The Circulating Furan Fatty Acid Metabolite CMPF Directly Enhances Hepatic FGF21 Secretion and Lipid Metabolism

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Physiology
University of Toronto

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2017

Abstract

Elevation of the fish oil metabolite 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) induces metabolic remodeling within the β-cell to a preferential use of fatty acids. Although detrimental to β-cell function, this energetic switch could benefit metabolic tissues like the liver, where obesity increases hepatic fat accumulation. CMPF primarily utilizes the Organic Anion Transporter (OAT) family, with OAT2 expressed in the liver. Following treatment, CMPF enters the hepatocyte. To determine a direct effect on liver function, isolated murine hepatocytes were treated for 24-hours with CMPF. CMPF significantly increased hepatic fatty acid oxidation and lipogenesis, without altering fatty acid uptake or gluconeogenesis. CMPF improved hepatic insulin signaling following lipid overload and enhanced FGF21 expression. Enhanced lipid oxidation occurred without ACC/AMPK phosphorylation, CMPF impaired enzymatic activity of isolated ACC, and direct ACC inhibition similarly increased FGF21 expression, suggesting an ACC inhibitor like effect of CMPF. These results demonstrate a potential therapeutic target to prevent hepatic steatosis.
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<td>ACC</td>
<td>Acetyl-CoA Carboxylase</td>
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<td>AKT</td>
<td>Protein Kinase B (PKB)</td>
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<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
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<tr>
<td>AMPK</td>
<td>AMP-activated Protein Kinase</td>
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<tr>
<td>ApoB</td>
<td>Apolipoprotein B</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<tr>
<td>cAMP</td>
<td>Cyclic AMP</td>
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<tr>
<td>CMPF</td>
<td>3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid</td>
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<tr>
<td>ChREBP</td>
<td>Carbohydrate-responsive element-binding protein</td>
</tr>
<tr>
<td>CREBP</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
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<tr>
<td>DIO</td>
<td>Diet induced obesity</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
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<tr>
<td>EtoAc</td>
<td>Ethyl acetate</td>
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<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
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<tr>
<td>FoxO1</td>
<td>Forkhead box protein O1</td>
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<td>G6P</td>
<td>Glucose 6-phosphate</td>
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<td>G6Pase</td>
<td>Glucose 6-phosphatase</td>
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<td>GLUT2</td>
<td>Glucose transporter 2</td>
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<td>GSK-3</td>
<td>Glycogen synthase kinase 3</td>
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<td>HFD</td>
<td>High fat diet</td>
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<tr>
<td>HMG-coA Reductase</td>
<td>3-hydroxy-3-methyl-glutaryl-coenzyme A reductase</td>
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<td>IL-6</td>
<td>Interleukin-6</td>
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INSIG  Insulin-induced gene 1 protein
IRS  Insulin receptor substrate
LPL  Lipoprotein lipase
LXR  Liver X receptor
MAPK  Mitogen-activated protein kinase
MRP Transporter  Multi-Drug resistance protein transporters
mTOR  Mechanistic target of rapamycin
MTP  Microsomal triglyceride transfer protein
NAFLD  Non-Alcoholic Fatty Liver Disease
NASH  Non-Alcoholic Steatohepatitis
OAT  Organic anion transporter
PKA  Protein Kinase A
PCR  Polymerase chain reaction
PEPCK  Phosphoenolpyruvate carboxykinase
PKC  Protein Kinase C
PP1  Protein phosphatase 1
PP2A  Protein phosphatase 2
PPAR  Peroxisome proliferator- activated receptor
PF-05175157  1,4-Dihydro-1’-[(2-methyl-1H-benzimidazol-6-yl)carbonyl]-1-(1-methylethyl)-spiro[5H-indazole-5,4’-piperidin]-7(6H)-one, 1-Isopropyl-1-(2-methyl-1H-benzo[d]imidazole-6-carbonyl)-1,4-dihydrospiro[indazole-5,4-piperidin]-7(6H)-one
qPCR  Quantitative polymerase chain reaction
S-2E  S-(+)-4-[1-(4 tert-Butylphenyl)-2-oxo-pyrrolidin-4-yl]methoxybenzoic acid
SCD1  Stearoyl-CoA desaturase-1
SREBP-1  Sterol regulatory element binding protein 1
TOFA  5-(Tetradecyloxy)-2-furoic acid
TNF-α  Tumor necrosis factor α
VLDL  Very low-density lipoprotein
Chapter 1: Introduction

1.1 Energy Metabolism Within the Liver

Every activity performed in our day to day lives requires the use of energy in the form of ATP. Nutrients that are absorbed from the diet in the form of carbohydrates, lipids, and proteins, are metabolized through a series of biochemical reactions and used to drive the internal processes of each cell (Berg, Tymoczko, & Stryer, 2002). The tissues within the body each perform their own functions and as a result have their own metabolic needs that must be met to ensure proper functionality. In order for sufficient energy substrates to be available and for energy homeostasis to be maintained, a strict regulation of the levels of available energetic metabolites in the body is required and the liver plays a vital role in the regulation of nutrient homeostasis (Berg TM. 2002). Within the liver, there are two distinct circulatory routes that supply the liver with blood, one delivers blood from the circulatory system and the other directly from the small intestines (Berg et al., 2002). As a result of this, the liver is able to sense the energetic needs of the body and tightly regulate most nutrient levels within the circulation (Postic, Dentin, & Girard, 2004; Rui, 2014). Functioning as a master regulator of nutrient homeostasis, the liver partakes in all aspects of glucose and lipid metabolism. The liver has the ability to assess the nutritional needs of the body and produce, store, or release energy substrates in the form of glucose and triglyceride rich lipoprotein particles called VLDL particles, into the circulation as they are required (Postic et al., 2004; Rui, 2014).

1.1.1 Glucose Metabolism

For the majority of tissues within the body, the preferred source of ATP is glucose (Houten & Wanders, 2010; Magistretti & Allaman, 2013). In particular, the brain is one of the most energy demanding organs and relies almost entirely on glucose as its main energy source (Magistretti & Allaman, 2013). Extreme fluctuations in the levels of glucose within the circulation can be quite harmful to organ function, making it essential that the levels of glucose be maintained within a narrow range. Allosteric regulation from metabolic substrates, in combination with hormones such as insulin and glucagon released from the pancreas, work to control the uptake, storage, and release of glucose into the circulation. This regulation occurs largely through their action on the liver in
order to maintain glucose homeostasis (Fig 1A) (Cherrington, Moore, Sindelar, & Edgerton, 2007; Ramnanan, Edgerton, Kraft, & Cherrington, 2011).

Figure 1: Overview of Glucose Metabolism in the Liver. (A) Glucose transport into and out of the hepatocyte occurs through the GLUT2 transporter. Glucose homeostasis within the hepatocyte is maintained through a tight regulation of the production, storage, and utilization of glucose. When glucose is in excess the hepatocyte works to store this excess glucose as glycogen through glycogenesis, or the hepatocyte utilizes this glucose to produce ATP through glycolysis. If the capacity for glycogenesis and glycolysis within the hepatocyte is reached, excess citrate from the TCA cycle will exit the mitochondria and be converted back into acetyl-coA to be converted into triglyceride stores through lipogenesis. In contrast, when glucose levels in the body are low, glucose is produced from the breakdown of glycogen stores through glycogenolysis, and the de-novo production of glucose known as gluconeogenesis. (B) Post-prandial glucose metabolism: When levels of glucose within the circulation are high following a meal the hepatocyte in part utilizes that glucose to produce ATP, but is primarily responsible for the storage of this excess energy as glycogen and triglycerides to be used during periods of nutrient deprivation. (C) Pre-prandial glucose metabolism: When blood glucose levels are low, the liver releases glucose into the circulation through the breakdown of glycogen and the de novo synthesis of glucose.
1.1.1a Post-Prandial

During the post-prandial state, when levels of glucose and consequently insulin have increased within the circulation, the liver primarily works to reduce blood glucose levels and convert excess energy substrates not immediately required by the body into energy stores that may be used later during periods of fasting (Fig 1B) (Berg et al., 2002; Houten & Wanders, 2010; Postic et al., 2004). Glucose that is in excess, enters the liver from the portal vein through the use of the GLUT2 transporter (Fig 1B) (Berg et al., 2002; Postic et al., 2004; Rui, 2014). This bidirectional transporter functions independently of insulin and instead is dependent on the concentration of glucose, allowing for movement down its concentration gradient (Postic et al., 2004). This gradient is maintained, as glucose that enters the cell is quickly phosphorylated by glucokinase to produce glucose-6 phosphate (G6P), resulting in a lowered intracellular glucose concentration and allowing for constitutive uptake (Ramnanan et al., 2011; Rui, 2014).

Although glucose uptake into the hepatocyte occurs independently of insulin, insulin does signal to the hepatocyte to store any excess glucose through a process called glycogenesis, in which glucose is condensed into the multi-branched polysaccharide glycogen (Fig 1B) (Postic et al., 2004; Rui, 2014). The rate of glycogen production is primarily controlled through the activity of two key enzymes: glycogen synthase and glycogen phosphorylase, that are responsible for the production and degradation of glycogen respectively (Han et al., 2016). During the fed state, binding of insulin to the hepatic insulin receptor activates an intracellular signaling cascade that results in the activation of protein kinase B (AKT) (Saltiel and Kahn, 2001). Activated AKT phosphorylates glycogen synthase kinase 3 (GSK-3) resulting in its inhibition, once inhibited GSK-3 is no longer able to phosphorylate either glycogen synthase or glycogen phosphorylase (Han et al., 2016). The phosphorylation status of glycogen synthase and glycogen phosphorylase is also influenced by protein phosphatase 1 (PP1) which is activated by insulin signaling and works to dephosphorylate both glycogen synthase and phosphorylase (Han et al, 2016). In the absence of phosphorylation, glycogen synthase is activated while glycogen phosphorylase is deactivated, allowing for glycogen production to occur (Han et al, 2016). Regulation of glycogen production during the fed state is additionally controlled through allosteric regulation. An abundance of glucose increases the accumulation
of G6P, which allosterically activates glycogen synthase in order to enhance glycogen production (Han et al., 2016). As the liver has a limited capacity for glycogen storage, excess glucose that is taken up by the liver is also converted into fatty acids that are then esterified onto glycerol-3 phosphate to make triacylglycerol (TAG), also referred to as triglycerides (Fig 1B) (Postic et al., 2004; Rui, 2014).

In addition to promoting glucose storage within the liver, the presence of insulin in the fed state promotes the inhibition of gluconeogenesis. (Berg et al., 2002; Postic et al., 2004; Rui, 2014). Activation of the intracellular insulin signaling cascade leads to phosphorylation of the transcription factor forkhead box protein O1 (FoxO1) that is responsible for the transcription of key rate limiting gluconeogenic enzymes such as phosphoenol pyruvate carboxykinase (PEPCK) and glucose–6-phosphatase (G6Pase) (Han et al., 2016). Once phosphorylated, FoxO1 is removed to the cytosol where it is degraded, thereby decreasing gluconeogenic gene transcription (Han et al., 2016). In addition, the activity of FoxO1, as well as the activity of the transcriptional co-activator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1a), is dependent on acetylation status, as post-translational acetylation interferes with their ability to bind to the promoter region (Han et al., 2016). Enhanced insulin signaling decreases both the abundance and activity of the deacetylase sirtuin-1 (SIRT1), thereby increasing transcription factor acetylation and further decreasing transcription of gluconeogenic genes (Puigserver et al., 2003). Through this inhibition of de novo glucose synthesis from non-carbohydrate carbon substrates, insulin works within the liver to limit the post prandial increase in blood glucose levels.

