the circulation, lipoprotein lipase (LPL) hydrolyses the lipoprotein TG. LPL resides on the luminal surface of endothelial cells lining capillaries that perfuse adipose, cardiac, and skeletal muscle tissue. Released free fatty acids are taken up by the peripheral tissues, a processes requiring apoCII as an LPL cofactor. As already mentioned, hydrolysis of the core TG within chylomicrons and VLDL results in the formation of a smaller and denser particle referred to as chylomicron remnants and IDL, respectively. IDL can be further metabolized by hepatic lipase to form CE-rich LDL. These lipoproteins remain in the circulation until being cleared by endocytosis at the liver and peripheral tissues. LDL is cleared by the LDL receptor (LDLR) using apoB as the receptor ligand while IDL is cleared by LDLR-related proteins using apoE as the receptor ligand (37, 38, 42, 46).

Circulating HDL has two ways of delivering cholesterol back to the liver for reverse cholesterol transport. First, hepatic scavenger receptor B1 (SRB1) binds to and removes CE from HDL particles. Otherwise, cholesterol ester transfer protein (CETP) mediates a lipid exchange between HDL and TRLs. Specifically, CE from HDL is exchanged for TG from TRLs. This results in CE-rich apoB-containing lipoproteins (such as chylomicron remnants, IDL and LDL) which can subsequently be taken up by the liver. HDL gains TG from the TRLs which can be hydrolyzed by hepatic lipase. TG-rich HDL has reduced
affinity for apoA1 favoring their dissociation. The lipid-poor apoA1 can now be lipidated again by ABCA1 to form new HDL, creating a cycle of HDL-CE transport from peripheral tissues to the liver (13, 37, 38).

1.4 Hepatic Fatty Acid and Cholesterol Metabolism

Hepatic *de novo* lipogenesis of cholesterol and TG both use cytosolic acetyl-CoA as their initial substrate. Acetyl-CoA is not only a substrate for several anabolic processes, it can be derived from a variety of catabolic ones such as glycolysis and fatty acid β-oxidation (47). From acetyl-CoA the lipogenesis pathways diverge and acetyl-CoA enters either the cholesterol or fatty acid biosynthetic pathways.

1.4.1 Cholesterol synthesis

The cholesterol biosynthetic pathway begins with the formation of acetoacetyl-CoA from two acetyl-CoA molecules followed by the addition of another acetyl-CoA molecule to create 3-hydroxy-3-methylglutarate-CoA (HMG-CoA). The next step has a clinical significance that is difficult to overstate. The conversion of HMG-CoA to mevalonate by HMG-CoA reductase (HMGCR) represents both the rate-limiting and first committed step of cholesterol and isoprenoid biosynthesis. This made way for the discovery of HMGCR inhibitors, known as statins that are in widespread clinical use today (reviewed in section 1.7.1). Mevalonate is converted into isoprene units which are subsequently polymerized into a 30-carbon long squalene chain. Finally, squalene is cyclized followed by additional modifications to yield a 27-carbon cholesterol molecule (48, 49).

1.4.2 Fatty acid synthesis

Fatty acid synthesis by *de novo* lipogenesis also begins with acetyl-CoA which is carboxylated by acetyl-CoA carboxylase (ACC) to form malonyl-CoA. The fatty acid synthase (FAS) complex adds additional units of acetyl-CoA to malonyl-CoA to generate an acyl-CoA. With subsequent additions of acetyl-CoA, FAS generates the 16-carbon acyl-CoA known as palmitic acid. Palmitic acid can undergo unsaturation or further elongation followed by esterification with glycerol to form monoacylglycerol. The further esterification of one or two more fatty acids results in the formation of
diacylglycerol and triacylglycerol, respectively. Triacylglycerol, or TG is the primary means of fatty acid storage and transport, as the hepatocyte can store them in lipid droplets or export them as VLDL (2, 50, 51).

1.4.3 Sterol response element-binding proteins

Another important layer in the regulation of lipid synthesis is the sterol response element-binding protein (SREBP) pathway. SREBP is a transcription factor encoded by two genes, SREBP1 and SREBP2. The former generates two isoforms due to alternative splicing, SREBP-1a and SREBP-1c. Generally, the active SREBP-1c protein regulates genes involved in the synthesis of fatty acids while SREBP-2 deals more so with cholesterol synthesis gene expression and SREBP-1a regulates both (52).

SREBPs are translated into their full length, endoplasmic reticulum-bound precursor form where they remain in proximity to the SREBP-cleavage activating protein (SCAP) and are held in place by the insulin-induced gene (Insig). When intracellular sterols are low or depleted, Insig releases SCAP which translocates the membrane-bound SREBP to the Golgi apparatus. Here proteolytic processing releases the SREBP protein from the Golgi membrane in its active form. It can now translocate to the nucleus where it promotes lipogenic gene expression by recognizing sterol receptor elements (SREs) within the promotors of target genes. Additionally, active SREBPs can further upregulate their own gene expression due to SREs located within their own promoter regions (52, 53).

1.4.4 Fatty acid oxidation

Hepatocytes process intracellular free fatty acids by both esterification into TG (described above) and also through fatty acid β-oxidation. In a process similar to the reverse of fatty acid synthesis, β-oxidation breaks down free fatty acids in cycles, where the fatty acid is shortened by one acetyl-CoA unit at a time. This acetyl-CoA can then be fully broken down to carbon dioxide and water in the tricarboxylic acid cycle or condensed into ketone bodies and secreted into the circulation (54).
To protect cells against fatty acid-induced lipotoxicity (see section 1.6.3.1), fatty acid β-oxidation is tightly-regulated in conjunction with esterification into TG for either storage or export as VLDL. β-oxidation can only occur inside the mitochondria matrix and while short and medium chain fatty acids can freely diffuse through the mitochondrial membrane, long-chain fatty acids (such as palmitoyl-CoA) cannot. Instead, carnitine palmitoyl transferase (CPT)-1α must first convert the long-chain acyl-CoA to an acyl-carnitine to allow for transfer across the mitochondria membrane. Once inside the mitochondria matrix CPT2 converts the acyl-carnitine back into a long-chain acyl-CoA, which can now undergo β-oxidation (16, 55).

The rates of de novo fatty acid synthesis and fatty acid β-oxidation are coordinated in such a way as to prevent futile cycling where a free fatty acid is broken down into acetyl-CoA subunits just to be re-synthesized back into a free fatty acid. This regulation is mediated by malonyl-CoA, the product of ACC from the first step of fatty acid synthesis (section 1.4.2). CPT1α is inhibited by malonyl-CoA, thus suppressing β-oxidation by preventing long-chain acyl-CoA from entering the mitochondria matrix (54, 56). This coordination between the rates of fatty acid synthesis and β-oxidation is important for proper regulation of energy flux, in instances such as transitioning from fasting to feeding.

In addition to the mitochondria, peroxisomes are a second site of β-oxidation. Very long-chain fatty acids, typically greater than 20 carbons, must be metabolized by peroxisomal β-oxidation. This process generates a shorter acyl-CoA which can subsequently undergo mitochondrial β-oxidation. Acyl-CoA oxidase (ACO) is the rate-limiting enzyme in peroxisomal β-oxidation and ACO deficiency results in the accumulation of very long chain fatty acids (57–59).

1.5 Hepatic Insulin Signaling

Insulin is a peptide hormone essential for driving the body’s metabolic response following the ingestion of a meal. It is secreted by pancreatic β-cells into the circulation after feeding and signals to peripheral insulin-sensitive tissues such as adipose, liver, and muscle. Insulin signaling is initiated when insulin binds to and activates the insulin
receptor (IR) activating its tyrosine kinase and promoting phosphorylation of the insulin receptor substrate (IRS). This allows for docking of proteins containing Src homology 2 (SH2) domains such as phosphoinositol 3-kinase (PI3K) which becomes active. PI3K activates phosphoinositide-dependent kinase 1 (PDK1) which phosphorylates and activates Protein Kinase B (AKT), a central mediator of the metabolic effects of insulin. Active AKT phosphorylates different targets relating to glucose and lipid metabolism (Figure 1-3, sections 1.5.1, 1.5.2) (50, 60, 61).

1.5.1 Regulation of glucose metabolism

The primary function of insulin on the liver is to suppress hepatic gluconeogenesis during the fed state. Active AKT phosphorylates and inhibits the transcription factor forkhead box-O1 (FOXO1) resulting in its exclusion from the nucleus. This prevents FOXO1-mediated transcription of gluconeogenic genes such as glucose 6-phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PEPCK) (Figure 1-3). However, this model does not explain how insulin can acutely regulate gluconeogenesis since it has been known for some time that insulin rapidly suppresses hepatic glucose output well before any changes in both G6P or PEPCK protein expression are observed (60). Very recent evidence suggests that this could be explained by effects of insulin outside the liver. An additional function of insulin is to inhibit adipose tissue lipolysis, thus reducing the flux of free fatty acids and glycerol to the liver. Perry, et al. (62) demonstrate that adipose tissue-derived acetyl-CoA (from free fatty acid β-oxidation) and glycerol serve as the primary gluconeogenic substrates used by the liver. Thus, in response to insulin administration, rapid inhibition of adipose tissue lipolysis was responsible for the acute suppression of hepatic glucose production. More simply put, insulin acutely suppresses hepatic gluconeogenesis through an extra-hepatic mechanism.

Another function of hepatic insulin signaling relating to glucose metabolism is the stimulation of glycogen synthesis. AKT phosphorylates and inhibits glycogen synthase kinase-3 (GSK3), which is an inhibitor of glycogen synthase (GS). Therefore, GS is active and under fed conditions will convert glucose into glycogen for storage (63) (Figure 1-3).
**Figure 1-3 Hepatic insulin signaling**

Insulin binds to the insulin receptor (IR) leading to activation of insulin receptor substrates (IRS) by tyrosine phosphorylation (pY). This leads to activation of phosphoinositol 3-kinase (PI3K) which recruits Protein Kinase B (AKT) inducing its serine (pS) and threonine (pT) phosphorylation by phosphoinositide-dependent kinase 1 (PDK1). Active AKT phosphorylates several targets which regulate glucose and lipid metabolism. Forkhead Box-O1 (FOXO1) phosphorylation results in its nuclear exclusion preventing it from upregulating gluconeogenic gene expression. Proline-rich AKT substrate of 40 kDa (PRAS40) and tuberous sclerosis complex 2 (TSC2) phosphorylation blocks inhibition of mammalian target of rapamycin complex 1 (mTORC1) which can activate the lipogenic transcription factor sterol regulatory element binding protein-1c (SREBP1c). Additionally, AKT also promotes glycogen synthesis by phosphorylating and inhibiting glycogen synthase kinase-3 (GSK3), which is an inhibitor of glycogen synthase (GS).
1.5.2 Regulation of lipid metabolism

Hepatic insulin signaling also triggers *de novo* lipogenesis (section 1.4) through the P13K-AKT pathway, especially during periods of excess nutrient availability. When AKT is phosphorylated and activated by insulin it phosphorylates a number of targets ultimately leading to increased activity of the lipogenic transcription factor SREBP-1c (64). Insulin increases *SREBP-1c* expression through the activation of the mammalian target of rapamycin complex (mTORC)-1. This is done by AKT phosphorylating and inhibiting two upstream inhibitors of mTORC1, proline-rich AKT substrate of 40 kDa (PRAS40) and tuberous sclerosis complex 2 (TSC2) (Figure 1-3) (64).

Additionally, insulin inhibits lipoprotein secretion, even while stimulating *de novo* lipogenesis via inhibition of FOXO1. Besides promoting gluconeogenic gene expression, FOXO1 also promotes the transcription of *MTTP* and *APOC3*. The MTP protein (described in sections 1.3.2 and 1.3.3) is responsible for the assembly of lipoproteins by loading TG onto apoB. Reducing MTP expression thus limits lipoprotein synthesis and secretion. ApoCIII is thought to play a role in subsequent lipoprotein synthesis and secretion, so limiting its expression can further inhibit lipoprotein production (65, 66).

1.5.3 Insulin resistance and Type 2 Diabetes

Insulin resistance is one of the components of the metabolic syndrome and it describes the state where tissues fail to appropriately respond to insulin. The pancreas compensates for this by increasing insulin production, creating a state known as hyperinsulinemia. With time this can develop into Type 2 Diabetes. The body now cannot regulate glucose homeostasis due to an inability to synthesize insulin. Extreme hyperglycemia ensues which is life-threatening and must be managed with exogenous insulin injections (67).

The result of insulin resistance with hyperinsulinemia depend on the organ system effected. In adipose tissue, insulin fails to suppress TG lipolysis, thus increasing circulating free fatty acids. In muscle, insulin fails to upregulate glucose transporter type 4 (GLUT4) translocation to the cell membrane limiting muscle glucose uptake resulting in hyperglycemia. Most notably, in liver insulin fails to suppress gluconeogenesis while continuing to stimulate lipogenesis, creating the phenotype seen in patients with diabetes.
of hyperinsulinemia, hyperglycemia, and hyperlipidemia (68). This paradoxical effect was suggested to be due to a bifurcation in the insulin signaling pathway, where AKT fails to inhibit FOXO1 and gluconeogenic gene expression while continuing to stimulate the mTORC1-SREBP1c lipogenic pathway (69-72). However, insulin resistance is typically thought to occur by serine phosphorylation of IRS1, an inhibitory event which dampens insulin signaling through the PI3K-AKT pathway, which should in theory effect all targets of AKT, not just FOXO1. Insulin resistance characterized by IRS-1 serine phosphorylation occurs via multiple mechanisms such as MAPK signaling (a negative feedback effect of insulin signaling) and protein kinase C-ε (PKCε) activation by lipotoxic intermediates such as diacylglycerol (DAG) (2, 50, 67, 73). More recent evidence suggests that the observed stimulation of both gluconeogenesis and lipogenesis despite reduced insulin sensitivity may be a result of increased nutrient flux to the liver, as is seen with insulin resistance (60, 62, 74, 75). Indeed, the increased adipose tissue-derived free fatty acid flux to the liver seen in insulin resistance has been shown to be the major driver of hepatic TG synthesis (75) and glucose production (62) independent of insulin signaling.

1.6 Hepatic Steatosis and Inflammation

Tightly linked to conditions such as insulin resistance, obesity, and dyslipidemia is the process of hepatic steatosis, or the accumulation of fat in the liver. Also factoring into these metabolic conditions and CVD risk factors is inflammation, although the role this plays is much more difficult to define.

1.6.1 Normal liver biology and morphology

The liver is responsible for many functions vital for survival. In addition to regulating glucose, lipid, and lipoprotein metabolism (discussed in previous sections), other functions involve synthesis (bile acids, albumen, clotting factors, hormones, other proteins), storage (glycogen, vitamins), and metabolism (prodrugs, toxins, ammonia, bilirubin). While not fully comprehensive, this list does demonstrate some of the functions that the liver is involved in such as digestion, oncotic pressure maintenance,
clotting, nutrient storage, drug metabolism, detoxification, the urea cycle, and the removal of other waste (76, 77).

So far, when referring to the liver it is usually the hepatocyte being described. Hepatocytes make up approximately 80% of the liver by mass and are responsible for most functions attributed to the liver. However, the liver has a very complex morphological structure which contains several additional cell types organized into lobules (Figure 1-4). The hepatic artery and portal vein deliver blood from the arteries and intestine, respectively. These arrange alongside bile ducts forming what are called portal triads. Blood flow from both the hepatic artery and portal vein enters the region known as the sinusoid, which drains to the central vein and exits the liver. Liver lobules are organized in hexagonal structure with six portal triads surrounding a central vein (Figure 1-4).

The lobule structure seen in the liver consists of several cell types in addition to hepatocytes, shown in Figure 1-4. First, sinusoid endothelial cells make up the lining of the vasculature, separating hepatocytes from the sinusoid, where blood flow occurs (78). The resulting area between the hepatocytes and sinusoid endothelial cells is referred to as the space of Disse. Cholangiocytes are biliary epithelial cells lining the bile ducts into where hepatocytes secrete bile acid and cholesterol for digestion by facilitating micelle formation and lipid absorption (79). Kupffer cells (or Ido cells) are liver-specific macrophage thought to originate from circulating monocytes and thus are for host immune defense (80). Hepatic stellate cells are important for the storage of most of the body’s retinoids (such as vitamin A). Additionally, during liver injury they transition into an “activated” state characterized by increased proliferation, contractility, and collagen secretion, contributing to liver fibrosis (81). These are the major cell types involved in liver function but there are several others including lymphocytes, neuroendocrine cells, blood cells, and smooth muscle cells (76).

Due to the highly lipogenic nature of the liver, it can store excess lipid as TG-rich lipid droplets. Hepatocyte lipid balance, or lipid droplet content is determined by four main processes (82):
1) Delivery of exogenous lipid to the liver (section 1.3.1). This would include lipid from circulating free fatty acids and apoB-containing lipoproteins.

2) *De novo* synthesis of lipid within the liver (section 1.4.2).

3) Export of lipid by VLDL secretion (section 1.3.2).

4) Metabolism of lipid by fatty acid β-oxidation (section 1.4.4).

The rates of 1) and 2) contribute to hepatic lipid storage which is opposed by lipid removal through the rates of 3) and 4). Hepatic TG is relatively inert and not particularly harmful. In contrast, free fatty acids and other lipid intermediates can result in lipotoxicity, so hepatocytes will promote their esterification in TG. However, hepatocytes have a limited capacity to store TG-rich lipid droplets before hepatic dysfunction can start to occur.
Figure 1-4 Liver cell types and structure

The liver and its various cell types are arranged into structures known as lobules. The hepatic artery and portal vein deliver blood from the arteries and intestine, respectively, which flows into the sinusoid. The bile duct is located near the hepatic artery and portal vein to form a portal triad. Blood flow through the sinusoid drains to the central vein and exits the liver. Liver lobules are organized in hexagonal structure with six portal triads surrounding a central vein (74-77).
1.6.2 Non-alcoholic fatty liver disease

Hepatic steatosis occurring in the absence of alcohol is clinically referred as non-alcoholic fatty liver disease (NAFLD). If the rates of lipid delivery to the liver and de novo lipogenesis within the liver are not matched by rates of fatty acid β-oxidation and VLDL secretion, then hepatocytes will increase lipid droplet formation and NAFLD will ensue. NAFLD is thought to be the hepatic manifestation of the metabolic syndrome due to its tight association with obesity, dyslipidemia, and type 2 diabetes (83). NAFLD encompasses a spectrum of disorders from simple steatosis to steatohepatitis (covered in the next section) which can progress to more severe conditions such as cirrhosis, which is irreversible and greatly increases the risk of developing hepatocellular carcinoma (83).

NAFLD is largely asymptomatic in its early stages, especially when present as only simple steatosis. The gold standard for diagnosis is a liver biopsy, despite efforts to develop less invasive diagnostic methods. Liver biopsy is subject to sampling error, subjective interpretation of results, and a small chance of complications (83–85). Developing means of non-invasive NAFLD diagnosis has been met with varying degrees of success and include imaging modalities, plasma markers, and scoring algorithms. The major drawbacks of these methods is both low sensitivity (false negatives) and specificity (false positives). Imaging can be done with ultrasound, computed tomography (CT) or most accurately with magnetic resonance imaging (MRI) (86) as well as derivations of these technologies such as transient elastography, or FibroScan® (87). Plasma markers that can be looked at to assess liver function include alanine and aspartate transaminases (ALT and AST, respectively), albumen, platelet count, glucose, insulin, TG, and cholesterol (85, 86). There are also many scoring algorithms which take these plasma measurements into account, in addition to age, gender, body mass index, insulin resistance and glycemia, hypertension, and the results from the imaging tools previously mentioned (85, 86). All of these methods for the diagnosis of NAFLD, or more severe steatohepatitis and fibrosis, are susceptible to giving false positive results (especially with comorbidities) or simply failing to detect NAFLD, especially when in its earliest stages. By the time clinicians are presented with enough evidence to warrant a biopsy, the liver
disease could have progressed to much more advanced and serious stages, described in the next section.

1.6.3  Non-alcoholic steatohepatitis and inflammation

NAFLD progression to its most serious form, termed nonalcoholic steatohepatitis (NASH), is in most general terms hallmarked by increased inflammation and fibrosis (86). Histologically it presents as simple steatosis (as is the case for NAFLD) with the additions of hepatocyte ballooning, monocyte infiltration, and collagen deposition (not seen with early NAFLD) (88). The increase in fibrosis and inflammation seen with NASH greatly increases one’s risk to progress to hepatocellular carcinoma and liver failure. The features of NASH stem from the lipid imbalances which characterized NAFLD but when left unchecked these further lead to NASH through several potential mechanisms. These include lipotoxicity, endoplasmic reticulum (ER) stress, oxidative stress, immune activity, and endotoxin and cytokine signaling (86), each described briefly below.

1.6.3.1  Lipotoxicity

When the liver’s mechanisms for mitigating the accumulation of free fatty acids become overwhelmed, lipotoxicity ensues. Excess fatty acids can induce ER stress, reactive oxygen species (ROS) production and oxidative stress, and insulin resistance from lipotoxic intermediates such as diacylglyceride (DAG) and ceramides (89, 90). These mechanisms collectively increase hepatic inflammation, cellular apoptosis and necrosis, fibrosis, and the progression of NAFLD to NASH.

1.6.3.2  ER stress

Membrane and secreted proteins are synthesized and folded within the ER lumen. When the accumulation of unfolded proteins exceeds the ER’s capacity, ER stress ensues. The ER stress response, or unfolded protein response (UPR) is an adaptive mechanism for cells to restore intracellular homeostasis. Activation of the UPR results in an overall reduction in protein synthesis, increased lipogenesis for ER membrane expansion,
induction of autophagy to recycle unfolded proteins, and the activation of various inflammatory cascades (89, 91).

1.6.3.3 Reactive Oxygen Species

An increase in reactive oxygen species (ROS) can lead to oxidative stress. ROS can be generated by lipotoxicity and the UPR which add to the already high ROS production by hepatocyte fatty acid oxidation (89). Excess oxidative stress can lead to nuclear and mitochondrial DNA damage and elevated proinflammatory cytokine production (89).

1.6.3.4 Immune responses

The innate immune system plays a major role in NASH development and progression to more serious diseases of the liver. The temporal relationship of the key events that culminate in NASH are not well understood. During the development of NASH, there is an increase in infiltration of circulating immune cells while endogenous Kupffer cells transition to a proinflammatory or “activated” phenotype. Possibly driving this is the chronic, low-grade inflammation and elevations in circulating cytokines seen during obesity, insulin resistance, and the metabolic syndrome (80). Additionally, dietary components (such as fats and fructose) can augment the microbiota within the gut and increase the production of endotoxins such as lipopolysaccharide (LPS). The same dietary factors can increase gut permeability and lead to elevations in circulating endotoxin concentrations (80, 92). Via the portal vein, endotoxins from the gut will first go to the liver, where LPS activates cell surface receptors triggering an inflammatory response (93).

The inflammatory responses of Kupffer cells and other immune cell types throughout the body are best characterized by an increase in the production and secretion of cytokines and chemokines (94). Secreted cytokines trigger further inflammatory signaling in liver cells, the effects of which depend on the cell type. Kupffer cells will increase cytokine production creating a feed-forward effect. Hepatic stellate cells will enter into their activated state where proliferation and collagen synthesis is increased, leading to fibrosis. In hepatocytes, cytokines can promote lipid accumulation and apoptosis. Importantly, hepatocyte apoptotic bodies and damage-associated molecular pattern
molecules (DAMPs) will further trigger inflammatory signaling in the surrounding cells. Chemokines promote the recruitment circulating immune cells so in the context of NASH, chemokines with increase the hepatic infiltration of immune cells (80, 94, 95).

1.6.4 Inflammatory signaling pathways

The many stimuli described in the previous section can trigger intracellular signaling pathways within hepatocytes, Kupffer cells, and other liver cell types which can further elevate the liver’s inflammatory status. These pathways are sensitive to the various stimuli implicated in NAFLD and NASH (lipotoxicity, ER and oxidative stress, cytokine signaling, etc.) and govern stimuli responses (apoptosis, cytokine production, etc.). The primary molecular pathways that will be discussed here are mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB).

1.6.4.1 MAPK pathways

The MAPK pathways are integral to a large number of cellular processes due to the vast number of signals they detect, and responses they elicit. The MAPK proteins represent a family of serine and threonine kinases which are often described according to one of three subgroups: extracellular signal-regulated kinases (ERKs), c-Jun N–terminal kinases (JNKs), and p38 MAPKs. Each subgroup contains multiple isoforms, as shown in Figure 1-5.

MAPK stimuli include hormones, growth factors, cytokines, DAMPs, pattern associated molecular pattern molecules (PAMPs), and environmental stress. They are activated by upstream MAPK kinase (MAP2K) phosphorylation of conserved threonine/tyrosine residues, which follows prior activation by MAPK kinase kinases (MAP3Ks). This three tier kinase hierarchy represents what is be the classic model of MAPK signaling. The numerous functions of MAPK signaling will not be discussed in detail but how the ERK, JNK, and p38 pathways pertain to inflammation and metabolic dysfunction will be briefly described below.

The ERK proteins were the first MAPKs to be described and exist in two isoforms, ERK1 and ERK2. As their name suggests, they were originally found to have a role in mitogen
signaling including some of the mitogenic effects of insulin. In addition to their key role in growth and differentiation, ERKs have since been found to play a role in innate immunity and inflammation. The JNK proteins exist in three isoforms, JNK1/2/3 and were discovered for their ability to phosphorylate the c-Jun transcription factor, regulating the function of the activating protein 1 (AP1) family of transcription factors. Compared to ERK1/2, JNK1/2/3 is much more sensitive to environmental stresses and proinflammatory signaling. Lastly, the p38 MAPKs exist as α, β, γ, and δ isoforms and like the JNKs, have a greater sensitivity to stress and inflammatory signaling.

Collectively, the MAPKs pathways respond to inflammatory stimuli and environment stresses to augment insulin sensitivity and inflammatory responses. Insulin sensitivity is reduced by IRS-1 serine phosphorylation by MAPKs (see section 1.5.3). Inflammatory responses are augmented though transcriptional regulation via AP1 heterodimers. Specifically, AP1 dimers can consist of a number of combinations of proteins such as ATF2, ELK1, SAP1a, c-Jun, and c-Fos. These dimer combinations determine the promoter targets of AP1, allowing AP1 to regulate a number of processes such as proinflammatory gene expression. MAPKs can phosphorylate these proteins, altering their activity or dimer stability (Figure 1-5) (96, 97).