**1.1.1b Pre-Prandial**

In contrast to the post-prandial state, when blood glucose levels are low after a period of fasting or in between meals, the liver releases energy substrates from its internal energy stores into the circulation to provide fuel for the other organs and tissues of the body (Fig 1C) (Houten & Wanders, 2010; Postic et al., 2004; Rui, 2014). Glucagon, released from the pancreatic alpha cell, in combination with the absence of insulin in the fasted state, leads to the activation of glycogen phosphorylase and the inhibition of glycogen synthase (Ramnanan et al., 2011). Regulation of these two enzymes, allows for the inhibition of glycogenesis and
the breakdown and release of glucose from glycogen stores within the liver, known as glycogenolysis (Fig 1C) (ChERRINGTON et al., 2007; RAMNANAN et al., 2011).

Once hepatic glycogen stores have been exhausted, the liver works to synthesize glucose via gluconeogenesis (RUI, 2014). Through this process, glucose is produced from non-carbohydrate carbon substrates such as glucogenic amino acids like alanine, breakdown products of triglycerides like glycerol, and intermediate components of metabolism such as pyruvate and lactate (Fig 1C) (POSTIC et al., 2004; RUI, 2014). These non-carbohydrate substrates are either generated within the liver or released from the muscle and adipose in response to low levels of nutrients in the circulation. The first rate limiting step of gluconeogenesis is the conversion of pyruvate to oxaloacetate, catalyzed by the enzyme pyruvate carboxylase (PC) (HAN et al., 2016). Following the production of oxaloacetate, there are two additional rate limiting reactions involved in the de novo production of glucose, that are catalyzed by PEPCK and G6Pase (RUI, 2014). The regulation of flux through the gluconeogenic pathway primarily occurs through alterations in the activity of transcription factors such as cyclic AMP response element-binding protein (CREB) and FoxO1 that control the expression of these rate limiting enzymes (HAN et al., 2016). During the fasted state, elevated glucagon increases the level of intracellular cyclic AMP (cAMP) (HAN et al., 2016). Elevated intracellular cAMP enhances protein kinase A (PKA) activity which in turn activates CREB (OH et al., 2013; HAN et al., 2016). In addition, the absence of insulin in the fasted state allows FoxO1 to remain localized to the nucleus, and enhances activity of the deacetylase SIRT1 to remove FoxO1 acetylation, thereby promoting binding of FoxO1 to its target promoter region to enhance gluconeogenic gene transcription (HAN et al., 2016). Release of newly-synthesized glucose into the circulation allows the liver to continuously supply organs such as the brain with glucose.

1.1.2 Lipid Metabolism

Lipid metabolism encompasses the breakdown and storage of lipids through fatty acid oxidation and lipogenesis respectively (IQBAL & HUSSAIN, 2009; NGUYEN et al., 2008). The breakdown and storage of lipids for the release and preservation of energy are not processes that are specific to the liver, but the liver acts as the central hub in lipid metabolism. By regulating production, oxidation,
secretion, and storage of lipids, the liver functions to control the levels of circulating triglycerides within the body.

**Figure 2: Overview of Lipid Metabolism in the Liver.** (A) Post-prandial lipid metabolism: Following a meal, dietary lipids are packaged into lipoprotein particles called chylomicrons that deliver lipids throughout the body prior to reaching the liver. Triglycerides within the liver, produced from dietary lipids or excess glucose are either stored in cytoplasmic lipid stores or packaged into very-low density lipoprotein particles and delivered to the adipose tissue. (B) Pre-prandial lipid metabolism: The hepatocyte raises blood glucose levels through the production of glucose from internal lipid stores through gluconeogenesis. In addition, the liver utilizes free fatty acids as its primary energy source through β-oxidation within the mitochondria. Ketone bodies produced from the excess oxidation of fatty acids are released into the circulation to be used as an additional energy source in tissues like the brain.

### 1.1.2a Post-Prandial

Following a meal, triglycerides ingested from the diet are packaged together with cholesterol, phospholipids, and apolipoproteins into chylomicron particles (Iqbal & Hussain, 2009; Zhang et al., 2014). These chylomicron particles that are generated within the small intestine are released into the lymphatic system and eventually find their way into the circulation via the thoracic duct (Riccardi, Bozzetto, & Annuzzi, 2006). The single layer phospholipid shell of these lipoprotein particles causes them to be hydrophilic. This allows them to transport the hydrophobic triglycerides contained within their core throughout the body (Cox & Garcia-Palmieri, 1990; Iqbal & Hussain, 2009). The tissues of the body utilize lipoprotein lipase (LPL) to hydrolyze the triglycerides within the chylomicron into free fatty acids that easily diffuse across the cell membrane (Cox & Garcia-Palmieri, 1990; Riccardi et al., 2006). As the triglycerides within the chylomicron are hydrolyzed throughout the body, a cholesterol-ester...
rich chylomicron remnant is left behind that is eventually taken up by the liver (Fig 2A) (Feingold & Grunfeld, 2015; Riccardi et al., 2006). In addition to dietary sources, the liver in the fed state generates triglycerides from excess glucose and proteins through lipogenesis (Fig 2A) (Postic et al., 2004). When in abundance, the liver utilizes glucose for fuel, but excessive flux through the TCA cycle results in the release of citrate from the mitochondria (Rui, 2014). Within the cytoplasm, citrate is converted back into acetyl-coA where it is then carboxylated by acetyl-coA carboxylase 1 (ACC1) to form malonyl-coA and initiate the process of lipogenesis (Fig 2A). Lipogenesis is primarily regulated through an abundance of carbohydrates, but it is in part stimulated hormonally by insulin and through activation of lipogenic transcription factors such as carbohydrate responsive element binding protein (ChREBP), sterol regulatory element- binding protein (SREBP), and liver X receptor (LXR) (Kersten, 2001). During the fed state, glucose becomes oxidized to form xylulose 5-phosphate which in turn activates protein phosphatase 2 (PP2), leading to the dephosphorylation and subsequent activation of ChREBP (Rui, 2014). In addition, an abundance of intracellular glucose promotes ChREBP acetylation in order to further enhance its transcriptional activity (Rui, 2014). Once active, ChREBP increases production of lipogenic genes such as fatty acid synthase (FAS), ACC, stearoyl-coA desaturase 1 (SCD1) and elongation of very long chain fatty acid proteins (Elovls), all of which enhance triglyceride production (Rui, 2014). The transcription factor SREBP is regulated by the presence of insulin (Eberle et al., 2004). Binding of insulin to the hepatic insulin receptor activates the mechanistic target of rapamycin (mTOR) pathway leading to phosphorylation of lipin1 (Rui, 2014). Phosphorylated lipin1 is translocated to the cytoplasm where it can no longer interfere with SREBP activity in the nucleus (Rui et al, 2014). Insulin’s activation of the AKT pathway also stimulates SREBP activity and suppresses the activity of insulin-induced gene protein 1 (INSIG), a protein that when active is in part responsible for keeping SREBP localized to the endoplasmic reticulum (Eberle et al., 2004). SREBP that is activated and localized to the nucleus enhances transcription of genes that are required for fatty acid, triglyceride, and cholesterol synthesis (Rui, 2014). Finally, the nuclear receptor LXR is a ligand activated nuclear receptor that responds to the levels of cholesterol within the cell (Calkin and Tontonz, 2012). Binding of cholesterol derivatives allows for recruitment of co-activators in order to increase transcription of genes involved in cholesterol and lipid metabolism such as FAS, LPL, SREBP and ChREBP (Calkin and Tontonz, 2012).
Triglycerides within the hepatic cytoplasm that are acquired either from chylomicron remnants or de novo lipid synthesis, are either stored within the cytoplasm of the hepatocyte as lipid droplets or they are packaged along with cholesterol and lipoproteins into another form of hydrophilic molecule known as very-low density lipoprotein (VLDL) particles (Fig 2A) (Bradbury, 2006). These VLDL particles are secreted into the circulation so that they may be taken up and stored as fat deposits within the adipose tissue.

1.1.2b Pre-Prandial

During a period of fasting when glucose is less abundant, the body prioritizes which tissues will utilize the glucose that is available and reserves it primarily for use in the brain (Houten & Wanders, 2010). As a result, rather than utilizing the newly synthesized and broken-down glucose for its own energy demands, the liver oxidizes fatty acids as its primary source of fuel (Fig 2B) (Houten & Wanders, 2010; Postic et al., 2004). Non-esterified fatty acids that have been released from the adipose tissue and taken up by the liver, as well as free fatty acids released from triglyceride stores within the hepatocyte, are transported into the hepatic mitochondria where they are oxidized through beta-oxidation (Fig 2B) (Houten & Wanders, 2010). Transport of fatty acids into the mitochondria requires active carnitine palmitoyltransferase 1 (CPT1) (Rui, 2014). In a fasting state when intracellular ATP levels are low and AMP levels are elevated, AMP-activated protein kinase (AMPK) becomes activated and phosphorylates ACC resulting in its inhibition (Mihaylova and Shaw, 2011). As ACC is responsible for the production of malonyl-coA, its inhibition causes the levels of malonyl-coA within the cell to decline (Rui, 2014). As malonyl-coA inhibits CPT1 activity, a decrease in malonyl-coA enhances fatty acid uptake into the mitochondria in order to promote fatty acid oxidation (Rui, 2014). Transcription factors such as peroxisome proliferator-activated receptor α (PPARα) are also responsible for the control of genes involved in mitochondrial β oxidation, fatty acid uptake, ketogenesis, gluconeogenesis, and triglyceride turnover through enhancing expression of genes such as CPT1 (Varga et al, 2011). Activation of PPARα primarily occurs during periods of nutrient deprivation from the binding of ligands such as fatty acids (Jump, 2011).

ACC inhibition not only stimulates fatty acid oxidation but additionally prevents the initiation of lipogenesis as malonyl-coA is the initial building block required for fatty acid chain
synthesis (Rui, 2014). Inhibition of lipogenesis that occurs during the fasting state is primarily regulated at the transcriptional level. Stimulation of PKA by glucagon increases phosphorylation and subsequent inhibition of ChREBP (Kersten, 2001). Additionally, activated AMPK phosphorylates SREBP thereby preventing its translocation to the nucleus (Kersten, 2001). Both of these events inhibit the production of genes that are required for triglyceride production (Kersten, 2001).