1.6.4.2 NF-κB pathway

The NF-κB pathway transcriptionally regulates anti-apoptotic and proinflammatory processes. Most notably, active NF-κB directly increases the cytokine and chemokine gene expression. Extracellular signals such as cytokines, PAMPs, and DAMPs bind to cell surface receptors including toll-like receptors (TLRs) and tumor necrosis factor receptors (TNFRs). Signaling through these receptors can activate MAPK and NF-κB pathways. NF-κB is normally sequestered within the cytoplasm by inhibitor of NF-κB-α (IκBα) where it is unable to bind the promoters of target genes. Inflammatory signals from TLRs or TNFRs activate the inhibitor of IκBα kinase (IKK) complex, consisting of α, β, and γ subunits. Activation of IKKβ through phosphorylation leads to it phosphorylating IκBα. Phosphorylated IκBα leads to its ubiquitination and degradation, thus releasing NF-κB which can migrate to the nucleus and promote inflammatory gene expression (98, 99) (Figure 1-5).
Figure 1-5 General overview of inflammatory signaling pathways

Intracellular signaling pathways activated in response to different types of inflammatory stimuli. Extracellular stimuli can include cytokines such as tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and foreign DNA, damage associated molecular patterns (DAMPs) such as oxidized LDL (oxLDL) and ATP, free fatty acids (FFAs), and various growth factors. Receptors for these stimuli include the IL-1β receptor (IL-1βR), TNF receptor (TNFR), and toll-like receptor (TLR) families. Intracellular inflammatory stimuli and damage includes FFAs, diacylglyceride (DAG), ceramides, reactive oxygen species (ROS), endoplasmic reticulum (ER) stress, and ultraviolet (UV) light. Both intracellular stimuli and extracellular-receptor mediated stimuli can activate the mitogen activated protein kinase (MAPK) pathways. The MAPK pathways are activated at the levels of the MAPK kinase kinases (MAP3Ks) which activate the MAPK kinases (MAP2Ks) by serine/threonine phosphorylation. MAP2Ks activate the MAPK proteins by threonine/tyrosine phosphorylation. These include three main families: p38 MAPKs, c-Jun N–terminal kinases (JNKs), and extracellular signal-regulated kinases (ERKs). When activated these can regulate many transcription factors by serine/threonine phosphorylation. These factors form activating protein 1 (AP1) heterodimers (such as Jun-Jun, Jun-Fos, and Jun-ATF) which can regulate inflammatory gene expression. Activated MAPKs can also phosphorylate the insulin receptor substrate (IRS) at serine residues, leading to decreased activity and insulin resistance. Another major pathway is through the nuclear factor-κB (NF-κB) transcription factor, which is normally bound to inhibitor of NF-κB-α (IκBα) and sequestered in the cytosol. The inhibitor of IκBα kinase (IKK) family phosphorylates IκBα, causing its ubiquitination and degradation, releasing NF-κB, allowing it to translocate to the nucleus and regulate inflammatory gene expression. Figure created using references (80, 96–114).
**Ligands:**
- Cytokines (TNFα, IL-1β)
- PAMPs (LPS, foreign DNA)
- DAMPs (ox-LDL, ATP)
- FFAs, Growth Factors

**Receptors:**

![Diagram of immune responses and signaling pathways](image)

- **IL-1βR**
- **TNFR**
- **TLR**

**Pathways:**
- **IKKαβγ**
- **MAP3Ks**
- **MAP2Ks**
- **MAPKs**
  - p38
  - JNK 1/2/3
  - ERK 1/2

**Signaling Consequences:**
- **NF-κB**
- **AP1 heterodimers**

**Nucleus**

**Inflammatory gene expression**

**Cytokines, Chemokines**
1.7 Modulating Lipoprotein Metabolism - Current and Potential Drug Targets

Efforts to lessen the onset of atherosclerotic CVD have primarily led to the development of therapies to lower LDL-C levels. An understanding of the molecular pathways that determine how the body maintains lipid homeostasis has offered clues as to how these pathways can be manipulated for therapeutic benefit. Indeed, this has allowed for target-based drug design to develop several different therapies – some very successful while others not, and still many more whose clinical potential have yet to be determined.

1.7.1 HMG-CoA reductase

As mentioned in section 1.4.1, HMGCR catalyzes the conversion on HMG-CoA to mevalonate, the rate-limiting step of cholesterol biosynthesis. Statins inhibit hepatic HMGCR and thus decrease the intracellular concentrations of cholesterol. This results in the activation of the sterol-sensing SREBP-2, which upregulates genes involved in cholesterol synthesis and uptake such as \textit{HMGCR}, \textit{PCSK9}, and most importantly \textit{LDLR}. The increase in membrane expression of the LDLR enhances the clearance of apoB100-containing lipoproteins and thus lowers plasma LDL-C levels (52, 115). Clinically, statins have demonstrated robust efficacy in preventing CVD and death in several large cardiovascular outcomes trials and thus are currently the standard of care (8, 10, 116).

1.7.2 Proprotein convertase subtilisin/kexin-9

Proprotein convertase subtilisin/kexin 9 (PCSK9) has gone from its initial discovery to late stage clinical development all in the last 15 years (117). It was first identified in patients who inherited a gain-of-function mutation resulting in hypercholesterolemia and a reduction in LDLR expression (118). Conversely, it was also shown that patients with loss-of-function mutations had much lower LDL-C and showed protection from CVD (119). The PCSK9 protein is secreted predominantly from hepatocytes into the circulation, where is binds to the LDLR. When the LDLR is internalized while bound to PCSK9, it is degraded instead of being recycled back to the cell membrane surface. Therefore, total LDLR expression is decreased causing an increase in circulating LDL-C (120, 121) Since its discovery, monoclonal antibodies have been developed against the
PCSK9 protein which have been shown to be very effective in lowering LDL-C, even as much as 60% (122). Interestingly, statins are known to induce SREBP2 activation causing an increase in PCSK9 and LDLR expression, where inhibition of the latter with the former likely serving as a negative feedback mechanism. Therefore, PCSK9 inhibition could negate this effect of increased PCSK9 expression and thus work synergistically in combination with statins to further increase hepatic LDLR expression and lower LDL-C (117, 121). Large cardiovascular outcomes trials are currently underway to test if PCSK9 inhibition can protect against CVD.

1.7.3 Niemann-Pick C1-like protein 1

Dietary and biliary cholesterol absorption in the gut takes place at the brush border of small intestinal cells. Mediating this process is the enterocyte membrane protein Niemann-Pick C1-like protein 1 (NPC1L1) (123). Ezetimibe inhibits NPC1L1 and thus reduces intestinal cholesterol absorption and transport to the liver. In the liver this results in increased LDLR expression, a mechanism similar to that of statins. Ezetimibe as monotherapy does not lower LDL-C as much as a statin, however it can further lower LDL-C when used as an add-on to statin therapy (124). Ezetimibe has been in use for some time but whether its incremental benefit in LDL-C lowering as a combination therapy translates into improved cardiovascular outcomes was not known until recently. Compared to simvastatin monotherapy, ezetimibe plus simvastatin gave a small but significant reduction in LDL-C and improved cardiovascular outcomes (125).

1.7.4 Cholesterol ester transfer protein

As mentioned in section 1.3.4, CETP facilitates the transfer of CE from HDL to TRLs in exchange for TG. Preventing this exchange results in an elevation in HDL-C while also lowering LDL-C, so several efforts have been made to therapeutically inhibit CETP. The first of these attempts was with Pfizer’s torcetrapib, which indeed did increase HDL-C while also lowering LDL-C in up to Phase III clinical trials. However, in late 2006 Pfizer announced that it was terminating all further development of torcetrapib and in 2007 it was revealed that torcetrapib increased CVD events by 25% compared to placebo (126). This effect was attributed to an increase in blood pressure caused by torcetrapib and not a
result of CETP inhibition, *per se* (127). It was still an open question whether the so-called “HDL hypothesis” (raising HDL-C) was still valid and specifically if CETP inhibition could still be viable for the prevention of CVD. Further development of three other CETP inhibitors continued after confirming the absences of any effects on blood pressure (128). In 2012 Hoffmann-La Roche halted development of dalcetrapib (129) and in 2015 Eli Lilly did the same with evacetrapib (130) both citing futility due to a lack of efficacy in preventing CVD. This leaves Merck’s anacetrapib as the remaining CETP inhibitor still in late stage clinical development (131) although there is a lack of consensus about whether it should or will remain so (132, 133).

These disappointing clinical findings have led to investigators taking a closer look at the evidence for CETP inhibition. Strong genetic evidence exists for loss of function variants resulting in increased HDL-C. However, it is still not known if HDL concentrations play a causal role in CVD development or if it is just a risk marker, and if raising HDL-C via CETP inhibition translates into CVD protection (133). Early studies showed that CETP gene transfer in mice increased atherosclerosis (134, 135) while CETP inhibition in rabbits was atheroprotective (136–138). However, other studies have found the opposite to be true (139, 140). In light of the inconsistencies in the animal studies it is important to note that mice do not express CETP and studies where mice express transgenic human CETP are unlikely to be representative of normal human metabolism. Additional studies have looked at plasma CETP function and concentration and its relationship with CVD risk. The six prospective studies that have looked at this have all showed an inverse relationship between CVD incidence and CETP activity (141–146). So if low CETP activity actually increases cardiovascular risk, it is hard to see the rationale for continued development of CETP inhibitors and has led some to argue that continuing clinical trials poses a risk to patients (147, 148).

### 1.7.5 AMP-activated protein kinase

AMP-activated protein kinase (AMPK) is a cell-autonomous sensor of cellular energy status and regulator of metabolic homeostasis. It is a highly conserved heterotrimeric serine/threonine kinase. Each heterotrimer consists of an α catalytic subunit, β scaffolding subunit, and γ regulatory subunit, with multiple isoforms existing for each (α1, α2, β1,
β2, γ1, γ2, γ3). These isoforms allow for the formation of up to 12 potential heterotrimers, and expression patterns differ in a tissue and species dependent manner. For example, α1β2γ1 and α2β2γ1 are the predominant isoforms in human liver and muscle, respectively, while α2β1γ1 predominates in rat liver (149, 150). AMPK is acutely sensitive to changes in intracellular energy status due to its ability to detect changes in AMP:ADP:ATP ratios. In response to low intracellular energy status under conditions such as hypoxia or nutrient deprivation, AMPK is activated by threonine-172 phosphorylation of the α subunit along with AMP binding to the γ subunit. Active AMPK phosphorylates a myriad of targets, triggering several metabolic responses (Figure 1-6).
Figure 1-6 Hepatic AMPK targets

Activated AMPK phosphorylates many targets to regulate cellular energy balance. Activated catabolic processes include autophagy, mitochondrial biogenesis, and fatty acid \( \beta \)-oxidation. Inhibited anabolic processes include cholesterol synthesis, fatty acid synthesis, protein synthesis, and gluconeogenesis. ACC, acetyl-CoA carboxylase; CRCT, cAMP response element binding protein-regulated transcription coactivator; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; mTORC1, mammalian target of rapamycin complex 1; PGC-1\( \alpha \), peroxisome proliferator-activated receptor \( \gamma \) coactivator 1-\( \alpha \); RAPTOR, regulatory associated protein of mTOR; SREBP, sterol regulatory element binding protein; TSC, tubular sclerosis complex; ULK, unc-5 like kinase.
Ultimately, AMPK signaling augments cellular metabolism towards catabolic, ATP-producing pathways, while concomitantly inhibiting non-essential anabolic, ATP-consuming pathways. Therefore, AMPK activation can promotes processes such as fatty acid β-oxidation and glycolysis while suppressing fatty acid synthesis and gluconeogenesis, making it a compelling target for treating conditions relating to cardiovascular disease, diabetes, inflammation, and even cancer (151–153). However, there are no therapies currently in clinical use that directly target AMPK. There is evidence that AMPK plays a role in the mechanism of action of several widely used drugs such as the aspirin metabolite salicylate (154–156) and metformin (157–160).

Of the many targets of AMPK, the most notable is possibly acetyl-CoA carboxylase (ACC) due to its key roles in fatty acid synthesis and β-oxidation (sections 1.4.2 and 1.4.4, respectively). Specifically, AMPK phosphorylation of ACC reduces its ability to produce malonyl-CoA, thus providing less substrate for fatty acid synthesis while also relieving inhibition on fatty acid β-oxidation. Malonyl-CoA inhibits Cpt1α and β-oxidation, and decreases in malonyl CoA relieves this inhibition. Another target is HMGCR, which like ACC is also inhibited by AMPK phosphorylation. However, although these two proteins were the first discovered targets of AMPK (161, 162), there is still very little understanding of the physiological relevance of HMGCR inhibition by AMPK phosphorylation (163). The dual inhibition of ACC and HMGCR by AMPK likely explains its ability to limit both fatty and cholesterol synthesis, respectively.

1.7.6 ATP-citrate lyase

ATP-citrate lyase (ACL) is responsible for the synthesis of cytosolic acetyl-CoA, a central metabolite in many metabolic pathways. It utilizes cytosolic citrate, CoASH, and ATP to generate acetyl-CoA plus oxaloacetate. Cytosolic citrate can come from transport out of the mitochondria, since it as a citric acid cycle intermediate, or can be synthesized within the cytosol from glutamate. As mentioned in section 1.4, cytosolic acetyl-CoA is a substrate for both fatty acid and cholesterol synthesis (Figure 1-7). This has been demonstrated with ACL knockout models and inhibitors resulting in suppressed de novo
fatty acid and cholesterol synthesis. Additionally, ACL can be nutritionally regulated by two mechanisms. First, its gene is an SREBP target and so it is upregulated in response to insulin. Second, it has been shown to be phosphorylated and activated by AKT (164–168).
Figure 1-7 Mitochondrial and cytosolic acetyl-CoA as both a substrate and product

Hepatic acetyl-CoA exists in multiple intracellular pools. The mitochondrial and nucleo-cytosolic pools are the most significant but there does exist peroxisomal and intrareticular pools as well (not shown). Mitochondrial acetyl-CoA can be derived from many sources including pyruvate (from glycolysis), free fatty acids (from β-oxidation), and amino acid metabolism. The majority of this mitochondrial acetyl-CoA is metabolized in the tricarboxylic acid (TCA) cycle or used for ketone body synthesis. To migrate between the mitochondrial and nucleo-cytosolic pools, acetyl-CoA must either be converted to citrate or utilize the carnitinepalmitoyl transferase system (not shown). Most cytoplasmic acetyl-CoA is synthesized from citrate by ATP-citrate lyase but can also be synthesized from acetate by acyl-CoA synthetase. The nucleo-cytosolic pool can be used for the synthesis of fatty acids or cholesterol or for protein acetylation. Acetyl-CoA carboxylase (ACC) catalyzes the conversion of acetyl-CoA to malonyl-CoA which is used by fatty acid synthase (FAS) to generate acyl-CoAs. Acetyl-CoA acetyltransferase creates acetoacetyl-CoA which is converted by 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS) to generate HMG-CoA for cholesterol synthesis.
1.7.6.1 Bempedoic acid

Bempedoic acid (BA; also known as ETC-1002 or 8-hydroxy-2,2,14,14-tetramethylpentadecanedioic acid) is an ACL inhibitor in clinical development for the treatment of hypercholesterolemia. It resembles a fatty acid analogue and was identified in a drug screen for its ability to inhibit cholesterol and fatty acid synthesis in vivo (169). Results from up to Phase IIb clinical trials have consistently shown a 30% lowering of LDL-C in hypercholesterolemic patients and an unanticipated 40% lowering in high sensitivity C-reactive protein (hsCRP) with BA monotherapy (168, 170). To date clinical studies have been completed in over 1000 subjects with more than 700 receiving BA across 10 clinical trials (3 Phase I, 7 Phase II). The majority of patients studied had hypercholesterolemia but BA has also been studied in patients with Type 2 Diabetes, statin intolerance, hypertension, or on stable statin or ezetimibe therapy (171–176). There is evidence in rodent models that BA may improve metabolic dysregulation associated with the metabolic syndrome, including insulin resistance, hepatic steatosis, atherogenic dyslipidemia and tissue inflammation (177–179).

BA appears to target the liver, where it inhibits ACL and may also activate AMPK (Figure 1-8) (177). To inhibit ACL, BA must be formed into a coenzyme-A thioester by acyl-CoA synthase (ACS). It was recently shown that the specific ACS isoform that catalyzes BA-CoA formation has high expression in human liver while showing no expression in muscle, explaining how BA’s mechanism is liver-specific (180). BA-CoA binds to and inhibits ACL in a manner competitive for coenzyme-A, resulting in a rapid decrease in acetyl-CoA and HMG-CoA while raising cytosolic citrate both in vitro and in vivo (168, 177). Activation of AMPK by BA phosphorylates and inhibits ACC and HMG-CoA reductase likely contributing to the observed inhibition of fatty acid synthesis and cholesterol synthesis, respectively, both in vitro and in vivo (Figure 1-6). Inhibition of ACC depletes the malonyl-CoA pool for fatty acid synthesis by FAS and relieves the inhibition of CPT1α-mediated β-oxidation by malonyl-CoA. Increased β-oxidation was observed in primary rat hepatocytes and in vivo results demonstrated increased plasma β-hydroxybutyrate, suggesting increased hepatic β-oxidation (168, 169).
Figure 1-8 Hepatic effects of bempedoic acid.

Bempedoic acid (BA) must first be converted to a CoA thioester (BA-CoA) to inhibit ATP-citrate lyase (ACL). This reduces cytosolic acetyl-CoA providing less substrate for sterol and fatty acid synthesis. BA can also activate AMPK by threonine phosphorylation of the α subunit. This results in the reduction of ACC activity and thus malonyl-CoA synthesis. As a result, fatty acid synthesis is reduced and β-oxidation is increased.
Studies in mice, rats, and hamsters have shown that BA increases hepatic protein expression of peroxisome proliferator activated receptor (PPAR) γ-coactivator 1α (PGC1α), a regulator of mitochondrial biogenesis, and elevates plasma levels of β-hydroxybutyrate (168). This occurred while also decreasing plasma cholesterol, TG, glucose and insulin, as well as hepatic fat content, fatty acid synthesis, and cholesterol synthesis (169, 177, 178, 181). In cultured human macrophages and murine adipose tissue, BA down-regulated proinflammatory MAPK signaling pathways and decreases cytokine and chemokine secretion through activation of AMPK signaling (178). The effect that BA has on atherosclerotic lesion development in Ldlr−/− mice fed a high-fat, cholesterol-containing diet, remains to be elucidated.

1.8 Model to be used

Mouse models are very common for studying several of the abnormalities associated with the metabolic syndrome. The C57BL/6 mouse is by far the most utilized strain due to its increased susceptibility to metabolic diseases when compared to other inbred strains of mice (182, 183). Using the C57BL/6 background, knockout of the LDLR gene (Ldlr) results in some susceptibility to atherosclerosis when fed a low fat chow diet, but plaques usually do not develop beyond small fatty streaks. Ldlr−/− mice fed a high fat, cholesterol containing diet however, develop far more severe atherosclerotic lesions over a reasonably short period of time due to the large increase in plasma cholesterol concentrations. These lesions are usually characterized as being large lipid-rich and foam cell-predominant and readily develop in the aortic sinus and aorta. This is a widely used model for studying atherosclerosis (184, 185). Additionally, this mouse models exhibits obesity, dyslipidemia, NAFLD development and inflammation when fed a diet with added fat and cholesterol (186–189).

1.9 Hypothesis and Scope of Thesis

In the current study, the ability of BA to protect against CVD through the prevention of CVD-associated risk factors was investigated. Specifically, using a murine model of the metabolic syndrome and atherosclerosis, this work sought to determine if targeting hepatic ACL and AMPK with BA could prevent the onset of abnormalities associated the
metabolic syndrome such as increased adiposity, insulin resistance, dyslipidemia, hepatic steatosis, inflammation and atherosclerosis. These abnormalities are linked to the process of atherogenesis. Therefore, the hypothesis to be tested was that in Ldlr⁻/⁻ mice fed an atherogenic diet high in fat and cholesterol, BA treatment would prevent the onset of obesity, dyslipidemia, hepatic steatosis, and insulin resistance, attenuate inflammation, and thus prevent the development of atherosclerosis.
Chapter 2

2 Methods

2.1 Animals and diets

Male, 8-week old Ldlr−/− C57BL/6 mice (B6.129S7-Ldlrtm1Her/J; Stock# 002207, Jackson Laboratory, Bar Harbour, ME) were maintained at 23°C on a 12-hour dark/light cycle and cared for in accordance with the Canadian Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Animal Care Committee at the University of Western Ontario (protocol number: 2012-028). 120 mice (n = 24/group) were fed ad libitum either control diets or diets supplemented with Bempedoic acid (BA, also known as ETC-1002; Ricerca Biosciences, Concord, OH) for 12 weeks. Diet compositions are shown on Table 2-1. Five groups of mice (see Figure 2.1) received either a purified rodent chow control diet (14% of calories from fat, Harlan Teklad T8604, Madison, WI), a high-fat cholesterol-containing diet (HFHC; 42% calories from fat and 0.2% cholesterol, Harlan Teklad TD09268) or HFHC supplemented with approximately 3, 10 or 30 mg of BA per kg body weight per day (mg/kg BA). BA was solubilized in ethanol and mixed with HFHC at the appropriate dose, followed by air drying of ethanol. Fresh food was provided twice per week with updated BA doses, which were based on the caloric intake and body weight measurements determined the previous day. Daily caloric intake was measured as the difference between the mass of food provided minus the mass of food remaining, which was then divided by change in time and multiplied by the caloric density of the food (chow = 3.1 kcal/g, HFHC = 4.5 kcal/gram).
**Table 2-1: Mouse diets**

<table>
<thead>
<tr>
<th>Formula</th>
<th>Chow Diet TD 8604</th>
<th>HFHC Diet TD 09268</th>
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</thead>
<tbody>
<tr>
<td>Casein</td>
<td>195</td>
<td></td>
</tr>
<tr>
<td>DI-Methionine</td>
<td>*Exact amounts not specified, see represented ingredients 341.46</td>
<td>3</td>
</tr>
<tr>
<td>Sucrose</td>
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<td>150.75</td>
</tr>
<tr>
<td>Corn starch</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anhydrous Milkfat</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>Lard, (Pork)</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Cellulose</td>
<td>35.13</td>
<td></td>
</tr>
<tr>
<td>Mineral Mix</td>
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<td></td>
</tr>
<tr>
<td>Zinc Carbonate</td>
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<td></td>
</tr>
<tr>
<td>Vitamin Mix</td>
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<td></td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Ethoxyquin</td>
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**Macronutrients % weight (% calories)**

<table>
<thead>
<tr>
<th></th>
<th>% weight (% calories)</th>
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</thead>
<tbody>
<tr>
<td>Protein</td>
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</tr>
<tr>
<td>Carbohydrate</td>
<td>40.2 (54)</td>
</tr>
<tr>
<td>Fat</td>
<td>4.7 (14)</td>
</tr>
<tr>
<td>kcal/g</td>
<td>3.0</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.005%</td>
</tr>
</tbody>
</table>

*Chow Diet (TD 8604) Represented Ingredients: Dehulled soybean meal, wheat middlings, flaked corn, ground corn, fish meal, soybean oil, brewers dried yeast, cane molasses, dried whey, dicalcium phosphate, calcium carbonate, iodized salt, choline, chloride, magnesium oxide, Vitamin A acetate, Vitamin D3, Vitamin E, niacin, pyridoxine hydrochloride, menadione, sodium, bisulfite, Vitamin B 12, manganous oxide, ferrous sulfate copper sulfate, zinc oxide, calcium, iodate, cobalt carbonate, chromium, potassium sulfate, kaolin
Blood samples were taken at baseline and weeks 4, 8, and 12. Metabolic cage experiments were performed at week 9. Micro (μ) CT scans were performed at week 10. Glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed at week 11.
2.2 Blood and tissue collection

All mice were fasted 6 hours before sacrifice or blood collection. Blood was collected at baseline and weeks 4, and 8 via a small nick in the tail vein at which time blood glucose was measured by glucometer (Bayer Contour Blood Glucose Monitoring System, Bayer Healthcare, Mississauga, ON). At sacrifice, mice were anesthetized with ketamine-xylazine (100 μg/g Ketamine Hydrochloride: Bioniche Animal Health Canada Inc. Belleville ON and 10 μg/g Xylazine: Bayer Healthcare, Animal Health Division, Bayer Inc. Toronto ON) and tissue dissections were performed via midline incision. A small section of liver tissue was harvested first by freeze-clamping, using pliers chilled in liquid nitrogen (190). Blood was collected in EDTA syringes by cardiac puncture, and plasma collected after centrifugation at 12 000 RPM at 4°C. Plasma samples were stored at -20°C. Epididymal adipose tissue, remaining liver, heart and aorta where then dissected and cleaned. For histological analyses, samples of liver and heart (containing the aortic root) were placed in Optimum Cutting Temperature (OCT) Compound (Sakura Finetek, USA Inc. Torrance, CA USA), frozen on dry ice and stored at -80°C. Remaining tissues were snap-frozen in liquid nitrogen and stored at -80°C.

2.3 Plasma analyses

Plasma was analyzed for cholesterol (Wako, VWR, Mississauga, ON) and TG (Roche TG/GB, glycerol blanked, Roche Diagnostics, Laval QC) concentrations by enzymatic assays as described previously (191). Concentrations of insulin (ALPCO Diagnostics, Windham, NH) and serum amyloid-A (SAA) (Invitrogen, Life Technologies, Mississauga, ON) were determined by mouse specific ELISA as per manufacturer’s instructions. Fast performance liquid chromatography (FPLC) was performed on fresh EDTA-plasma using an AKTA purifier and a Superose 6 column as previously described (191). FPLC separates plasma contents on the basis of size, thus allowing for the separation and identification of the lipoproteins present in a sample. Cholesterol and TG measurements were performed on samples from each eluted fraction as described above. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed using enzymatic kinetic rate reactions (Roche Diagnostics) performed at the London Health Sciences Centre Core Laboratory.
2.4 Glucose and insulin tolerance tests

Glucose and insulin tolerance testing were performed after 11 weeks on diet, to measure glucose disposal and insulin sensitivity, respectively. For glucose tolerance tests (GTTs), mice were fasted for 6 hours prior to receiving 1g/kg glucose by i.p. injection of a 15% glucose, 0.9% NaCl solution. For insulin tolerance tests (ITTs), mice were fasted for 5 hours prior to injection of 0.6 IU/kg Novolin ge Toronto (Novo Nordisk) i.p. Blood glucose measurements by glucometer were determined on samples taken up to 120 minutes (GTT) or 60 minutes (ITT) post-injection and reported as blood glucose vs time or glucose change from baseline vs time.

2.5 Metabolic analyses

Oxygen consumption (VO$_2$, L/kg/hour), carbon dioxide production (VCO$_2$, L/kg/hour), and respiratory exchange ratio (RER, VCO$_2$/VO$_2$) were measured using the Comprehensive Laboratory Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH). Mice had continuous access to food and water and after a 24-hour acclimatization period data was collected for up 48 hours.

For fatty acid oxidation, fresh liver was homogenized in 0.1 M phosphate buffer containing 0.25 M sucrose and 1 mM EDTA. Liver homogenates were incubated for 30 min at 37°C in a buffer containing 150 mM KCl, 10 mM Hepes (pH 7.2), 5 mM Tris malonate, 10 mM MgCl$_2$, 1 mM carnitine, 0.15% Fatty-Acid Free Bovine Serum Albumen (Sigma Aldrich, Oakville, ON), 5 mM ATP and 50 μM (µCi) $^3$H-palmitate (Perkin Elmer, Waltham, MA) complexed with FAF-BSA. Reactions were stopped with 200 μL 0.6 N perchloric acid and unreacted fatty acids extracted with n-hexane. $^3$H$_2$O in the aqueous phase was measured by liquid scintillation counting (191).