The liver is also responsible for the production of ketone bodies which occurs from the oxidation of fatty acids in the mitochondria if excessive gluconeogenesis is occurring (Houten & Wanders, 2010). These ketone bodies are released into the circulation and can be utilized by the brain as an additional source of fuel if sufficient glucose is not available (Houten & Wanders, 2010).

1.1.3 Protein Metabolism and Additional Liver Functions

In addition to regulating glucose and lipid homeostasis during periods of nutrient intake and fasting, the liver also plays a major role in the metabolism of proteins, primarily through its ability to interconvert amino acids and remove toxic ammonia from the body (Charlton, 1996). Amino acids are the building blocks of proteins and can be classified as being either essential, or non-essential (Charlton, 1996). An essential amino acid cannot be synthesized in the body and must be acquired from the diet, but non-essential amino acids can be generated in the liver, and to a smaller extent in the kidney. The liver expresses a group of enzymes called aminotransferase’s that catalyze the transfer of an amino group onto a keto acid to generate a new amino acid (Charlton, 1996). Transamination allows for the inter-conversion of amino acids and the generation of non-essential amino acids from intermediates of the Krebs cycle and other metabolic pathways. During the breakdown of proteins and amino acids within the body, the toxic by-product ammonia is produced (Charlton, 1996). Ammonia is not easily cleared from the body and must be first converted to urea by the liver so that it can be excreted in the urine. In addition, the liver is responsible for the production of plasma proteins such as albumin (Charlton, 1996; Rui, 2014). The liver is also essential in the detoxification of blood, the degradation of hormones such as insulin allowing for their clearance from the circulation, the release of bile which is critical to the absorption of dietary fats, and the production of blood clotting factors (Fabbrini, Sullivan, & Klein, 2010; Rui, 2014). The liver also plays an essential role in the body’s immune system through the
use of Kupffer cells, which are specialized macrophages located within the liver that work to eliminate various bacteria, parasites, and cellular debris from the blood (Fabbrini, Sullivan, & Klein, 2010; Rui, 2014).

The liver is a critical organ in the regulation of energy homeostasis within the body. Within the next section we will highlight the detrimental impact that a deregulation of lipid metabolism within the liver has on multiple components of health within the body.

## 1.2 Deregulated Lipid Metabolism

Within the western world, an increased propensity towards a sedentary lifestyle combined with excessive caloric intake has resulted in an epidemic of obesity. This obesity, particularly when centralized around the abdominal region is commonly believed to be an initiator of a number of metabolic disturbances (Eckel, Grundy, & Zimmet, 2005; Samson & Garber, 2014). Abdominal obesity, also commonly referred to as visceral fat, is more metabolically active than subcutaneous fat leading to enhanced lipolysis and release of free fatty acids into the circulation (Nesto, 2005). As well, as fat mass increases a pro-inflammatory state is initiated through the release of cytokines including TNF-α and IL-6 from adipocytes into the circulation (Nesto, 2005). Inflammation within an adipocyte is initiated either from an inability of the vasculature to keep up with the growing fat mass resulting in hypoxia, or as a result of adipocytes swelling to the point of almost bursting and initiating cell death (Nesto, 2005). The combination of increased circulating free fatty acids and cytokines impairs insulin sensitivity within the hepatocyte (Roberts, Hevener, & Barnard, 2013). As insulin plays an essential role in the regulation of nutrient metabolism within the liver, the development of hepatic insulin resistance is commonly found in association with a number of additional metabolic disturbances including high blood glucose, high blood pressure, high circulating triglycerides, low levels of high-density lipoprotein, and abdominal obesity (Saltiel & Kahn, 2001). In addition, abdominal obesity leads to the development of a procoagulant state, in which the expression of plasminogen activator inhibitor-1, a factor that inhibits the degradation of fibrin clots, and the expression of clotting factors such as factors VII and X are all increased, enhancing the risk of thrombosis formation (Blokhin & Lentz, 2013; Sakkinen, Wahl, Cushman, Lewis, & Tracy, 2000). As a result, when abdominal obesity in combination with a number of these metabolic disturbances occur together, it greatly increases the risk of developing a secondary
condition such as heart disease, Type 2 Diabetes or Non-Alcoholic Fatty Liver Disease (Eckel et al., 2005).

1.2.1 Non-Alcoholic Fatty Liver Disease (NAFLD)

The most common liver disease is a spectrum of disorders known as Non-Alcoholic Fatty Liver Disease (NAFLD) and is a condition that affects approximately 20-30% of the Western population (Fabbrini et al., 2010; Smith & Adams, 2011). The development of NAFLD is often associated with the presence of the metabolic syndrome (Smith & Adams, 2011). The metabolic syndrome is a syndrome that is defined as the presence of a cluster of metabolic abnormalities that include high blood glucose, high blood pressure, high circulating triglycerides, low high-density lipoprotein (HDL) levels and abdominal obesity, and the presence of which greatly increases the risk of developing a secondary condition such as cardiovascular disease and Type 2 Diabetes (Smith & Adams, 2011). In addition, NAFLD has been found to have a strong correlation with the presence of obesity and the development of Type 2 Diabetes (Smith & Adams, 2011). NAFLD, a spectrum of liver disorders that arises from a deregulation to lipid metabolism within the liver and ranges from a simple excessive accumulation of intrahepatic fat known as hepatic steatosis, to the presence of fibrosis and inflammation as a result of hepatic cell injury known as Non-Alcoholic Steatohepatitis (NASH) (Fabbrini et al., 2010; Ress & Kaser, 2016; Tiniakos, Vos, & Brunt, 2010). Of individuals that have been diagnosed with NAFLD, approximately 15-20% will present with NASH which equates to approximately 3-5% of the general population (Zezos & Remer, 2014). The hepatocellular injury that occurs during NASH puts individuals at high risk for developing liver cirrhosis, in which the liver is no longer able to function as a result of scar tissue completely replacing the healthy liver tissue, and resulting in the requirement of a liver transplantation (Zezos & Remer, 2014). NASH related liver failure is currently the 3rd most common cause of liver transplantation, with estimations that it will soon surpass hepatitis C and alcoholic fatty liver disease as the primary cause (Zezos & Remer, 2014).

1.2.1a Obesity and NAFLD

The excess accumulation of fat seen in hepatosteatosis is the result of an imbalance between lipid acquisition, accrued through lipogenesis and hepatic fatty acid uptake and esterification, and lipid removal, via beta oxidation and lipoprotein secretion (Fabbrini et al., 2010; Smith &
Adams, 2011). One of the primary contributors to the development of this imbalance is excessive caloric intake (Galbo & Shulman, 2013). When the dietary intake of lipids exceeds the metabolic capacity of the body and the capacity of the adipose tissue to expand, the body is no longer able to compensate and lipid accumulation begins to occur within the hepatic cytoplasm (Galbo & Shulman, 2013; Ress & Kaser, 2016). In individuals that are not considered obese, the prevalence of simple steatosis is only about 15% with more severe forms of NAFLD such as NASH and liver cirrhosis, only occurring in approximately 3% of the non-obese population (Fabbrini et al., 2010). The occurrence of NAFLD increases dramatically with an increase in body mass index (BMI), a measure of body fat based on an individuals’ weight and height. Individuals with a BMI ranging between 18.5-25.0 are considered to be of normal weight (Fabbrini et al., 2010). If an individual’s BMI falls between 25.0 – 29.9 they are considered to be overweight but are not yet obese (Fabbrini et al., 2010). Once an individual’s BMI surpasses 30 they are classified as obese and can be further divided into 3 classes (Fabbrini et al., 2010). Class 1 or low risk obesity is an individual with a BMI between 30.0 – 34.9, Class 2 or moderate-risk obesity is any individual with a BMI of 35.0-39.9, and Class 3 or high-risk obesity is any individual with a BMI greater than or equal to 40.0 (Fabbrini et al., 2010). Simple steatosis occurs with a frequency of 65% in individuals that are considered to have class 1 or 2 obesity and in 85% of individuals with class 3 obesity (Fabbrini et al., 2010). More severe forms of NAFLD occur with a frequency of 20% to 40% in class 1 and 2 obese individuals and class 3 obese individuals respectively (Fabbrini et al., 2010).

1.2.1b Association between NAFLD, Obesity, and Insulin Resistance

Obesity often coincides with the presence of insulin resistance and there is a correlation that exits between the development of insulin resistance and the presence of NAFLD (Birkenfeld & Shulman, 2014; Boden, 2003; Galbo & Shulman, 2013; Gariani, Philippe, & Jornayvaz, 2013; Ress & Kaser, 2016). A definitive conclusion has not yet been reached as to whether insulin resistance contributes to the development of NAFLD, or if insulin resistance is a secondary condition arising from the presence of NAFLD. One of the more common schools of thought regarding this association is that the excessive caloric intake associated with obesity initially leads to an increase in adipose tissue mass and the eventual development of insulin
resistance within the adipose tissue (Galbo & Shulman, 2013; Gariani et al., 2013; Kumashiro et al., 2011). As a result of an increase in adipose tissue mass, more surface area is available to undergo lipolysis and release free fatty acids into the circulation (Birkenfeld & Shulman, 2014; Boden, 2003). Combined with the development of insulin resistance, in which insulin is no longer able to inhibit the breakdown and release of non-esterified fatty acids, the levels of free fatty acids within the circulation become elevated (Boden, 2003). The liver responds to this increase in circulating free fatty acids by enhancing fatty acid uptake and leading to an increase in hepatic diacylglycerol (DAG), and ceramide concentration, both of which influence the development of insulin resistance (Fig 3) (Birkenfeld & Shulman, 2014; Galbo & Shulman, 2013; Kumashiro et al., 2011; Roberts et al., 2013). An increase in hepatic DAG activates protein kinase C isoforms such as PKC\(_e\), which phosphorylate serine residues of the insulin receptor substrate (IRS) protein. This inhibits tyrosine phosphorylation via the tyrosine kinase insulin receptor and thus impairs activation of insulin signaling within the hepatocyte (Fig 3) (Boden, 2003; Galbo & Shulman, 2013; Kumashiro et al., 2011). Through a similar mechanism, ceramides activate atypical PKC and mitogen activated protein kinases (MAPK) to phosphorylate serine residues of IRS proteins (Roberts et al., 2013). In addition, ceramides activate protein phosphatase 2A leading to the de-phosphorylation and subsequent inactivation of AKT (Fig 3) (Roberts et al., 2013). In the absence of downstream insulin signaling in the liver, additional glucose within the circulation is no longer converted into glycogen, gluconeogenesis is no longer impaired, and secretion of triglyceride rich VLDL particles is no longer inhibited, further increasing the levels of glucose and lipids within the circulation (Boden, 2003; Kumashiro et al., 2011).
Figure 3: Hepatic Lipid Accumulation Impairs Insulin Signaling. Enhanced adipose tissue lipolysis increases delivery and accumulation of fatty acids within the liver. Accumulation of intracellular fatty acids increases concentrations of DAG and ceramide which activates kinases such as PKC, MAPK and PP2A. Both PKC and MAPK, phosphorylate serine residues of the IRS proteins, thereby inhibiting tyrosine phosphorylation and subsequent activation by the insulin receptor. PP2A dephosphorylates and subsequently inactivates AKT. Both of these functions impair downstream activation of the insulin signaling pathway. DAG: diacylglycerol, MAPK: mitogen-activated protein kinases, PP2A: protein phosphatase 2, IRS: insulin receptor substrate, AKT: protein kinase B

1.2.1c Initiation of Hepatic Fat Accumulation

As the BMI and overall fat mass of an individual increases, insulin resistance commonly develops within the adipose tissue leading to a direct increase in adipose tissue lipolysis and the release of non-esterified fatty acids into the circulation, thereby increasing the delivery of fatty acids to the liver (Fig 4) (Galbo & Shulman, 2013; Ress & Kaser, 2016). The rate of fatty acid uptake into the liver is dependent on both the concentration of fatty acids present in the circulation, and on the hepatocytes capacity to uptake those lipids (Bradbury, 2006; Fabbrini et al., 2010). As the delivery of fatty acids to the liver is enhanced, the limiting factor becomes the hepatocellular capacity for uptake, which is in part dependent on the number and activity of fatty acid transporters located on the sinusoidal plasma membrane (Bradbury, 2006). In obese individuals that have developed NAFLD, the levels of these transporters are elevated.
when compared to the levels in obese individuals without NAFLD, indicating that lipid uptake is enhanced and contributing to lipid accumulation (Fig 4) (Kohjima et al., 2007; Nakamuta et al., 2008).