Fatty acid and cholesterol synthesis were measured following i.p. injection of radiolabeled acetic acid (Amersham Canada Ltd). Briefly, mice fasted for 6 hours were injected with 20 μCi of [1-$^{14}$C]-acetic acid and sacrificed 1 hour later. Tissues (500 mg) were extracted in chloroform:methanol and incorporation of [1-$^{14}$C]-acetic acid into fatty acid and cholesterol were assayed as described previously (191).
2.6 Micro-computed tomography imaging

Whole-mouse composition analysis was done by micro-computed tomography (μCT) imaging using a Locus Ultra μCT scanner (GE Healthcare, London, ON) after mice were on diet for 10 weeks. Total adipose volume was measured using MicroView 2.2 with threshold values of -380 to -30 Hounsfield Units to identify adipose tissue (192, 193).

2.7 Tissue sectioning and histology

Tissue histological and morphological analysis were performed using hearts or liver frozen in OCT and stored at -80°C (194, 195). Frozen serial sections were prepared with a Leica CM 3050S cryostat to cut 10 and 8 μm thick sections for heart and liver, respectively. Slides were stained with hematoxylin and Eosin (H&E), oil red-O (ORO, Sigma Aldrich, Oakville, ON), or picrosirius red. Adjacent slides were analyzed by immunohistochemistry (IHC) for CD68, cleaved caspase 3 (CC3), or smooth-muscle α-actin (SMA) as described previously (195), see Table 2-2 for antibody information. Briefly, slides were fixed in acetone followed by air drying, rehydrating in phosphate buffered saline (PBS) and blocking with 2% Fatty-Acid Free Bovine Serum Albumin (Sigma Aldrich, Oakville, ON) in PBS. Primary (Table 2-2) and biotinylated secondary (Table 2-3) antibody incubations were performed in blocking solution. Peroxidase blocking with 3% hydrogen peroxide was followed by incubation with the ABC reagent (ABC Elite Standard Kit, Vector Laboratories, Burlington, ON). Slides were exposed to the DAB substrate (Peroxidase substrate kit, Vector Laboratories) and counterstained in hematoxylin (Sigma-Aldrich).

Within atherosclerotic lesions, areas positive for ORO, CD68 and SMA were measured with ImageJ 1.50 (National Institutes of Health) and expressed as percent of total lesion area. Areas positive for picrosirius red were measured with ImageJ using MRI Fibrosis Tool (196) and expressed as percent of total lesion area. Lesion CC3-positive nuclei and total nuclei were counted with ImageJ using Color Deconvolution (197, 198) and expressed as percent CC3 positive nuclei.
2.8 Tissue lipid analysis

Liver and aortic tissues were weighed and lipids extracted using the chloroform:methanol (2:1) method of Folch, *et al* (199). Following extraction, samples and standards were dried under N₂ gas and solubilized in chloroform with 1% Triton X-100. Following drying under N₂ gas again, samples were resolubilized in water and levels of triacylglycerol (TG), total cholesterol (TC) and free cholesterol (FC) were measured by enzymatic assay. Lipid measurements were normalized to wet tissue weight. Cholesteryl ester (CE) was determined by the difference between TC minus FC (194).

2.9 Tissue gene expression

Quantitative real-time PCR (qRT-PCR) was performed on RNA isolated from liver or aorta using TRIzol® (Life Technologies, Burlington, ON). Reverse transcription into cDNA was performed using the High Capacity Reverse Transcription kit (Applied Biosystems, ABI). All primer and probe sets were from Applied Biosystems (Streetsville, CA) except Srebf1c (200). The probe and primer sequences for Srebf1c were designed using Primer Express 2.0 (ABI). Primers were obtained from Sigma-Genosys (Forward Primer: CAGGCCCGGGAAGTCACT; Reverse Primer: GACCACGGAGCCATGGATT) and the probes from ABI (Probe: FAM-ATTTGAAGACATGCTCCA-MG). All qRT-PCR assays were previously optimized and known to work for murine liver and aortic tissues.

2.10 Tissue protein expression

Frozen full-length aorta or freeze clamped liver samples (~50mg) were homogenized as previously described (195). Proteins were separated on 4-15% acrylamide gels (Bio-Rad) by SDS-PAGE and transferred to Immobilon-FL (EMD Millipore, Darmstadt, Germany) PVDF membranes. Primary antibodies were used for phosphorylated and total AMPK, ACC, ERK1/2, p38, JNK1/2, IKKαβ, and IκBα along with β-actin. Detailed information of primary antibodies is shown in Table 2-2. Phosphoproteins were analyzed using both phospho-specific and total antibodies on the same blot, with antibodies raised in different species (rabbit, mouse, or goat). Appropriate secondary antibodies (Table 2-3) conjugated to fluorophores of different emission wavelengths (IRdye-800CW or IRdye680RD, LI-
COR Biosciences, Lincoln, NE) were multiplexed to allow for simultaneous detection of phosphorylated and total proteins. A LI-COR Odyssey Fc scanner (LI-COR Biosciences) was used to detect the near-infrared emission signals (700 and 800 nm wavelength) produced after laser excitation of the conjugated fluorophores. Image Studio 5.0 (LI-COR Biosciences, Lincoln, NE) was used for signal quantitation. Phosphoproteins were normalized to loading control (total protein of interest) and expressed as fold change compared to chow-fed mice. B-actin was also probes for each blot a visual determination of equal loading. Blots were optimized to ensure signal was in a linear range.

### 2.11 Statistical analyses

All data are expressed as means +/- SEM. Statistical analyses were performed using GraphPad Prism 6. A One-way ANOVA was used to test for differences between groups of three or more followed by Tukey’s post-hoc test. A two-tailed t-test was used to test for differences between two groups. A Two-way ANOVA was used to test for differences between groups when data was collected over a series of timepoints, followed by a Tukey’s post-hoc test. $P < 0.05$ was deemed statistically significant as represented by letters comparing three or more groups.
Table 2-2 Primary antibodies used for Western blotting and IHC

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Table 2-3 Secondary antibodies used for Western blotting and IHC

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<td>LI-COR Biosciences, Lincoln, NE</td>
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<td>Goat</td>
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3 Results

3.1 Bempedoic acid attenuates the HFHC-induced elevation in body weight and adiposity

Male, 8 week old Ldlr−/− mice showed no differences in daily caloric intake between any groups fed HFHC ± BA for 12 weeks, while chow-fed mice had significantly lower caloric intake compared to all groups (Figure 3-1A). Any group receiving the HFHC diet showed a significantly increased gain in body weight when compared to chow-fed mice (Figure 3-1B). Compared to HFHC feeding alone, mice fed 3 and 10 mg/kg BA displayed no difference in body weight while mice treated with 30mg/kg BA had a significantly lower gain in body weight compared to mice fed HFHC (Figure 3-1BC). 30 mg/kg BA appeared to reduce body weight through a reduction in adiposity as epididymal fat pad mass measured at sacrifice was significantly less than HFHC alone while 3 and 10 mg/kg BA showed no change (Figure 3-1D). Additionally, μ-CT measurements of body composition after 10 weeks on diet showed an attenuation in whole body adiposity with 30 mg/kg BA with no effect on lean or skeletal tissue mass (Figure 3-1E).

3.2 Bempedoic acid prevents hyperlipidemia

Plasma cholesterol and TG concentrations were elevated (5-fold and 4-fold, respectively) in mice fed the HFHC diet compared to mice fed chow. Hypercholesterolemia was significantly attenuated with both 10 and 30 mg/kg BA but not 3 mg/kg BA (Figure 3-2A) and hypertriglyceridemia was significantly attenuated with all three doses of BA (Figure 3-2B). Cholesterol and TG concentrations in plasma lipoproteins separated by FPLC revealed that the HFHC diet greatly elevated the VLDL to LDL fractions compared to chow. Reductions in plasma lipids with 30 mg/kg BA took place primarily in the VLDL fractions with some effects observed on IDL, LDL, and HDL cholesterol fractions (Figure 3-3).
Figure 3-1: Effects of BA on body weight and adiposity.

Average daily caloric intake based on grams of food over the course of the 12-week study (A) (n=22-24). Weekly body weight measurements taken throughout the 12-week study (B) (n=22-24). Change in body weight from baseline to week 12 (C) (n=22-24). Epididymal fat pad mass taken at sacrifice (D) (n=22-24). Total whole body adipose, lean, and skeletal tissue mass measured by micro-CT scanning (D) (n=8). Data are means ± SEM, different letters indicate statistical differences by one-way (A, C-E) or two-way (B) ANOVA, $P < 0.05$. Statistical differences in (B) are for the whole curve.
Figure 3-2: Effects of BA on plasma lipids

Plasma cholesterol (A) and triacylglycerol (B) concentrations from samples taken every 4 weeks (n=12). Data are means ± SEM, different letters represent statistical differences of whole curves by two-way repeated measures ANOVA, $P < 0.05$. 
Figure 3-3: FPLC analysis of the effects of BA on plasma lipids.

Fast-performance liquid chromatography (FPLC) fractions from plasma collected after 12 weeks on diet, analyzed for cholesterol (A) and triacylglycerol (B) concentrations (n=5-12). Area under the curve (AUC) values for very low-density lipoprotein (VLDL) (fractions 5-9) and low-density lipoprotein (LDL) (fractions 10-19) analyzed by one-way ANOVA, $P < 0.05$. All data are means ± SEM.
3.3 Bempedoic acid prevents hepatic lipid accumulation

The HFHC diet caused significant increases in hepatic TG, TC, FC, and CE compared to chow feeding alone. This effect was mirrored with the 3 mg/kg BA group which significantly elevated all measured lipids compared to chow-fed mice while showing no significant differences compared to the HFHC-fed group. TG, TC, FC, and CE were significantly lowered by both 10 and 30 mg/kg BA compared to HFHC. Interestingly, BA at 10 and 30 mg/kg showed no significant differences in liver lipid content compared to those in chow-fed mice (Figure 3-4A-D). These results were reflected in the liver histology when stained for neutral lipids with ORO (Figure 3-4E).

3.4 Bempedoic acid influences multiple metabolic pathways in the liver

It was observed that there was a dose-dependent increase in liver weight despite the large reductions in lipid content (Figure 3-5A). Plasma ALT and AST concentrations showed no significant changes between all groups suggesting normal liver function (Figure 3-5BC).

To further understand the mechanism of action of BA in the liver, analyses of gene and protein expression were carried out. BA has been previously demonstrated to activate the AMPK pathway in rodent liver (177). This was assessed in mice treated with 30 mg/kg BA by Western blotting for phosphorylation (p) of both AMPK and its target ACC. Both pAMPK and pACC were suppressed in the liver of HFHC-fed mice compared to mice fed chow. Treatment with 30 mg/kg BA reversed this effect resulting in significantly increased pAMPK and pACC relative to the HFHC diet alone (Figure 3-6). Additionally, compared to chow and 30 mg/kg BA, the HFHC diet unexpectedly appeared to increase total AMPK protein levels. This increase was not quantified and how this should be interpreted remains uncertain.

Hepatic expression of genes involved in fatty acid β-oxidation were analyzed by qRT-PCR. Ppara and its target genes, Cpt1a and Acox were generally increased with both 10 and 30 mg/kg BA groups (Figure 3-7A). There was a particularly strong effect on Acox
expression, which is entirely regulated by PPARα activity (57). The HFHC diet induced a large elevation in the lipogenic transcription factor Srebf1c compared to the chow diet and this was significantly attenuated in 30 mg/kg BA-treated mice (Figure 3-7B). Interestingly, the hepatic expression of other lipogenic genes was generally increased with BA treatment compared to the HFHC diet alone. Specifically, when compared to livers from HFHC-fed mice alone, 30 mg/kg BA significantly increased the expression of Acacb and Fasn while trends were present for the remaining genes (Srebf2, Acaca, Hmgcr, and Pcsk9) with the exception of Acly (Figure 3-7B). This is possibly due to a compensatory feedback response resulting from inhibition of ACL, reduced intracellular sterols, and increased SREBP-2 activity.

3.5 Bempedoic acid improves hepatic lipid metabolism

Hepatic lipid metabolism was assessed biochemically by measuring rates of hepatic fatty acid β-oxidation and fatty acid synthesis. Compared to chow-fed mice, HFHC feeding resulted in a small nonsignificant reduction in β-oxidation of 3H-palmitic acid and a large significant increase in 14C-acetic acid incorporation into fatty acids (Figure 3-8AB). 30 mg/kg BA significantly enhanced β-oxidation compared to chow and HFHC feeding while also significantly reducing fatty acid synthesis when compared to HFHC alone (Figure 3-8AB). These metabolic improvements are consistent with activated AMPK and reduced SREBP-1c gene expression. Metabolic cage experiments were performed to measure respiratory exchange ratio (RER, a measure of glucose versus lipid fuel utilization) and respiration rates (VO₂ and VCO₂). Chow-fed mice had a significantly higher RER during the dark cycle compared to the HFHC and 30 mg/kg BA groups. The high RER in the mice fed chow indicates a preference towards carbohydrate metabolism reflecting their carbohydrate-based diet. The lower RER values observed in the HFHC and 30 mg/kg BA mice were not different from one another and reflect the high lipid content in the diets of these groups. Respiration rates (VO₂ and VCO₂) displayed small trends towards reduced rates in HFHC-fed mice compared to the chow and 30 mg/kg BA groups but no changes were significant (Figure 3-8CD). This suggests that any potential differences in energy expenditure were not large enough to be detected.
Figure 3-4: Effects of BA on hepatic lipids.

Liver free cholesterol (FC), cholesteryl ester (CE), total cholesterol (TC), and triacylglycerol (TG) (A-D) (n=14). Oil red-O (ORO) staining of liver tissue (E). Data are means ± SEM, different letters represent statistical differences by one-way ANOVA, $P < 0.05$. 
Figure 3-5: Effects of BA on liver size and function

Liver mass measured at sacrifice (A) (n=24). Plasma ALT (B) and AST (C) values (n=8).
Data are means ± SEM, different letters represent statistical differences by one-way
ANOVA, $P < 0.05$. 
Figure 3-6: Effects of BA on the AMPK signaling pathway.

Representative immunoblots of biological replicates for phospho- and total AMPK and ACC (A) with quantitations by densitometry shown below (B) (n=7-8). Data are means ± SEM, different letters indicate statistical differences by one-way ANOVA, *P* < 0.05.
Figure 3-7: Hepatic effects of BA on metabolic gene expression.

Hepatic gene expression of peroxisome-proliferator receptor alpha (Ppara) and its targets carnitinepalmitoyl transferase 1a (Cpt1a) and acyl-CoA oxidase (Acox) (A) and lipogenic genes sterol response element-binding protein (SREBP)-1c (Srebf1c), SREBP-2 (Srebf2), acetyl-CoA (ACC)-1 (Acaca), ACC-2 (Acacb), ATP-citrate lyase (Acly), fatty acid synthase (Fasn), 3-hydroxy-3-methylglutary-CoA reductase (Hmgcr), and proprotein convertase subtilisin/kexin type 9 (Pcsk9) (B) (n= 8-12). Data are means ± SEM, different letters indicate statistical differences by one-way ANOVA, P < 0.05.
Figure 3-8: Effects of BA on hepatic lipid metabolism.

Hepatic β-oxidation (FAO) of $^3$H-palmitic acid into $^3$H$_2$O (A) and hepatic fatty acid synthesis (FAS) measured by incorporation of $^{14}$C-acetic acid into $^{14}$C-fatty acids (B) (n=8). Metabolic cage measurements of light and dark cycle volume of oxygen consumed (VO$_2$) and carbon dioxide produced (VCO$_2$) (C), and respiratory exchange ration RER (D) (n=6-12). Data are means ± SEM, different letters represent statistical differences by one-way ANOVA $P < 0.05$. 
3.6 Bempedoic acid prevents impaired glucose homeostasis

Fasting blood glucose and plasma insulin were measured over the duration of the study at baseline and weeks 4, 8, and 12. By week 12, blood glucose for the chow-fed mice was significantly less than the HFHC-fed mice (Figure 3-9A). Mice treated with 30 mg/kg BA also showed a significant reduction in blood glucose compared to HFHC, while 3 and 10 mg/kg BA had no effect (Figure 3-9A). Plasma insulin concentrations were significantly elevated in the HFHC and 3 mg/kg BA groups compared to chow fed mice, whereas 30 mg/kg BA significantly attenuated plasma insulin to levels similar to those in chow-fed mice (Figure 3-9B).

Glucose and insulin tolerance tests showed reduced glucose and insulin sensitivity in HFHC-fed mice compared to chow-fed mice. In mice treated with 10 and 30 mg/kg BA, glucose and insulin tolerance improved (Figure 3-10AB). However, when the data was expressed as the change from baseline these effects were lost (Figure 3-10CD).

3.7 Bempedoic acid reduces inflammatory marker expression

To examine the effects of BA on inflammation, gene and protein expression analyses were used to assess proinflammatory signaling pathways in liver and aortic tissue. The MAPK (p38, ERK, and JNK) and NFκB (IKK and IκBα) signaling cascades link the effects of inflammatory stimuli to a cellular response, usually through a transcriptional mechanism. These inflammatory pathways have been suggested to play a role in pathologies associated with the metabolic syndrome, such as insulin resistance. In liver, the HFHC diet had little influence in MAPK signaling compared to chow. Mice treated with 30 mg/kg BA showed significantly decreased phosphorylation of p38 and ERK1/2 compared to both chow and HFHC alone (Figure 3-11). In the aorta, the HFHC diet significantly increased JNK phosphorylation compared to chow, an effect that was attenuated with 30 mg/kg BA (Figure 3-12). The remaining MAPKs showed minimal differences in phosphorylation between the chow, HFHC, and 30 mg/kg BA groups.
Phosphorylation of IKK and IκBα, regulators of NFκB activation, was minimal for all groups and also showed no differences (Figure 3-11, 3-12).

Under inflammatory conditions, such as those seen with HFHC feeding, tissues will display an increase in classically activated macrophage (M1, proinflammatory) gene expression and lower alternatively activated macrophage (M2, reparative and anti-inflammatory) gene expression. This was observed in the livers and aortae of HFHC-fed mice for several of the genes measured when compared to mice fed chow alone (Figure 3-13A). In liver, this effect was attenuated with 30 mg/kg BA for all M1 genes measured and was statistically significant for Tnf and Ccl3 (Figure 3-13A). In aortic tissue of HFHC-fed mice, M1 gene expression was elevated. Treatment with both 10 and 30 mg/kg BA decreased mean expression of these genes, but this effect did not reach statistical significance (Figure 3-13B). However, when the effects of BA treatments on all aortic M1 genes were analyzed there was a significant effect of the drug on overall M1 gene expression compared to the HFHC diet alone ($P < 0.001$) (Figure 3-13B). M2 gene expression showed that liver Cdl63 expression was significantly lower in HFHC-fed mice compared to both the chow and 30 mg/kg BA groups. Expression of M2 genes Arg1 and Il10 were not different among groups in aorta and liver (Figure 3.14-A).

Additionally, Adgre1 expression, an estimation of total macrophage content, was increased in the liver and aorta of HFHC-fed mice and was not further affected by BA treatment (Figure 3-14A).

Chronic inflammation was assessed by measuring plasma Serum amyloid-A (SAA) levels. This liver derived acute-phase protein is rapidly increased during exposure to inflammatory stimuli and is also mildly elevated under conditions of low-grade chronic inflammation (201, 202). After 12 weeks on diet, the change in plasma SAA concentration from baseline was significantly greater in mice fed HFHC when compared to mice fed chow (increase of 52% and 13%, respectively). Mice fed the HFHC diet also had a significantly greater change in SAA concentrations compared to mice treated with 30 mg/kg BA (decrease of 19%) (Figure 3-15).
Figure 3-9: Effects of BA on glycemia.

Fasting blood glucose (A) (n=24) and plasma insulin (B) (n=12) sampled every 4 weeks. Data are means ± SEM, different letters indicate statistical differences of whole curves by two-way ANOVA $P < 0.05$
**Figure 3-10: Effects of BA on glucose homeostasis.**

Glucose tolerance test (GTT) (A) and incremental-GTT (iGTT) (B) with area under the curve (AUC, inset) (n=12-15). Insulin tolerance test (ITT) (C) and iITT (D) with AUC (inset) (n=19-20). Data are means ± SEM, different letters indicate statistical difference by one-way ANOVA, $P < 0.05$. 
Figure 3-11: Effects of BA on hepatic inflammatory signaling pathways.

Representative immunoblots of biological replicates for mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) pathway signaling (A) and quantitations by densitometry shown below (B) (n=7-8). Data are means ± SEM, different letters indicate statistical differences by one-way ANOVA, $P<0.05$. 
Figure 3-12: Effects of BA on aortic inflammatory signaling pathways.

Representative immunoblots of biological replicates for mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) pathway signaling (A) and quantitations by densitometry shown below (B) (n=5-8). Data are means ± SEM, different letters indicate statistical differences by one-way ANOVA, $P < 0.05$. 
Figure 3-13: Effects of BA on M1 inflammatory gene expression.

Hepatic M1 gene expression (n=11-12) (A). Full-length aortic M1 gene expression (n=5-8) (B). Data are means ± SEM, different letters indicated statistically significant differences by one-way ANOVA, $P < 0.05$. 
Figure 3-14: Effects of BA on M2 gene expression.

Hepatic M2 gene expression arginase 1 (Arg1) and cluster of differentiation 163 (Cd163) (n=11-12) (A). Full-length aortic M2 gene expression Arg1, Interleukin 10 (Il10), and Cd163 (n=5-8) (B). (C) Hepatic and aortic adhesion G protein-coupled receptor E1 (Adgre1) (macrophage) gene expression (n=6-12). Data are means ± SEM, different letters indicated statistically significant differences by one-way ANOVA, $P < 0.05$. 
Figure 3-15: Effects of BA on chronic inflammation.

Percent change in plasma concentration of serum amyloid-A (SAA) from baseline to 12 weeks on diet (n=10-16). Data are means ± SEM, different letters indicated statistically significant differences by one-way ANOVA, $P < 0.05$. 
3.8 Bempedoic acid prevents atherosclerotic lesion development

The aortic sinus was sectioned for histological examination of atherosclerotic lesion development and morphology. Lesion analysis of H&E or ORO stained sections of the aortic sinus revealed the development of small fatty streaks in chow-fed mice, whereas the lesions in HFHC-fed mice were significantly larger by 16-fold (Figure 3-16). Compared with HFHC-fed mice, treatment with 30 mg/kg BA robustly attenuated lesion size (-47%) as measured by lesion area stained with ORO (Figure 3-16). Lipid analysis of whole aortae revealed that TC and CE increased significantly in mice fed the HFHC diet, as compared to chow-fed mice, and CE was decreased significantly by treatment with 10 and 30 mg/kg BA. There was a trend to increased aortic FC and TG with the HFHC-diet and a trend to lower FC and TG values in mice treated with BA at 10 and 30 mg/kg (Figure 3-17). Lesion morphology was further analyzed by immunohistochemistry (IHC) using macrophage and smooth muscle cell-specific antibodies (CD68 and α-actin, respectively) and by Picrosirius Red staining for collagen content. Chow-fed mice were not included in further lesion analysis due to their small lesion size (Figure 3-16). In 30 mg/kg BA-treated mice, macrophage content as a percent of lesion area was significantly greater than in lesions from HFHC-fed mice, while percent smooth muscle α-actin was nonsignificantly lower (Figure 3-18). Total collagen content was unchanged between the HFHC and 30 mg/kg groups. Levels of lesion apoptosis, measured by percent of activated caspase-3 positive nuclei, were significantly lower with 30 mg/kg BA treatment compared to mice fed HFHC alone (Figure 3-19). Collectively, this suggests that in addition to smaller lesion size, BA-treated mice have more macrophages, fewer SMCs, and fewer apoptotic cells per lesion, consistent with earlier stage lesions. Collectively, this implies that BA suppresses the development of atherosclerosis in this model.
Figure 3-16: Effects of BA on atherosclerotic lesion development.

Representative images of H&E or ORO stained aortic sinus sections (A). Quantitation of lesion area from ORO images (B) (n=10-13). Data are means ± SEM, different letters indicate statistical differences by one-way ANOVA, $P < 0.05$. 
Figure 3-17: Effects of BA on aortic lipid accumulation.

Aortic free cholesterol (FC), cholesteryl ester (CE), total cholesterol (TC), and triacylglycerol (TG) content (n=11-12). Data are means ± SEM, different letters indicate statistical differences by one-way ANOVA, P <0.05.
Aortic Lipids

FC

CE

TC

TG

mg/g tissue

mg/g tissue

mg/g tissue

mg/g tissue

Chow  HFHC  +3 mg/kg BA  +10 mg/kg BA  +30 mg/kg BA
Figure 3-18: Effects of BA on atherosclerotic lesion morphology.

Representative immunohistochemistry stains for CD68, smooth muscle (SM) α-actin and Picrosirius Red (A) with quantitation shown below (B) (n=8-13). Chow mice were not included in the analysis due to very small lesion size (see Figure 3-16). Data are means ± SEM, different letters indicate statistical differences by unpaired two-tailed t test, $P$-value shown on graph.
Figure 3-19: Effects of BA on atherosclerotic lesion apoptosis.

Representative immunohistochemistry stains of cleaved caspase-3 with quantitation of proportion of positively stained nuclei shown on right (n=4-5). Chow mice were not included in the analysis due to very small lesion size (see Figure 3-16). Data are means ± SEM, different letters indicate statistical differences by unpaired two-tailed t test, P-value shown on graph.
Chapter 4

4 Discussion

CVD risk management has improved tremendously over the past few decades. However, CVD continues to be a major cause of death in developed countries, driven in part by the rapidly increasing prevalence of the metabolic syndrome and Type 2 Diabetes mellitus. Strong evidence from large-scale clinical trials support the notion that lowering LDL-C reduces CVD risk. Statins have shown proven efficacy in lowering LDL-C and preventing CVD events but despite this, many at-risk patients are unable to reach their target LDL-C levels leaving a large group of individuals with significant residual risk. As a result, novel therapeutic options are needed to treat cardiometabolic risk factors.

One significant barrier to managing lipid levels is the often overlooked condition referred to as statin intolerance. This has typically lacked a consensus definition and the reported prevalence rates can range from 7-29% (203). However, it appears to be gaining increasing attention from the medical community. Statin intolerance can be loosely defined as when a patient is unable to take a statin due to any number of side effects, thus preventing them from achieving their target LDL-C goal (204, 205). Of course, this is a major cause for concern due to the increased CVD risk seen with statin noncompliance and failure to reach target LDL-C goals (206, 207). Side effects most commonly include discomfort from the myopathy-associated muscle pain, along with a small risk for new onset of Type 2 Diabetes or a rise in liver function enzymes (205). While these don’t negate the CVD benefit seen with statins, it still has a great impact on patient quality of life and can reduce compliance.

Mechanistically, it is thought that the muscle side effects of statins are a result of the drug acting directly on muscle tissue (208). Within the skeletal muscle, myocyte dysfunction and damage has been suggested to be caused by myocyte HMG-CoA reductase inhibition and the resulting lowering of myocyte cholesterol synthesis (209) or off-target effects within muscle tissues by statins themselves (210). This myocyte damage can present as
muscle pain and myopathy, and perhaps even reduce glucose utilization by muscle, increasing blood glucose levels and Type 2 Diabetes risk.