Figure 4: Initiation of Hepatic Fat Accumulation in Obese Individuals. Increased adipose tissue lipolysis in obese individuals results in increased delivery and uptake of fatty acids into the hepatocyte. In addition, obese individuals have increased release of inflammatory cytokines, which in combination with the excessive caloric intake enhances lipogenesis. Although the liver attempts to compensate for the excess hepatic triglycerides through enhanced VLDL secretion, excessive accumulation of intrahepatic fat still occurs, putting stress on the metabolic capacity of the liver and resulting in the development of mitochondrial abnormalities.

Although non-esterified fatty acids that are taken up from the circulation do contribute to the majority of intrahepatic triglyceride content, approximately 26% of triglycerides that accumulate within the liver during NAFLD are alternatively derived from the de novo lipogenesis of fatty acids from glucose (Fig 4) (Kumashiro et al., 2011). This is in contrast to the less than 5% contribution that occurs in a healthy liver. De novo lipogenesis is primarily controlled at the transcriptional level. A diet particularly high in carbohydrates will activate the transcription factor ChREBP, which binds to the promoter region and enhances expression of lipogenic related genes (Sae-Lee, Moolsuwan, Chan, & Poungvarin, 2016). Additionally, endoplasmic reticulum stress initiated through excessive caloric intake leads to the activation
of transcription factors such as SREBP1c, which subsequently activates the lipogenic pathway and increases the abundance of fatty acids that can contribute to triglyceride stores (Dara, Ji, & Kaplowitz, 2011; Fabbrini et al., 2010; Kumashiro et al., 2011). Obesity is also associated with an alteration in adipocyte membrane lipid composition, resulting in an increase in unsaturated fatty acids such as palmitoleic and arachidonic acid that enhance vulnerability of the adipose to inflammation and release of inflammatory cytokines (Pietiläinen et al., 2011). The inflammatory cytokines TNF-α and IL-6 increase hepatic lipogenesis through an increase in citrate levels, which are converted into acetyl-coA thereby activating acetyl-coA carboxylase and the lipogenic pathway (Grunfeld et al., 1990). In an individual of normal weight with appropriate insulin sensitivity, insulin signaling following a meal will stimulate lipogenesis and the storage of triglycerides (Rui, 2014). The enhanced lipogenesis noted in obese individuals occurs paradoxically to what one may expect with the development of insulin resistance but is likely accounted for by the activation of lipogenic transcription factors like SREBP1c and ChREBP.

During a period of fasting, an estimated 90% of the livers’ fuel is derived from the oxidation of fatty acids (Houten & Wanders, 2010). With the enhanced fatty acid uptake and de novo lipogenesis that is occurring in obese individuals with NAFLD, the demand on the hepatic mitochondria becomes too great and subjects that have NAFLD display evidence of decreased fatty acid oxidation as a result of mitochondrial structural and functional abnormalities (Fig 4) (Bradbury, 2006; Gusdon, Song, & Qu, 2014; Kawano & Cohen, 2013).

Interestingly, in an apparent attempt to compensate for the enhanced fat accumulation, individuals with NAFLD that are obese appear to have increased triglyceride secretion from the liver in the form of very low-density lipoproteins (Fig 4) (Choi & Ginsberg, 2011). This may be attributed to the presence of insulin resistance in the hepatocytes, as functional insulin signaling would typically inhibit VLDL secretion (Kawano & Cohen, 2013). Increased triglyceride accumulation combined with decreased insulin signaling, causes apolipoprotein B (ApoB), the primary structural protein required for VLDL formation, to no longer be degraded, thereby promoting VLDL formation (Kawano & Cohen, 2013). As well, a decrease in insulin signaling results in the FoxO1 transcription factor remaining localized to the nucleus and enhancing expression of the microsomal triglyceride transfer protein (MTP), responsible for transferring lipids into maturing VLDL particles (Kawano & Cohen, 2013). The enhanced
VLDL secretion that is seen though is not enough to compensate for the enhanced rate of accumulation that is occurring resulting in the progression of steatosis (Kawano & Cohen, 2013).

1.2.1d Progression of NAFLD

NAFLD is a chronic condition that develops slowly and progressively over many years. The progression of NAFLD from simple steatosis to more severe forms like steatohepatitis, is most commonly explained by the “two-hit hypothesis” (Giorgio, Prono, Graziano, & Nobili, 2013; Paschos & Paletas, 2009). A healthy, fully functioning liver will never be completely devoid of lipid stores but the liver is not meant to function as a storage location for fat, so the lipid droplets that are present typically account for less than 5-10% of the liver’s total weight (Kawano & Cohen, 2013; Ress & Kaser, 2016). When the accumulation of fat within the liver exceeds 5-10% of the liver’s total weight, or if under visual examination the triglyceride accumulation exceeds 5% of the total hepatic area, then the individual is considered to have liver steatosis, the most basic form of NAFLD (Fabbrini et al., 2010; Kawano & Cohen, 2013). At the early stage of simple steatosis, in which there is only an excess accumulation of fat within the hepatocytes, the disease can remain relatively benign and without further insult the liver can continue to function normally with no adverse side effects for years (Fabbrini et al., 2010). This development in steatosis is often classified as the first “hit” and once the balance in lipid homeostasis in the liver has been disturbed, there is the risk that the disease will progress further and develop into a more severe form of NAFLD (Fig 5) (Paschos & Paletas, 2009).
Figure 5: Progression of Non-Alcoholic Fatty Liver Disease. Schematic representing the progression of NAFLD from a healthy liver devoid of excess lipid accumulation and fibrosis, to liver steatosis characterized by excessive lipid accumulation, to steatohepatitis in which fibrosis has begun to develop and finally to liver cirrhosis where scar tissue has taken over much of the liver. The histological sections are stained with Masson’s trichrome stain, all collagen fibers are stained in blue. (PT); portal triad, composed of the portal vein, bile duct and hepatic artery. (CV); central vein. Histological images are adapted from (Cohen, Horton, & Hobbs, 2011).

The excess fat that has accumulated may induce an inflammatory reaction within the hepatocytes, oxidative stress, and mitochondrial dysfunction, all of which are included as the second “hit” (Paschos & Paletas, 2009; Smith & Adams, 2011). The inflammation, in combination with liver cell death, leads to the activation of stellate cells and the formation of scar tissue or fibrosis as the liver attempts to heal itself (Tiniakos et al., 2010). This manifestation of inflammation and fibrosis in combination with steatosis is known as Non-Alcoholic Steatohepatitis (NASH), a more severe form of NAFLD (Fabbrini et al., 2010; Tiniakos et al., 2010). NAFLD may not progress further than NASH but in approximately
20% of patients that have developed NASH the disease continues to progress to the development of liver cirrhosis, where permanent damage and scaring continue to replace normal liver tissue, preventing blood flow and impairing liver function (Gusdon et al., 2014; Tiniakos et al., 2010). If the formation of this scar tissue continues, eventual end stage liver failure is likely (Tiniakos et al., 2010). In addition, NAFLD that has progressed to the stage of cirrhosis, and even occasionally before cirrhosis has developed, poses a risk for the development of liver cancer (Baffy, 2013; Michelotti, Machado, & Diehl, 2013; Starley, Calcagno, & Harrison, 2010). Hepatocellular carcinoma is the 5th most common cancer worldwide. In about 2% of individuals diagnosed with NAFLD, hepatocellular carcinoma will develop. This risk is comparable to the 4% prevalence seen in patients with hepatitis C, a known instigator of hepatocellular carcinoma (Baffy, 2013).

1.2.1e Symptoms and Complications of NAFLD

Although the early stages of NAFLD are reversible, it is difficult to catch NAFLD before it has progressed. Most individuals remain asymptomatic until the disease has reached cirrhosis and damage is no longer reparable (Adams & Angulo, 2006; Ratziu, Goodman, & Sanyal, 2015; Tiniakos et al., 2010). During the early stages, a patient may experience a loss of appetite, frequent nausea, weakness, fatigue, and/or weight loss (Adams & Angulo, 2006; Tiniakos et al., 2010). Although not specific to NAFLD, individuals may also experience pruritus; an intense itching as a result of bile salt accumulation under the skin (Tiniakos et al., 2010). As the liver continues to lose function, production of essential proteins such as albumin and proteins that are required for blood clotting may decline, resulting in edema of the extremities and a buildup of fluid in the peritoneum known as ascites, and increased bruising and bleeding (Tiniakos et al., 2010). During the later stages of NAFLD, an accumulation of toxins within the blood may present as personality changes such as a neglect of one’s appearance, forgetfulness, or changes in sleep habits as the liver loses its ability to properly filter the blood. Impairment to blood flow as a result of scar tissue formation may cause the liver and spleen to swell, ascites to form, and force blood supplies to find a new route, forming varices throughout the stomach and esophagus which run the risk of rupturing if blood pressure is high (Adams & Angulo, 2006; Tiniakos et al., 2010). Unfortunately, the majority of the symptoms that may present are indirect and common amongst other diseases, causing
NAFLD to go largely unnoticed and requiring a necessity for preventative treatment (Tiniakos et al., 2010).

1.2.1f Diagnosis of NAFLD

If an individual is at risk for developing NAFLD, or has begun to experience symptoms that may be related to NAFLD development, their doctor will typically begin by ordering a blood test to measure liver function (Adams & Angulo, 2006; Ratziu et al., 2015; Tiniakos et al., 2010). The most common liver functional blood test examines the levels of enzymes within the circulation that are required for liver function. These include, aspartate transaminase (AST) and alanine transaminase (ALT) (Adams & Angulo, 2006; Ratziu et al., 2015). Once the liver has become damaged, high levels of these enzymes, particularly AST which is specific to the liver, may be released into the circulation. If the ratio of AST: ALT within the circulation is 2:1 or greater this is an indication that damage in the form of NAFLD, hepatitis C, or Alcoholic Fatty Liver Disease, has occurred but further testing is required to determine which is the culprit (Adams & Angulo, 2006; Ratziu et al., 2015). If the patient has not consumed greater than 30 grams (male) or 20 grams (female) of alcohol per day (a regular sized beer contains approximately 14 grams), then alcoholic fatty liver disease can often be eliminated as a potential cause of this damage (Adams & Angulo, 2006). To further validate the presence of NAFLD, non-invasive imaging techniques such as ultrasound, tomography, proton magnetic resonance spectroscopy and magnetic resonance imaging can be used (Khov, Sharma, & Riley, 2014). Although these methods are relatively non-invasive, they are less effective at detecting the disease if steatosis accounts for less than 30% of the liver, therefore although invasive and costly the gold standard for diagnosing NAFLD is through a liver biopsy (Khov et al., 2014; Tiniakos et al., 2010). To perform a liver biopsy, a small needle is inserted into the liver and a sample of the liver is obtained in order to grade the degree and progression of the disease (Adams & Angulo, 2006; Ratziu et al., 2015; Tiniakos et al., 2010). Although there is no single scoring system that has been universally accepted, NAFLD is typically graded based on the presence or absence of five main histological features. The primary feature that is assessed is the extent and type of steatosis that is present, whether that be microvesicular or macrovesicular. (Tiniakos et al., 2010) Microvesicular steatosis is the presence of small lipid vacuoles within the cytoplasm with no displacement to the nucleus,
whereas macrovesicular steatosis is the presence of large lipid vacuoles that displace the hepatic nucleus (Tiniakos et al., 2010). A second feature that is examined is the presence of fibrosis and its primary location within each hepatocyte, whether that be perisinusoidal or periportal (Tiniakos et al., 2010). A third feature is the presence of microgranulomas, large lipogranulomas, or inflammatory foci that indicate inflammation within the hepatocyte (Tiniakos et al., 2010). The fourth feature is the presence of liver cell injury indicated by hepatocellular ballooning, a form of hepatocyte cell death. The final feature is the presence of other abnormal hepatic findings such as Mallory’s hyaline or glycogenated nuclei (Tiniakos et al., 2010).

1.2.1g Treatment of NAFLD

At this time, there are no clinically approved therapies for the direct treatment of NAFLD and most therapies are aimed at lifestyle modifications or treatment of the secondary conditions that may also be present. If an individual is obese the most effective treatment is a change in lifestyle with enhanced exercise and caloric restriction to gradually and naturally induce weight loss (Adams & Angulo, 2006; Ratziu et al. 2015). If the disease is still in the early stages, life style modification and weight loss have been shown to be very effective at reducing fat accumulation within the liver (Ratziu et al. 2015). Other lifestyle modifications may include a more specific diet, combined with a combination of medications if a secondary condition such as diabetes is also present. An example medication is pioglitazone, a thiazolidinedione derivative, that is used in individuals with hyperglycemia to improve insulin sensitivity, and improve glucose and lipid metabolism through activation of the nuclear transcription factor peroxisome proliferator activated receptor gamma (PPARγ) in order to enhance genetic transcription of genes involved in fatty acid storage and glucose metabolism (Belfort et al., 2006). If the disease has progressed to include the presence of inflammation and fibrosis, medications may be administered to target the pathophysiological mechanism such as the lipase inhibitor orlistat that helps to promote weight loss, metformin that helps to stimulate beta oxidation and antioxidants such as vitamin E that may decrease inflammation and prevent the activation of hepatic stellate cells (Adams & Angulo, 2006; Ratziu et al. 2015).

Recently, fish oil supplementation has emerged as a potential therapeutic treatment to reduce fat accumulation in the liver associated with NAFLD (Di Minno et al., 2012; Eslick, Howe,
Fish oil has been shown to regulate the expression and activity of key transcription factors involved in hepatic lipid metabolism. These include, activation of PPARα and PPARγ to promote fatty acid oxidation and improve insulin sensitivity, and inhibition of SREBP-1 and ChREBP to inhibit hepatic glycolysis and lipogenesis (Di Minno et al., 2012; Eslick et al., 2009; Sidhu, 2003).

Fish oil supplementation is now commonly prescribed to treat hyperlipidemia and reduce hepatic steatosis through a lowering of plasma triglyceride levels and an enhancement to hepatic fatty acid oxidation (Di Minno et al., 2012; Eslick et al., 2009). The active components of fish oil are believed to be the omega-3 poly unsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), however the presence of additional active components that may be playing a role are still under investigation (Eslick et al., 2009). This study aims to investigate the role of a particular furan fatty acid metabolite produced from the furan fatty acid contaminants found within fish oil supplementation that shows potential in the reduction of hepatic steatosis. The next section will provide a more in-depth introduction into this fish oil metabolite 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF).

1.3 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF)

1.3.1 Discovery of CMPF

The furan fatty acid metabolite 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) was initially identified as a uremic toxin elevated in the serum of individuals with uremia (Fig 6) (Deguchi et al., 2004; Miyamoto et al., 2012; Niwa, Takeda, Maeda, Shibata, & Tatematsu, 1988; Sassa et al., 2000). Uremia is a condition that is characterized by impaired kidney function resulting in the abnormal accumulation of toxic waste products that would typically be cleared in a healthy individual (Deguchi et al., 2004). Included amongst these toxic waste products are urea, creatinine, and organic anions like indoxyl sulfate, indoleacetate, hippurate, and CMPF (Deguchi et al., 2004; Miyamoto et al., 2012). Elevation of these toxic waste products causes patients with uremia to
suffer from a wide variety of symptoms such as exhaustion, nausea, irregular heartbeat, shortness of breath, and neurological symptoms like psychosis, seizures, and coma (Deguchi et al., 2004). An elevation of organic anions occurs due to impairment of the organic anion transporters within the kidney responsible for their clearance and is particularly problematic as organic anions bind with a strong affinity to plasma proteins (Deguchi et al., 2004; Niwa et al., 1988). By binding to plasma proteins, organic anions prevent the binding of drugs to albumin within the circulation, thereby interfering with the effectiveness of uremic drug therapy (Lim et al., 1993; Niwa et al., 1988). Uremia is not the only pathological condition in which CMPF is elevated within the circulation. In 2014, Prentice et al. found the furan fatty acid metabolite CMPF to be elevated in the serum of individuals with Gestational Diabetes, Type 2 Diabetes and Pre-Diabetes.

Diabetes Mellitus, encompasses a group of diseases that are characterized by a prolonged elevation in blood glucose levels (American Diabetes, 2010). This elevation in blood glucose arises as a result of either an impairment to insulin biosynthesis and secretion from the β-cell, or from an inability of the β-cell to adapt to the development of peripheral insulin resistance (American Diabetes, 2010). Recent studies have suggested that the β-cell dysfunction responsible for the development of diabetes is in part influenced by elevated levels of metabolites within the circulation (Nolan, 2014; Schaffer, 2003). With the use of metabolomics screening to examine changes within the metabolic profile of diabetic individuals, Prentice et al (2014) identified the furan fatty acid metabolite: CMPF to be increased 7-fold in the plasma of patients with gestational diabetes, with a 2- to 3-fold increase in individuals with pre-diabetes and Type 2 Diabetes when compared to their matched normal glucose tolerant controls. These results suggest that an elevation in CMPF within the circulation may influence the progression of diabetes development.

1.3.2 CMPF is a Furan Fatty Acid Metabolite

Furan fatty acids are a naturally occurring class of fatty acids that are characterized by the presence of a furan moiety; a five-membered aromatic ring composed of 4 carbons and one oxygen atom (Fig 7) (Spiteller, 2005). With the oxygen at position one, a furan fatty acid is classified as having a saturated carboxylic acid.
chain extending from the second carbon that is typically either 7, 9, 11, or 13 carbons in length, a pentyl or propyl side chain attached to the 5th carbon and a methyl group on the 3rd and/or 4th carbons (Spiteller, 2005). When being metabolized, it is the straight carboxylic chain that will be broken down via beta carboxylation. The metabolism of a furan fatty acid generates a urofuran fatty acid like CMPF, composed of a dicarboxylic acid with 10-12 carbon atoms including those of the furan ring (D. Sand, Schlenk, Thoma, & Spiteller, 1983).

### 1.3.2a Furan Fatty Acids in the Diet

The primary producer of furan fatty acids in nature is algae, but furan fatty acids are also produced naturally by other plants and microorganisms (Spiteller, 2005). Fish and larger mammals acquire furan fatty acids from the ingestion of these plants and algae and incorporate them into their phospholipids and cholesterol esters (Glass, Krick, & Echardt, 1974; Spiteller, 2005). In a study performed by Sand et al. (1984), it was demonstrated with the use of C\(^{14}\) radiolabelled acetate that fish do not possess the ability to synthesize either the furan ring or the alkyl side chain and must acquire them from the consumption of plant material. In humans, one of the primary sources of furan fatty acids comes from the consumption of fish oils (Scorletti & Byrne, 2013; Vetter & Wendlinger, 2013). Fish oils are primarily known to contain omega-3 fatty acids in the forms of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) but they also contain trace amounts of ‘contaminants’ which include furan fatty acids. (Scorletti & Byrne, 2013). Of the furan fatty acids consumed within our diet, there are five that occur most frequently; 9M5, 9D5, 11D3, 11M5, and 11D5 (Spiteller, 2005) (Fig 8). In this furan fatty acid nomenclature, the initial number represents the length of the carboxylic side chain extending from carbon 2 of the furan ring, the ‘M’ or ‘D’ represents the presence of a methyl or di-methyl group respectively on carbons 3 and 4, and the final number represents the length of the alkyl side chain extending from carbon 5. Following ingestion, it is the straight carboxylic side chain that is metabolized to a minimum of 3 carbons. As the furan fatty acid 11D3 has the closest initial structure to CMPF, with two methyl groups on carbons 3 and 4 and a propyl side chain on carbon 5, it is the most likely precursor for the generation of CMPF (Spiteller, 2005) (Fig 8).
Figure 8: Metabolism of CMPF from fish oil. Although primarily composed of ω-3 fatty acids, fish oil additionally contains furan fatty acids. Of the 5 most commonly occurring furan fatty acids, CMPF is most likely metabolized from 11D3 due to its two methyl groups at position 3 and 4 of the furan ring and the propyl side chain at position 5.

Furan fatty acids are essential components of fish oil as they are radical scavengers that protect against the peroxidation of the poly unsaturated fatty acids (Okada, Kaneko, & Okajima, 1996; Vetter & Wendlinger, 2013). Furan fatty acids can also be obtained in lesser extents from the fat of milk and plant products (Spiteller, 2005). Once ingested, these furan fatty acids are broken down into urofuran fatty acids such as CMPF and are typically excreted within the urine (Spiteller, 2005).
1.3.2b CMPF Elevation Following Fish Oil Supplementation

When untargeted metabolomics was performed on both the urine and serum of individuals following Lovaza; a prescription fish oil supplementation, the major metabolic by-product was found to be CMPF (Prentice et al., Unpublished) (Fig 9). It is important to note, that CMPF was not detected within the initial supplementation, indicating that CMPF is produced and becomes elevated within the circulation following high dose fish oil consumption.