Bempedoic acid (BA, also known as ETC-1002) is in clinical development for the treatment of hypercholesterolemia in patients at a high risk for CVD (details of BA, including mechanism of action, are reviewed in section 1.7.6.1). In up to phase IIb clinical trials, BA has consistently demonstrated LDL-C lowering of up to 30% as monotherapy (171), 50% as an add-on to ezetimibe therapy (174), and 24% as an add-on to statin therapy (175). Whether or not this translates into protection from CVD is not known.

The current study was undertaken to address the question of whether or not BA could prevent the development of atherosclerosis in Ldlr-/- mice fed a HFHC diet, along with other components of the metabolic syndrome such as obesity, dyslipidemia, insulin resistance, and chronic inflammation. Mice were randomized to receive a chow diet, a HFHC diet, or BA added to the HFHC diet at the doses of 3, 10, or 30 mg/kg. In most cases, the metabolic abnormalities assessed were reduced with the dose of 30 mg/kg BA over a 12-week period. For example, when compared to mice fed the HFHC diet alone, mice treated with 30 mg/kg BA showed reduced body weight and adiposity, reduced lipids in the plasma, liver, and aorta, improved glycemia, reduced chronic inflammation, and reduced atherosclerotic burden. This effect was often mirrored in mice receiving 10 mg/kg BA for several, but not all parameters measured. Mice receiving the lowest dose of 3 mg/kg showed little difference compared to mice receiving HFHC alone for almost all the parameters measured. One exception was plasma TG concentration where 3 mg/kg BA significantly lowered plasma TG compared to HFHC, with similar efficacy seen with the higher two doses of BA. BA at 30 mg/kg was effective in preventing atherosclerosis in Ldlr-/- mice fed a HFHC diet.

4.1 Interpretation and evaluation of results

4.1.1 Hyperlipidemia and tissue lipid accumulation

In terms of lipid-based parameters, mice responded as expected to receiving the HFHC diet for 12 weeks. Using the Ldlr-/- mouse model in combination with the HFHC diet is a
very reliable and robust model of lipid-induced atherosclerosis. The large increase in
dyslipidemia and atherosclerotic lesion size seen in the HFHC-fed mice attest to this.
Other lipid-related parameters also demonstrate this, as hepatic and aortic lipids, along
with adiposity and total body weight, are greatly elevated in mice when comparing
HFHC feeding to chow. The sheer magnitude of effect size exemplifies the strength of
this model for studying hyperlipidemia and other components of the metabolic syndrome
and atherosclerosis.

Overall, BA showed robust efficacy when it came to preventing the hyperlipidemia and
tissue lipid accumulation induced by the HFHC diet. Plasma cholesterol and TGs were
increased several fold in HFHC-fed mice compared to chow. Separation of plasma
lipoproteins by FPLC analysis revealed that this took place in the VLDL, IDL, and LDL
cholesterol fractions along with the VLDL TG fractions. 30 mg/kg BA attenuated
hypercholesterolemia primarily in the VLDL and possibly IDL fractions. 10 mg/kg BA
also significantly reduced plasma cholesterol, which appeared to take place in the VLDL
fraction but not to a great enough extent to reach statistical significance. 3 mg/kg did not
significantly lower plasma cholesterol concentrations compared to HFHC, as was the
case with most parameters measured. However, it was very interesting to observe that all
three doses of BA did elicit a statistically significant reduction in plasma TG. This was
unusual in that it was one of the only parameters for which the 3 mg/kg BA had a
statistically significant effect compared to the HFHC diet. FPLC analysis showed that
plasma TG reduction took place in the VLDL fractions, as most plasma TG is found here
in fasted mice. Although not measured directly, reductions in VLDL cholesterol and TG
by BA were likely a result of reduced hepatic lipoprotein production.

4.1.2 Glucose homeostasis

Other parameters showed a moderate but still significant response to the HFHC diet in
comparison to chow, although not to the same extent as observed with the lipid
measurements. Glucose homeostasis was generally worsened with HFHC feeding, as
shown by increased fasting glucose and insulin, GTTs, and ITTs. When data from the
tolerance tests were normalized to baseline glucose value, there was still a significant
difference in glucose sensitivity but there was no significant effect on insulin sensitivity.
This can be interpreted a few different ways. Two important factors that need to be considered when interpreting the data are the baseline differences in both body weight and blood glucose. Differences in body weight will influence the dose of glucose or insulin received. Additionally, increased weight is likely due to increased adiposity while glucose disposal is primarily done by lean tissues, such as muscle, brain, and liver (211). In the current study, mice fed the HFHC diet received far greater doses of glucose and insulin due to their increased body weight. It is therefore possible that glucose tolerance was similar between groups and the perceived glucose intolerance in the HFHC-fed mice is the result of a larger dose of glucose. Alternatively, it has been suggested that mice should receive glucose based on the weight of their lean tissue, not total body weight, if such information is available (212). This can be determined by μCT scanning. Fixed glucose dosing is also a possibility and has been demonstrated in chow versus high fat fed mice (213). Additionally, baseline glucose values were not the same across the treatment arms of the study. Therefore, comparisons of the absolute GTT and ITT data would not be appropriate and data should be analyzed as glucose normalized to baseline or baseline-subtracted glucose (211), as shown in Figure 3-10B and 3-10D.

When looking at fasting glucose values taken over the course of the study, an interesting trend becomes apparent. Statistical analysis of the time course plots showed that mice in the chow and 30 mg/kg BA groups had significantly lower fasting glucose values than the HFHC and 3 and 10 mg/kg BA groups. It is stated in the results section that this is an attenuation of hyperglycemia, but closer inspection of the graphs suggests that the HFHC and lower dose BA groups had no change in glycemia while the chow and high dose BA groups displayed a reduction in glycemia. A likely explanation is that at baseline, mouse glucose values were slightly inflated due to a stress response from the tail bleed procedure. In fact, it is known that mice will increase glucose production under conditions of stress, likely through an increase in levels of catecholamines and cortisol (214, 215). As the study progressed, mice became more acclimatized to the glucose monitoring procedure so that by week 12, normoglycemic mice (chow and 30 mg/kg BA) showed a reduction while hyperglycemic mice (HFHC and 3 and 10 mg/kg BA) show no change in fasting blood glucose concentrations. If this scenario is true, then it would be
valid to suggest that chow and 30 mg/kg BA groups are normoglycemic relative to the other three groups which display hyperglycemia, as stated in section 3.6.

4.1.3 Metabolic measures

A number of metabolic measurements were taken including fatty acid β-oxidation, fatty acid synthesis, and metabolic cage analyses such as oxygen utilization, and carbon dioxide production. Fatty acid β-oxidation was measured using radiolabeled $[^{3}\text{H}]$-palmitic acid and tracking its conversion to deuterated water ($^{3}\text{H}_2\text{O}$). While most of this process takes place in the mitochondria, peroxisomes are another potential site of β-oxidation and both organelles contain similar enzymes but with different substrate specificities (216, 217). Specifically, all very-long-chained fatty acids (24-30 carbons) and some long-chain fatty acids (12-22 carbons) undergo β-oxidation exclusively in peroxisomes, most long-chain fatty acids and all medium-chain (6-12 carbons) and short-chain (<6 carbons) fatty acids undergo β-oxidation in the mitochondria (218). Since BA treatment had a large peroxisome proliferation effect, peroxisomal β-oxidation could account for much of the increased fatty acid β-oxidation of long-chain and very-long chain fatty acids. While the relative contribution of mitochondrial and peroxisomal β-oxidation was not determined, this could be estimated by performing the β-oxidation assay with or without a CPT1α inhibitor (such as etomoxir (219)). During inhibition of mitochondrial β-oxidation, the remaining β-oxidation would likely be peroxisomal, although this theory has yet to be validated.

Fatty acid synthesis was analyzed by measuring the incorporation of [1-$^{14}$C]-acetic acid into fatty acids. A very important consideration for using this method is the effect of radiolabeled substrate dilution within the endogenous cytosolic acetyl-CoA pool (220–224). Therefore, the assumption is made that acetyl-CoA pool sizes and thus radiolabel dilutions are equal between groups. However, the current study employs a specific inhibitor of acetyl-CoA synthesis and it is known that BA reduces intracellular acetyl-CoA concentrations (177), so these results should be interpreted cautiously. An alternative approach is to use tritium-labeled water ($^{3}\text{H}_2\text{O}$) as its specific activity should be similar between experimental groups (221). However, a major limitation of this approach is the very high amounts of radioactivity necessary to perform assays using
tritium-labeled water as substrate, especially when studying a large number of subjects (221).

Metabolic cages were used to measure volume of oxygen utilized (VO$_2$) and carbon dioxide produced (VCO$_2$). The analysis of murine energy expenditure has long been an area of controversy and different means of data analysis can yield very different results (225–231). It is common to find literature example of misinterpretations of data or unsubstantiated conclusions (231). One of the greatest confounding factors of energy metabolism in humans and mice is the role of body mass (225, 226). While it is known that energy expenditure increases with body mass, this relationship is certainly not linear. In models of diet-induced obesity, increased body mass is driven by increased adiposity. While adipose tissue does contribute to energy expenditure, the effect is not as great as the contribution from lean tissue. However, it is very common for investigators to normalize energy expenditure to total body mass, thus in a model of diet-induced obesity this will give the appearance of diminished energy expenditure in the obese group (225).

A better way to characterize energy expenditure when body weight differences are present is to normalize the data to each mouse’s lean body mass, which can be determined by μCT or nuclear magnetic resonance imaging. A much more robust method involves using analysis of covariance (ANCOVA), where energy expenditure is plotted in relation to body weight or lean body weight for each individual mouse. Displaying the data this way reveals if differences in energy expenditure are the result of different body weights, or if the treatment has an effect compared to the control group (225, 226). Another important consideration when doing energy expenditure analysis is that very subtle changes in metabolic rates can have a significant effect over a long period of time (229).

Taking the current study as an example, a small reduction in body weight seen in BA-treated mice compared to HFHC-fed controls (~ 2 grams) occurred over the course of the 12-week study. Assuming this is 2 grams of pure fat, this would represent 74 kJ of energy (2 x 37 kJ/gram fat) and thus a difference in energy expenditure of ~0.9 kJ per day over the 12-week study. Assuming there are no differences in caloric intake between mice fed the HFHC diet compared to BA-treated mice, and that an average mouse has an energy expenditure of 50-60 kJ/day(231), BA-treated mice only need to expend < 2% more energy to display the observed difference in body weight. Needless to say, such a small
difference in daily energy expenditure is far less than what indirect calorimetry can accurately detect (231).

4.1.4 Inflammation

The effects of BA on inflammation were assessed by measuring several markers of inflammation in the liver, aorta, and plasma. The HFHC diet induces chronic, metabolic inflammation, exacerbated by the addition of cholesterol. This effect is moderate, in contrast to the large effect seen in acute inflammation, such as when lipopolysaccharide (LPS) is administered to mice (232, 233). Analyses of inflammatory gene expression, intracellular signaling pathways, and plasma markers were carried out to try and detect the low grade inflammation seen with the HFHC diet and its resolution seen with BA treatment.

The effects of the HFHC diet can be seen on hepatic and aortic inflammatory gene expression in comparison to chow fed mice. One set of genes represents an M1 macrophage phenotype, which is indicative of classically activated, proinflammatory macrophages. This is in contrast to M2 genes which represent alternatively activated, reparative, anti-inflammatory macrophages. In liver there was a suppressive effect of the HFHC diet on M2 gene expression, but not in aortae. Additionally, total macrophages content was increased in the aortae and liver of mice fed the HFHC diet compared to chow-fed mice, as measured by Emr1 gene expression. Overall, BA attenuated this proinflammatory gene expression pattern. This was observed with 30 mg/kg BA-treated mice for most hepatic proinflammatory genes measured. Additionally, hepatic Nos2/Arg1 was reduced in 30 mg/kg BA-treated mice compared to HFHC-fed mice, suggesting that the population of macrophages present in the liver displayed more of an M2 phenotype.

In the aorta, BA at any dose did not significantly lower proinflammatory gene expression but trends can be observed in the data for the 10 and 30 mg/kg doses. Collectively, comparing expression of all aortic M1 genes between groups fed the HFHC diet +/- BA by two-way ANOVA revealed a significant effect of BA compared to HFHC-feeding alone ($P < 0.0001$). The lack of any statistically significant effect in the aorta on any individual gene is likely attributable to an insufficient sample size for these relatively low abundance mRNAs. Chow-fed mice showed relatively constant aortic M1 gene
expression while in HFHC-fed mice, expression was highly variable. The intermediate M1 gene expression seen in BA-treated mice was also highly variable. With this high standard deviation and a total 5 treatment groups, a much greater sample size would have been needed to detect statistically significant effects of BA on aortic inflammation.

Intracellular inflammatory signaling was also assessed by immunoblotting for phosphorylated MAPK and NF-κB pathway proteins. Overall there was a limited effect of the diet on the phosphorylation of these proteins, and in many instances there was no difference between chow and HFHC-fed mice. This was unexpected since the inflammatory gene expression data did show an effect of the HFHC diet. 30 mg/kg BA treated mice showed reduced phosphorylation of hepatic ERK and p38 and aortic JNK, compared to HFHC fed mice while other MAPK proteins and none of the NF-κB proteins showed any differences. A likely explanation is that the chronic low-grade inflammation seen with in HFHC fed mice has a very subtle effect on phosphorylation of MAPK and NF-κB proteins, below what phosphoprotein immunoblotting could accurately detect. This small effect size combined with the small sample size (8 or less) likely explains the limited differences seen in inflammatory signaling pathway phosphorylation.