1.3.2c Clearance of CMPF

CMPF exists at basal levels within the circulation of healthy individuals as it is typically cleared quite effectively from the kidney utilizing a group of organic anion transporters (OAT) (Burckhardt & Burckhardt, 2003; Deguchi et al., 2004). These organic anion transporters belong to a subfamily of amphiphilic solute transporters (SLC22) that belong to the larger major facilitator superfamily (Anzai, Kanai, & Endou, 2006; Kobayashi et al., 2005). They are responsible for the transport of a large range of organic compounds both into and out of the cell. The members of the OAT family are highly conserved, each containing 6 transmembrane domains connected by an intracellular loop, with both the amino and carboxyl terminus intracellularly located (Roth, Obaidat, & Hagenbuch, 2012). Although the prototypical OAT1 is capable of bidirectional transport, the majority of OAT family members are responsible for facilitated transport of organic anions into the cell through exchange with an intracellular compound such as α-ketoglutarate (Anzai et al., 2006). Within the kidney, the primary OAT responsible for CMPF clearance is OAT3 but as the members of the OAT family are known to share substrate specificity, and as the kidney expresses almost all members of the OAT family, it is likely that CMPF is also cleared with the help of additional OAT family members (Deguchi, Kouno, Terasaki, Takadate, & Otagiri, 2005; Deguchi et al., 2004).
1.3.3 CMPF Elevation Impairs Beta Cell Function

In patients that are considered pre-diabetic, as well as in patients that have gestational diabetes and Type 2 Diabetes, there is a significant elevation of CMPF seen within the circulation (Prentice et al., 2014). This information indicates that when levels of CMPF within the circulation become elevated they may contribute to the development of diabetes. To determine the effect that CMPF has on the development of Type 2 Diabetes, Prentice et al (2014) administered CMPF to mice through intraperitoneal injections for one week at a concentration that was comparable to the endogenous levels seen in diabetic patients. Mice that had received these injections of CMPF exhibited glucose intolerance, impaired glucose utilization within the beta cell, and an impairment to beta cell insulin secretion in vivo, all of which suggested an impairment to beta cell function (Prentice et al., 2014).

1.3.3a CMPF Impairs Glucose Stimulated Insulin Secretion

To further examine the mechanism behind this apparent beta cell dysfunction, human and mouse islets were isolated and the effects on insulin secretion in response to acute CMPF administration in-vitro was examined. Islets isolated from both mice and humans treated with CMPF had enhanced basal insulin secretion, with no significant change in secretion upon high glucose stimulation, demonstrating an impaired glucose stimulated insulin secretion response (Liu et al., 2016; Prentice et al., 2014; Retnakaran et al., 2016; S. Zhang et al., 2017). The impaired regulation of insulin secretion is a result of a significant reduction in insulin granule content within the islets after CMPF treatment, but with no change to insulin granule morphology (Prentice et al., 2014). Although naturally occurring furan fatty acids that are consumed in the diet have antioxidant properties, CMPF appears to have an opposite effect within the β-cell and in fact enhances the production of reactive oxygen species (ROS) (Miyamoto et al., 2012; Prentice et al., 2014). Once in the cell, CMPF directly interacts with superoxide anion radicals and peroxyl radicals, as would be expected from an antioxidant (Miyamoto et al., 2012). This reaction though leads to the production of CMPF radical adducts that react back with dissolved oxygen, leading to an over production of superoxide (Miyamoto et al., 2012). The direct impairment of beta cell function that is induced by CMPF treatment arises from an impairment to mitochondrial function that is due to this accumulation of reactive oxygen species (ROS) (Miyamoto et al., 2012; Prentice et al., 2014; Saadeh et al.,...
The increase in ROS within these islets leads to an alteration in insulin gene transcription, impairing the beta cells ability to properly synthesize insulin and reducing insulin content within the islet (Prentice et al., 2014).

1.3.3b CMPF Induces a Metabolic Switch

The impairment that occurs in insulin biosynthesis following CMPF treatment, does not on its own explain the exaggerated secretion of insulin that occurs in non-stimulatory glucose concentrations. Interestingly, along with the impairment in insulin biosynthesis, islets isolated from mice that had received CMPF injections also demonstrated an alteration in their energy metabolism leading to a decrease in their ability to properly sense and respond to glucose (Liu et al., 2016; Prentice et al., 2014). Through the measurement of changes to mitochondrial membrane potential as a result of substrate metabolism, it was shown that islets from mice treated with CMPF for one week following a 6-week high fat diet (HFD) (CMPF-DIO) displayed mitochondrial hyperpolarization in response to palmitate, with a significantly blunted mitochondrial hyperpolarization in response to glucose (Liu et al., 2016). This is in contrast to the robust mitochondrial hyperpolarization that was observed in response to glucose stimulation in islets isolated from both control CHOW and control HFD mice (DIO) (Liu et al., 2016). These results were further confirmed through examination of the oxygen consumption rate in response to substrate metabolism. In isolated islets from CMPF-DIO mice the oxygen consumption rate was significantly reduced following glucose administration, but significantly elevated in response to palmitate when compared to DIO controls (Liu et al., 2016). Additionally, using radiolabelling to measure the capacity for glucose oxidation, human islets treated with CMPF displayed significantly reduced glucose oxidation. Human islets pre-treated with palmitate had no alteration to their capacity for glucose oxidation when compared to vehicle controls (Liu et al., 2016). This indicates a metabolic switch from glucose metabolism to fatty acid oxidation following CMPF treatment, similar to what is seen in the body during a fasting state (Berg et al., 2002). This decrease in glucose metabolism occurs due to a reduction in glycolysis even though glucose uptake into these islets was enhanced, further validating CMPF’s impairment on glucose utilization and glucose stimulated insulin secretion (Liu et al., 2016).
1.3.4 CMPF Elevation Improves Hepatic Steatosis

Obesity is a growing epidemic within the western world and is not only associated with the presence of insulin resistance and Type 2 Diabetes development but also has a strong correlation to the incidence of non-alcoholic fatty liver disease, where the buildup of fat within the body as a result of excessive caloric intake impairs glucose metabolism and promotes the formation of insulin resistance (Smith & Adams, 2011). To determine what effect CMPF has on the development of obesity related Type 2 Diabetes, mice were injected with CMPF for one week prior to being placed on a HFD for 4 weeks (Fraulob, Ogg-Diamantino, Fernandes-Santos, Aguila, & Mandarim-de-Lacerda, 2010). A diet that is composed of 60 % fat is used in animal models to induce insulin resistance and simulate an obesity related type 2 diabetic model(Wang & Liao, 2012).

1.3.4a CMPF Improves Insulin Sensitivity

After 4 weeks of HFD feeding, mice that had been given intraperitoneal injections of CMPF had enhanced insulin secretion at basal levels with impaired glucose stimulated insulin secretion, as would be expected in the presence of beta cell dysfunction (Prentice et al., Unpublished). Interestingly though, mice that had received CMPF prior to HFD feeding, had improved insulin sensitivity when compared to control-HFD mice (Fig 10) (Prentice et al., Unpublished). The insulin sensitivity of these CMPF-HFD mice reached levels that were comparable to control CHOW mice, a finding that is contrary to what would be expected following a high fat diet.

![Figure 10: CMPF improves insulin sensitivity following a HFD.](image-url)

Blood glucose during ip insulin tolerance test in mice 4 weeks following the final injection. Mice that had received CMPF prior to a HFD had improved insulin sensitivity when compared to the HFD-controls. *P<0.05, **P<0.01. All error bars SEM. (Prentice et al. Unpublished).
1.3.4b CMPF Decreases Hepatic Triglyceride Accumulation

Excessive caloric intake and a lack of exercise in obese individuals will contribute to the development of a higher percentage of total adipose tissue (Fabbrini et al., 2010). As a result of this, the levels of free fatty acids released from the adipose and delivered through the circulation to the liver will be increased and will lead to an impairment in insulin signaling within the hepatocyte, thereby enhancing hyperglycemia and contributing to the development of whole body insulin resistance (Birkenfeld & Shulman, 2014; Galbo & Shulman, 2013; Gariani et al., 2013). In mice that had received CMPF injections prior to 4 weeks of a HFD, insulin sensitivity was significantly improved when compared to HFD controls and was restored to levels that were similar to the mice that had not received a HFD (Fig 11) (Prentice et al., Unpublished). As a result of this surprising observation, we examined the fat content within the livers of these mice. Morphologically, the livers of the mice that had received CMPF prior to a HFD resembled those of the non-HFD mice and were phenotypically a deep red colour rather than the characteristic pale colour that is associated with a fatty liver from a HFD (Fig 11A) (Prentice et al., Unpublished). Histological staining and quantification of triglyceride content revealed them to have a significant reduction in hepatic steatosis when compared to the livers of the HFD controls (Fig 11A and 11B) (Prentice et al., Unpublished). This evidence indicates that CMPF is affecting lipid metabolism and storage within the hepatocyte to prevent hepatic steatosis, but it is unclear as to whether this is a direct effect of CMPF on lipid metabolism in the liver or through an indirect action of CMPF elsewhere in the body.

![Figure 11: CMPF decreases triglyceride accumulation in the liver following HFD. (A) Representative liver morphology and Oil Red O staining and (B) quantification of triglyceride concentration in isolated livers from mice 4 weeks following their final injection demonstrating a reduction in hepatic triglyceride concentration in CMPF-HFD mice compared to HFD-controls (n=8/group). *P<0.05, **P<0.01. All error bars SEM. (Prentice et al. Unpublished)](image)
In chapter 1 we began with an introduction into energy metabolism within the liver and the essential role that the liver plays to regulate both glucose and lipid homeostasis within the body. Deregulation of energy metabolism within the liver can be quite detrimental and result in the development of a number of metabolic disturbances within the body including the development of hepatic steatosis, the most basic form of Non-Alcoholic Fatty Liver Disease. As there are no treatments currently available to specifically cure or prevent the development of NAFLD, research into potential therapeutics is essential. Of growing interest is the beneficial effect that fish oil supplementation appears to have to reduce hepatic steatosis, and whether these beneficial effects are solely a result of omega-3 poly-unsaturated fatty acids. In Section 1.3, we introduced an additional compound produced as a by-product of fish oil consumption that is the topic of interest for this study; CMPF. The furan fatty acid metabolite CMPF was initially discovered in relation to the development of diabetes, as a rapid elevation in circulating CMPF levels induces beta cell dysfunction and impairs glucose stimulated insulin secretion. Interestingly though, CMPF is additionally elevated, although to a lesser extent, following the consumption of fish oil as the furan fatty acid that CMPF is metabolized from is a ‘contaminant’ of the fish oil preparation. When administered to mice, CMPF appears to have a dual effect in that it additionally prevents the development of hepatic steatosis and improves insulin sensitivity following a HFD. With this information in mind, the next chapter will introduce the hypothesis of this study and outline the specific aims that will be addressed to assess the role of CMPF on hepatic function.
Chapter 2: Research Aims and Hypothesis

2.1 Rationale

Fish oil is a commonly recommended supplement for individuals who have hyperlipidemia, to aid in the reduction of circulating triglycerides, liver steatosis, and inflammation (Di Minno et al., 2012). It is thought that the omega-3 poly unsaturated fatty acids present in fish oil play the major role in its beneficial effects (Eslick et al., 2009). However, the metabolic effects of these fish oil metabolic by-products, as well as the additional components of fish oil are still under investigation. Following fish oil supplementation with the prescription fish oil Lovaza, the most abundantly elevated metabolite was the furan fatty acid metabolite CMPF (Prentice et al., Unpublished). Previously our lab discovered CMPF to impair beta cell function when significantly elevated within the circulation (Prentice et al., 2014). Impairment to beta cell function occurred from an impairment to glucose stimulated insulin secretion that results from a preferential use of fatty acid oxidation. In a cell type such as the beta cell, that relies heavily on its ability to sense and metabolize glucose in order to respond accordingly with the secretion of insulin, a switch to a preference for fatty acid oxidation is quite detrimental. However, in a cell type that plays a significant role in the regulation and storage of lipids such as the hepatocyte, an enhancement to lipid metabolism could be beneficial and combined with the evidence that CMPF is elevated following fish oil consumption indicates that CMPF may be an active fish oil metabolite and have a double-edged effect within the body.

From a recent study currently being submitted for publication, we have found that when administered to mice prior to a HFD, CMPF enhanced insulin sensitivity and significantly altered the hepatic phenotype resulting in a reduction in triglyceride accumulation compared to HFD-controls (See introduction section 1.3.4) (Prentice et al., Unpublished). These results demonstrate that CMPF alters hepatic function in-vivo, however, it remains to be determined whether these effects are the result of a direct influence on hepatic metabolism or through an indirect action of CMPF on another tissue within the body. Further investigation is required into the cellular mechanism that allows CMPF to prevent hepatic steatosis development and is important in helping to understand the effect that CMPF has on whole-body metabolism.
2.2 Objective and Hypothesis

The objective of this study was to confirm a direct effect of CMPF on the liver and to characterize the cellular mechanism that allowed CMPF to beneficially reduce hepatic steatosis in-vivo. We hypothesized that CMPF acts directly on the hepatocyte to enhance fatty acid oxidation, and decrease triglyceride accumulation, thereby preventing hepatic steatosis.

2.3 Scientific Aims

2.3.1 Direct Effect of CMPF on Liver Metabolism

To test our hypothesis primary hepatocytes were isolated from untreated c57/BL6 mice and treated for 24-hours with CMPF in-vitro in order to assess direct alterations to hepatic function or cellular signaling as a result of CMPF treatment.

2.3.1a Aim 1a: Direct Effect on Liver Function

The first part of our first aim was to examine whether CMPF directly influenced glucose and lipid metabolism. Based on the metabolic switch that CMPF induced within the hepatocyte, we hypothesized that CMPF would directly enhance hepatic fatty acid oxidation. To assess changes in hepatic function, primary hepatocytes were treated for 24-hours with CMPF and assessed on their ability to uptake, store, produce and utilize both glucose and fatty acids. These functional components were primarily assessed with the use of radiolabelling. Following CMPF treatment, we found there to be a significant increase in hepatic fatty acid oxidation.

2.3.1b Aim 1b: Changes to Hepatic Cellular Signaling

Within the second part of our first aim we were interested in characterizing signal transduction changes that were occurring in the affected metabolic pathways with the use of ELISA kits, western blotting and quantitative polymerase chain reaction (qPCR) to measure gene expression. Of particular interest was the insulin signaling pathway; assessed through AKT phosphorylation, and activation of the major regulatory pathway of fatty acid oxidation; assessed through phosphorylation of both AMPK and ACC. Due to the improvement in insulin
sensitivity following a high fat diet \textit{in-vivo} and the increase in hepatic fatty acid oxidation \textit{in-vitro}, we hypothesized that CMPF would improve lipid impaired phosphorylation of AKT and enhance phosphorylation of the AMPK/ ACC pathway. Following CMPF treatment, we found a trend towards improved AKT phosphorylation with no alteration to the phosphorylation of either AMPK or ACC. In addition, 24-hour treatment with CMPF was found to enhance both the expression and secretion of fibroblast growth factor 21 (FGF21), a protein known to alter energy metabolism within the body.

\textbf{2.3.1c  Aim 1c: Mechanism to Facilitate Hepatic CMPF Entry}

Finally, in the third portion of our first aim, we were interested in the mechanism that may be facilitating CMPF’s transport into the hepatocyte. We hypothesized that CMPF is able to enter the hepatocyte through the use of an organic anion transporter. To test this hypothesis, we began by confirming that CMPF is able to both enter and accumulate within the hepatocyte and then assessed both OAT expression within the liver and the effect of OAT inhibition on CMPF accumulation within the liver. Primary hepatocytes were found to express the organic anion transporter 2 (OAT2) and following 24-hour treatment, CMPF significantly accumulated within the hepatocyte.

\textbf{2.3.2 Underlying Mechanism Mediating CMPF’s Effect in the Liver}

In our second aim, we examined the underlying mechanism that allows CMPF to influence hepatic lipid accumulation. As a result of CMPF enhancing fatty acid oxidation in the absence of either AMPK or ACC phosphorylation, we hypothesized that CMPF was directly interacting with ACC in order to inhibit its activity. Primary hepatocytes were treated with compounds that structurally resembled that of CMPF, and whose methods of action to inhibit ACC were well known, in order to elucidate how CMPF may be enhancing whole body lipid metabolism. We found that when isolated ACC enzymes were treated with CMPF directly, there was a decrease in their enzymatic ability. In addition, treatment of primary hepatocytes with direct ACC inhibitors lead to a similar increase in both the expression and secretion of FGF21.
Chapter 3: Direct Effect of CMPF on Primary Hepatocytes In-Vitro

3.1 Introduction

A significant elevation in circulating levels of the furan fatty acid metabolite CMPF has previously been believed to be quite detrimental to the health of an individual, resulting in beta cell dysfunction and promoting the development of diabetes (Prentice et al., 2014). Recent evidence has counteracted this concept and shed light onto a double-edged effect that CMPF appears to have, in that it acts favourably in the prevention of hepatic steatosis (Prentice et al., Unpublished). Although the metabolic switch that CMPF induces within the beta cell impairs glucose sensing and results in beta cell dysfunction, it appears that within cell types responsible for the regulation of lipids this enhancement to fatty acid metabolism may in fact be beneficial. Following administration into mice prior to being fed a HFD, CMPF treatment improved whole body lipid metabolism and insulin sensitivity (Prentice et al., Unpublished) (See section 1.3.4). Within the liver, CMPF beneficially influenced the hepatic phenotype and resulted in a decrease to hepatic triglyceride accumulation compared to HFD controls. In an attempt to elucidate how CMPF is able to improve hepatic steatosis following a high-fat diet, a microarray was performed on the livers of mice following CMPF-HFD treatment (Prentice et al., Unpublished) (Fig 12A). Of particular interest was an increase in fibroblast growth factor 21 (FGF21) expression. This enhanced gene expression corresponded to a significant elevation in circulating FGF21 when compared to HFD controls (Prentice et al. Unpublished) (Fig 12B).

Figure 12: CMPF increases circulating levels of FGF21. (A) Microarray performed on the livers of mice following 1 week of injections and 4 weeks of HFD which shows an upregulation in expression of FGF21 (B) FGF21 levels in serum of mice following injection and 4 weeks of dietary intervention (Prentice et al. Unpublished).
Fibroblast growth factor 21 (FGF21), is a protein that is primarily produced within the liver and has been established to have beneficial effects on whole body fat utilization when elevated within the circulation, leading to a reduction in hepatic steatosis (Galman et al., 2008; X. Lin, Liur, & Hu, 2017). Levels of FGF21 are typically elevated during periods of fasting or in response to a ketogenic diet and are controlled through the activity of the ligand activated transcription factor peroxisome proliferator-activated receptor α (PPARα) (Erickson & Moreau, 2016). Following secretion into the circulation, FGF21 acts primarily on the adipose tissue and central nervous system to enhance glucose uptake, enhance energy utilization, decrease adipose tissue lipolysis, and increase secretion of metabolic hormones such as adiponectin (Lin et al., 2013) (Fig 13). It is likely through the release of hormones such as adiponectin and stimulation of the sympathetic nervous system, that enables FGF21 to decrease hepatic glucose production, decrease hepatic lipogenesis, and increase hepatic fatty acid oxidation (Fisher & Maratos-Flier, 2016). This increase in fatty acid oxidation occurs as a result of increased activity of the transcription factor PPARα and its co-activator PGC-1α, leading to enhanced transcription of genes that are required for fatty acid oxidation (Lin et al., 2013; Fisher & Maratos-Flier, 2016; Ge et al., 2012) (Fig 13). These effects of FGF21 on the liver are likely not direct as they are not observed following direct

![Figure 13: Summary of the major direct and indirect effects of FGF21 elevation within the body.](image) When elevated within the circulation CMPF acts directly on the adipose tissue and CNS to increase energy expenditure and decrease adipose tissue lipolysis. The effects of FGF21 on the liver are likely indirect but lead to enhanced transcriptional activity of fatty acid oxidation related genes, while simultaneously inhibiting gluconeogenesis and lipogenesis within the hepatocyte. **CNS: Central Nervous System**
treatment of FGF21 in isolated hepatocytes (Lin et al., 2013). When combined together, the effects of FGF21 on the central nervous system, adipose tissue, and liver, enable FGF21 to enhance whole body insulin sensitivity, reduce body weight, reduce hepatic steatosis and reduce both triglyceride and blood glucose levels in the circulation (Fisher & Maratos-Flier, 2016; Maratos-Flier, 2017). Although these three tissues are the primary locations of FGF21’s effects, FGF21 also acts on other tissues within the body such as the bone, to decrease growth hormone response and the pancreas, to increase insulin production (Fisher & Maratos-Flier, 2016).

To determine if CMPF is able to enter the liver, our lab utilized mass spectrometry to measure the concentration of CMPF within both the circulation and whole liver 1-hour following injection (Fig 14A). Post administration, CMPF was significantly elevated within both the circulation and whole liver tissue (Fig 14 B and C) (Prentice et al., Unpublished).

**Figure 14: CMPF accumulates in the liver 1-hour post injection.** (A) Elution profile of CMPF determined through SRM/MS. Quantification of CMPF concentration measured through mass spectrometry to show the (B) Concentration of CMPF in the serum 1-hour following injection (n=3). (C) Concentration of CMPF in whole liver tissue 1-hour following injection and normalized to protein content (n=3). ***P<0.01, ****P<0.001. All error bars SEM. (Prentice et al. Unpublished)

CMPF’s ability to accumulate within the liver, in combination with the beneficial effects it appears to have to reduce hepatic lipid accumulation and enhance hepatic FGF21 expression, suggest that CMPF is acting on the liver to enhance lipid metabolism. Further investigation is required to determine if the enhancement seen to lipid metabolism following CMPF treatment is direct or if it is an indirect result of CMPF acting elsewhere in the body, for example through its increase in
circulating levels of FGF21. With the use of primary hepatocytes, we aim to replicate the beneficial effects seen on liver metabolism in-vivo within an isolated setting in order to demonstrate a direct effect of CMPF within the hepatocyte.