A third marker of inflammation was performed by measuring plasma concentrations of the SAA protein. This acute-phase protein is rapidly secreted by the liver in response to inflammatory stimuli. Additionally, it is modestly elevated under conditions of chronic inflammation. Unexpectedly, several mice exhibited very large increases in SAA concentration (100-fold greater than group mean) independent of treatment group and study time point. In these cases, mice consistently exhibited signs of aggression and dominance behavior, occasionally having visible wounds. This is not atypical mouse behavior, especially among male mice and other investigators have made similar observations (234, 235). These mice were excluded and in the remaining mice the change in SAA from baseline to week 12 was analyzed. Mice fed chow showed little change in SAA concentration while in mice fed the HFHC diet plasma SAA was increased by approximately 50%. This was prevented in mice treated with 30 mg/kg BA and their SAA concentrations actually showed a slight decrease from baseline. This is likely owing
to the known anti-inflammatory properties of BA, as demonstrated by the CRP lowering seen in clinical trials.

4.1.5 Atherosclerosis

As mentioned in section 4.1.1, the Ldlr-/- mouse is a good model of atherosclerosis due to the severity of dyslipidemia that develops in response to the HFHC diet. As expected, the HFHC-fed mice developed very large lipid and foam cell-rich lesions within the aortic roots, while lesions in chow-fed animals were barely detectable. 30 mg/kg BA showed a robust reduction in lesion size compared to HFHC-fed mice an effect which was comparable to the degree of plasma cholesterol lowering. Further analyses were performed to study lesion composition and morphology. In addition to a reduced lesion size, BA treated mice had a higher macrophage content, slightly lower smooth muscle cell contents, and a reduced percentage of apoptotic cells, all indicative of a less advanced stage lesion.

4.2 The question of mechanism

Overall, these findings are consistent with BA inhibiting hepatic ACL and activating AMPK. Additionally, BA appeared to stimulate PPAR\(\alpha\) activation which offers a third mechanistic explanation of the results. The current study was not designed to determine a specific mechanism(s). However, comparing the data from BA-treated mice with literature data may offer insight into the relative contributions of the three potential mechanisms.

4.2.1 Comparison to related drugs and mouse models

Many previous studies have looked at genetic knockouts, overexpression, or chemical inhibition or activation of AMPK, ACL, PPAR\(\alpha\), or closely related pathways in mouse models of metabolic disease. These studies can be helpful when interpreting the data from the current study.
4.2.1.1.1 AMPK

Activation of AMPK under conditions of low intracellular energy results in an upregulation of catabolic, ATP-producing pathways and an inhibition of anabolic, ATP-consuming pathways (See section 1.7.5). Therefore, it is thought to be a promising drug target for the treatment of metabolic diseases. Most AMPK activators work via inhibition of the mitochondria respiratory chain, thus increasing cellular AMP levels and AMPK activation (236). Therefore, these are indirect activators and are not specific for AMPK. BA has been shown to activate AMPK without altering adenylate nucleotide levels (177), decreasing the likelihood of an AMP-mediated mechanism of AMPK activation. Resveratrol and other polyphenols are good examples of indirect AMPK activators that have been very well studied. While the mechanisms of polyphenols are complex and controversial (237) their cardioprotective potential has been demonstrated in several animal models (238–241). For example, the synthetic polyphenol S17834 has been shown to activate AMPK while inhibiting diet-induced hepatic steatosis, hyperlipidemia, and atherosclerosis in Ldlr/- mice (240). These effects are thought to involve AMPK-mediated inhibition of SREBP (242). Specifically, S17834 was shown to reduce hepatic expression of SREBP-2 and its target genes (242). In contrast, BA appeared to increase SREBP-2 target gene expression, which may be a feedback effect from cholesterol synthesis inhibition.

While compounds that activate AMPK indirectly have been well studied, direct AMPK activators have only been characterized relatively recently. Compound A-769662 was developed by Abbott Laboratories and was the first described direct AMPK activator (243). It was demonstrated to activate AMPK independently of upstream kinases (244) and cellular AMP levels (245) in a manner requiring the β-subunit of AMPK (245, 246). Additionally, A-769662 preferentially activated β1-containing complexes over β2 (246). In leptin-deficient (ob/ob) mice, a model of diet-induced obesity, A-769662 was effective in reducing plasma glucose, body weight, and plasma and liver TG, but displayed poor bioavailability (243). Salicylate was also found to bind the same β1-subunit site as A-769662 and had similar effects (154). Although it has better bioavailability, its effects on atherosclerosis are not yet known. Based on this information and the similar metabolic
effects observed with BA, it would be interesting to test if BA has similar AMPK β1-subunit binding properties.

Two additional compounds known as 991 (247) (also known as ex229 (248)) and MT 63-78 (249) have been recently developed and also show preference towards the AMPK β1-subunit (236). Compound C13 (and its metabolite C2) has been shown to directly target α1-containing AMPK complexes (250, 251). Lastly, CNX-012-570 has been shown to directly activate γ-containing subunits (252). Collectively, these direct activators have only been characterized very recently and several key aspects such as bioavailability and toxicology profile have not been described. It is difficult to make comparisons of these compounds with BA, until further information on these compounds are published. Therefore, while AMPK activation can certainly be observed with BA treatment (177, 178) the contributing role of AMPK activation in BA’s mechanism of action remains undetermined.

4.2.1.1.2 ACL

Besides BA, other inhibitors of ACL have been tested but none are currently in clinical development. Prior examples of ACL inhibitors include hydroxycitrate, a natural inhibitor (253–256), and the synthetic inhibitors BMS-303141 (257–259) and SB-204990 (260–262). However common issues with these compounds include poor membrane permeability and the high doses required to elicit an effect, resulting in off-target effects (170, 253, 263).

Previous studies of in vivo inhibition of ACL have reported very similar effects as seen with BA treatment. For example, treatment of rats with hydroxycitrate inhibited hepatic cholesterol and fatty acid synthesis (254–256, 264). Treatment of rats with SB-204990 reduced plasma cholesterol and TG (262) while treatment of high-fat fed mice with BMS-303141 reduced plasma lipids, glucose, and body weight gain (259).

Of particular interest are studies where ACL knockdown was performed specifically in the liver. Wang, et al. demonstrated that in leptin receptor-deficient (db/db) mice this reduced hepatic levels of acetyl-CoA, malonyl-CoA, lipogenesis, and TG while also
improving fasting glucose, and glucose tolerance (165). Interestingly, hepatic gene expression analysis revealed a small increase in SREBP-2 expression similar to what was seen with BA. However, expression of ACC and FAS were suppressed in the ACL deficient mice, in contrast to what was seen with BA in the current study. Additionally, ACL deficient mice showed a marked suppression of Ppara and Acox expression (165), in contrast to the large induction seen with BA treatment. This is likely owing to the fact that in the current study, BA treatment in mice likely inhibited ACL and activating PPARα while the study by Wang, et al. looked a hepatic ACL deficiency alone (165). A follow up study examined hepatic ACL knockdown in wild type mice fed either a low- or high-fat diet (166). Again, ACL knockdown reduced hepatic acetyl-CoA, malonyl-CoA and plasma TG on both diets, and while hepatic VLDL TG secretion was also reduced, hepatic TG was actually increased (166). The latter result is difficult to reconcile with all the other findings, and it is important to note that different diets and mouse models can sometimes produce very different effects. Overall, however, these examples are mostly consistent with the notion that the effects of BA in the current study are largely mediated by hepatic ACL inhibition.

4.2.1.1.3 PPARα

Peroxisome proliferator-activated receptor α (PPARα) is a transcriptional regulator of genes involved in lipid and lipoprotein metabolism, with a significant effect on regulating β-oxidation (265). It is the target of most fibrates, a class of drugs in clinical use as an add-on therapy for hyperlipidemia (265, 266). PPARα has been best studied for its role in the liver but it is also highly expressed in heart, kidney, and muscle (267–269). In the current study, there is a clear peroxisome proliferation effect of BA on murine liver, possible due to PPARα activation.

The effects of fibrates in mouse models of cardiovascular disease have been mixed. The fibrate ciprofibrate was shown to increase plasma cholesterol and atherosclerosis in Apoe-/- mice (270) and Ldlr-/- mice (271). Fenofibrate was also shown to increase plasma cholesterol but have no effect on atherosclerosis in Apoe-/- mice (272, 273) while conversely reducing plasma lipids and atherosclerosis in Ldlr-/- mice (274). Gemfibrozil decreased cholesterol and atherosclerosis in Apoe-/- mice (275, 276). Bezafibrate
decreased cholesterol and atherosclerosis in Ldlr-/- mice (277). Clearly there is difficulty in interpreting this data. However, if one looks at only the studies in Ldlr-/- mice where fenofibrate and bezafibrate were shown to reduce atherosclerosis, the data is very similar to the results of BA treatment in the current study. For example, similar to BA, fibrate treatment reduced body weight, fat pad mass, plasma cholesterol and TG, and atherosclerosis, while also increasing Aco gene expression (274, 277).

An important thing to note about PPARα agonists is that rodent models are highly sensitive to peroxisome proliferators. Even under short exposure to fibrates, rodents will display large increases in liver weight due to both hyperplasia and hypertrophy (278). This is precisely what was observed with BA treatment. Furthermore, under chronic exposure of peroxisome proliferators rodents will display an increased incidence of hepatocellular carcinoma (278, 279). It should be noted however, that this effect has not been observed in humans or primates and for several decades fibrates have been in clinical use for hypertriglyceridemia (279–281). Additionally, BA has successfully completed long-term carcinogenicity and toxicology studies in rats and mice as required by the Food and Drug Administration (282, 283).

In the current study, the large increase in fatty acid synthase (Fasn) gene expression seen with BA treatment could further play a role in PPARα activation. The Semenkovich lab previously demonstrated that knockout of Fasn in the liver of mice unexpectedly resulted in fasting hypoglycemia, hepatic steatosis, and other phenotypes similar to that seen in Ppara knockout mice (284). This suggested that FAS synthesizes an endogenous ligand for PPARα, which the group showed in a later study (285). These studies demonstrated a greater role for FAS beyond the storage of excess nutrients as fat. It appears that FAS can work though signaling mechanisms to impact liver physiology (286). Therefore, it is entirely possible that the large increase in Fasn expression seen with BA treatment could be at least in part responsible for the increased PPARα activation.

In summary, the metabolic benefits seen with BA appear similar to some (but not all) findings from studies of fibrates in mice. The strongest evidences for the involvement of PPARα in the mechanism of action of BA are both the large increase in hepatic Aco gene
expression and the increase in total liver mass. Authors of a previous study made very similar observations (including a lack of increased hepatic $Ppara$ expression), leading them to suggest a mechanism that involves PPARα activation (287). Additionally, increase fatty acid synthase activity (although not measured directly) could contribute to this PPARα effect.

### 4.2.2 Comparison to clinical data

It is difficult to fully know the clinical implications of a drug based solely on data from animal models. The discussion of PPARα and fibrates in the previous section exemplifies this as studies in rodents inconsistently demonstrate a benefit with fibrates, while also revealing side effects that appear to be clinically irrelevant. The current study demonstrated that in $Ldlr^{-/-}$ mice fed a HFHC diet, BA can lower TG and cholesterol in the plasma, liver, and aorta, increase hepatic lipid metabolism, improve glucose homeostasis, lower markers of inflammation, and reduce atherosclerosis. These data suggest a mechanism of action that very likely involves the inhibition of ACL, with a possible contribution of AMPK activation, and certain involvement of PPARα activation (based on increased liver size and $Aco$ expression). In comparison, human trials with BA have demonstrated robust efficacy in lowering LDL-C, non-HDL-C, apoB, and hsCRP with limited effects on plasma TG, glycemia, blood pressure, and body weight. The lack of any clinically significant effects on plasma TG in particular suggests that the human mechanism does not involve PPARα activation. Collectively, the published literature on the use of BA in clinical trials strongly suggests that in humans, BA’s primary mechanism of action in humans is the inhibition of hepatic ACL, a limited but undetermined effect of AMPK activation, and no effect on PPARα activation.

### 4.3 Conclusions

The results from this study demonstrate the clear efficacy of BA in preventing atherosclerosis and other aspects of the metabolic syndrome in $Ldlr^{-/-}$ mice fed a HFHC diet. Future studies should further expand both the current understanding of BA’s mechanism of action while also determining if there any side effects or additional benefits of chronic BA treatment. It remains to be determined what the contributions are
of ACL, AMPK, and PPARα in BA’s metabolic effects. This can be explored using knockout mice where each of these proteins are deleted in a whole-body, or preferably, a liver-specific manner. Additionally, to determine the effects of BA on inflammation, better *in vivo* inflammatory models could be utilized. As discussed already, using mice receiving a large inflammatory stimulus would be superior to the chronic inflammation seen in obesity for testing BA’s potentially anti-inflammatory effects. One additional question that remains to be answered is whether or not BA has any lipid-independent effects. Utilizing the inflammatory model just mentioned with acute BA treatment could help answer this question, especially if any effects of BA on inflammation can be detected prior to any changes in hepatic or plasma lipid levels.

In addition to further preclinical research, there remains much work to be done in human clinical trials which are already well underway. Moving forward, it will be very interesting to see how BA performs in future and ongoing clinical trials. Currently, patient enrollment has begun for two additional trials. First, a Phase 2b trail will look at the effects of BA when added to a high-dose (80 mg) of atorvastatin (NCT02659397). And next, a Phase 3 trial will look at the long-term safety and tolerability of BA in high-risk patients with hyperlipidemia and high CVD risk (NCT02666664). Results from these studies are eagerly anticipated.
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