The excessive fat accumulation that occurs in hepatocytes during steatosis is a result of a deregulation to one or more components of lipid homeostasis (Fabbrini et al., 2010). This can be the result of increased triglyceride uptake, increased triglyceride synthesis, decreased fatty acid oxidation or decreased fatty acid secretion. To assess a direct effect of CMPF on the hepatocyte we aim to assess both function and signaling changes within these aspects of lipid metabolism as well as glucose metabolism with the use of radiolabelling, in combination with western blotting and qPCR.

In addition, in order for CMPF to directly affect hepatic function it must either interact with a cell surface receptor or gain entry into the hepatocyte. Within the kidney, CMPF is effectively cleared from the circulation by members of the organic anion transporter (OAT) family, specifically OAT3 (Kobayashi et al., 2005). The individual members of the organic anion transporter family are known to have abundant substrate overlap and differ most between isoforms in their tissue distribution (Sekine, Cha, & Endou, 2000). Within the liver, the OAT isoform OAT2 has been reported to have the most abundant expression (Roth et al., 2012; Shen et al., 2015). As we have previously demonstrated that CMPF is able to accumulate within the liver 1-hour post injection, we aim to confirm accumulation of CMPF within the hepatocyte and to determine if CMPF is utilizing OAT2 to facilitate its entry.

With the use of these in-vitro assays, we predict that CMPF gains entry and accumulates within the hepatocyte where it has a direct effect on hepatic function in order to protect against the development of hepatic steatosis. Following entry into the hepatocyte, we predict that CMPF results in an increase to fatty acid oxidation thereby decreasing hepatic triglyceride accumulation and improving lipid impaired hepatic insulin signaling.
3.2 Materials and Methods

3.2.1 Reagent Preparation

CMPF was purchased from Cayman Chemicals (Cat# 10007133) and dissolved to a stock concentration of 100mM using 70% Ethanol. The ACC inhibitors, TOFA (Cat# T6575), S-2E (Cat# S6445) and PF-05175157 (Cat# PZ0299) were purchased from Sigma Aldrich and dissolved in DMSO to a stock concentration of 20mM, 100mM and 100mM respectively. CMPF and ACC inhibitors were all stored at 4°C. AICAR (Cat# A9978) was purchased from Sigma Aldrich and dissolved in ultrapure water to a stock concentration of 100mM and stored at -20°C. Prior to in-vitro treatment, CMPF was conjugated to free-fatty acid free BSA (Sigma Aldrich Cat# A8806) at a ratio of 3:1, for 4-hours at 37°C.

3.2.2 Animal Use

All mice used were male c57BL/6 mice purchased from Jackson Laboratory (Maine, USA). Following delivery, mice were allowed to acclimatize to our animal facility for 1-week prior to experimentation. Unless otherwise stated, all mice were fed a standard Rodent Diet. All animal studies were performed in accordance with the Canadian Council of Animal Care Guidelines and were approved by the Animal Care Committee at the University of Toronto.

3.2.3 Isolation and Culture of Primary Murine Hepatocytes

Primary hepatocytes were isolated from male 7-9-week-old c57BL/6 mice using a two step-collagenase perfusion method as described previously with some modifications (Dentin et al., 2007; W. Zhang et al., 2012). Briefly, blood flow through the thoracic vena cava was tied off and the abdominal vena cava was cannulated using a 25G butterfly needle (VWR Cat#CABD367341). A small incision was made within the hepatic portal vein to allow liquid flow through the liver. The liver was perfused for 5 min with perfusion buffer (GIBCO Cat# 17701-038), followed by an additional 3-4 min perfusion with digestion buffer composed of 0.25mg/ml collagenase IV (SIGMA Cat#C5138-1G) dissolved in low glucose Dulbecco’s Modified Eagle Medium (DMEM) (GIBCO Cat#11885-084). During the perfusion, both the perfusion and digestion buffer were warmed and maintained at a temperature of 42°C. Following digestion, the liver was excised,
broken apart and filtered through a 70-micron cell filter (FALCON Cat#352350) using ice cold high glucose DMEM (GIBCO Cat#11995-065) with p/s (GIBCO Cat#15140-122) and 10% FBS (SIGMA Cat#F1051-500ML). The filtered cells were centrifuged at 100g for 3 min at 4°C to generate a cell pellet of live isolated hepatocytes that were re-suspended and counted using Trypan Blue (GIBCO Cat# 15250-061) to allow an equal seeding density of 500 000 cells/ well. Primary hepatocytes were allowed to adhere to the plate in the presence of 10% FBS for 4-hours, after which the media was changed to warm high glucose DMEM with p/s and 0% FBS along with any additional treatment reagents.

3.2.4 Radioactivity

3.2.4a Fatty Acid Uptake

Fatty acid uptake was performed as previously described (Wilmsen et al., 2003) with some modifications. Briefly, primary hepatocytes were treated for 24-hours with 200µM CMPF in 0% FBS media. Following treatment, primary hepatocytes were washed with warm PBS and treated for 3-min with transport solution composed of 20µM Palmitate (SIGMA Cat#P9767-10G) dissolved in 70% Ethanol and 0.5µCi/ml 14C Palmitic Acid (Perkin Elmer, Boston, MA) conjugated to BSA for 4 hours in Hepes Buffer (see Table 1). Immediately following the 3-min incubation, primary hepatocytes were placed on ice and washed thoroughly with ice cold stop solution containing 0.5% free-fatty acid free BSA. A cellular lysate was created with the addition of 1M KOH. Radioactivity of the cellular lysate was counted and normalized to protein content measured through a Bradford Assay (SIGMA Cat#B6916-500ML).

<table>
<thead>
<tr>
<th>Hepes Buffer</th>
<th>Final Concentration</th>
<th>Per 50 ml (from stock)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>140mM</td>
<td>3.5ml (from 2M)</td>
</tr>
<tr>
<td>Hepes –Na pH 7.4</td>
<td>20mM</td>
<td>2ml (from 0.5M)</td>
</tr>
<tr>
<td>KCl</td>
<td>5mM</td>
<td>0.25ml (from 1M)</td>
</tr>
<tr>
<td>MgSO4</td>
<td>2.5mM</td>
<td>0.125ml (from 1M)</td>
</tr>
<tr>
<td>CaCl2</td>
<td>1.0mM</td>
<td>0.05ml (from 1M)</td>
</tr>
<tr>
<td>Fatty acid free albumin</td>
<td>0.1%</td>
<td>0.05g</td>
</tr>
</tbody>
</table>

*No sodium bicarbonate*
3.2.4b Fatty Acid Oxidation

Fatty acid oxidation was performed as described previously (Ceddia & Sweeney, 2004) with some modifications. Briefly, primary hepatocytes were treated for 24-hours with CMPF and 100μM TOFA (SIGMA Cat#T6575-5MG) in 0% FBS media. Following treatment, the media was replaced with a transport solution containing 1mM L-carnitine (SIGMA Cat#C0283), 1.5mM Oleic Acid (SIGMA Cat#O1008) and 1.0μCi/ml 14C Oleic Acid (PerkinElmer, Boston, MA) conjugated to BSA for 4 hours in Hepes Buffer (see Table 1). Incubation with the transport solution occurred for 90 min at 37°C, after which the media was collected into a scintillation vial and 1M KOH was added onto the cells to create a cellular lysate. As the radiolabelled oleic is being oxidized within the mitochondria, the radiolabelled carbons are released from the Krebs cycle into the media as radiolabelled CO₂. In order to release the dissolved CO₂ from the solution, a strong acid of 4M H₂SO₄ was added to the collected media following incubation and quickly covered with a strip of filter paper soaked in 2M NaOH in order to capture any released CO₂. The filter covered scintillation vials were incubated for 1 hour at 37°C. Following incubation, the filter paper was removed and allowed to air dry overnight, while the media and acid solution was discarded. The following day, the filter paper was soaked for 2 hours in ultrapure water. The entire mixture of filter paper and water was added to a scintillation vial with scintillation fluid in order to count the level of radiation. All data was normalized to protein content within the cellular lysate, determined through a Bradford Assay.

3.2.4c Lipogenesis

Lipogenesis was performed as described previously (Akie & Cooper, 2015). Briefly, primary hepatocytes were isolated and treated for 24-hours with CMPF, 100μM TOFA (Sigma Aldrich Cat# T6575), 10μM S-2E (Sigma Aldrich Cat# S6445) and 10μM PF-05175157 (Sigma Aldrich Cat# PZ0299) in 0% FBS media containing 100nM insulin (Eli Lilly Humulin R 100units/ml DIN 00586714). Following treatment, primary hepatocytes were incubated for 2-hours at 37°C with a transport solution containing 10μM acetate (SIGMACat#S2889) and 0.5μCi/ml 14C Acetic Acid (PerkinElmer, Boston, MA) in Hepes Buffer (see Table 1). Radiolabelled acetate that is taken up from the media and converted into radiolabeled malonyl-
coA to be incorporated into long chain fatty acids was then measured within the cellular lysate. Briefly, following incubation the media was removed and 0.1N HCL was added to create a cellular lysate. Using a sample of the cellular lysate, a chloroform: methanol mixture at a ratio of 2:1 was added, vortexed and allowed to sit at room temperature for 5 min. Ultrapure water was then added to the samples, vortexed and allowed to sit at room temperature for 5 min. The samples were then centrifuged for 10 min at 3000 RPM in order to separate the organic and aqueous layers. The bottom organic layer containing the dissolved lipids produced from the radiolabelled acetate was carefully removed and added to a vial of scintillation fluid in order to be radioactively counted.

3.2.4d Glucose Uptake

Glucose uptake was performed as previously described (Ceddia and Sweeney, 2004) with some modifications. Briefly, primary hepatocytes were treated for 24-hours with 200µM CMPF in 0% FBS media. Following treatment primary hepatocytes were treated with transport solution containing 10µM 2-deoxy glucose (SIGMA Cat #121649) and 0.5µCi/ml \(^{3}H\) 2-deoxy glucose (PerkinElmer, Boston, MA) for 5 min. Immediately following incubation, primary hepatocytes were placed on ice and washed thoroughly with ice cold saline solution (0.9% NaCl) and lysed with the addition of 1M KOH. Concentration of glucose taken up into the cell was measured through radioactive counting of the cellular lysate. To ensure assay functionality, L6WT muscle cells that were kindly supplied by Dr. Gary Sweeney at York University, were stimulated with 100nM insulin (Eli Lilly Humulin R 100units/ml DIN 00586714) for 15 min prior to treatment with the transport solution. All data was normalized to protein content within the cellular lysate, determined through a Bradford Assay.

3.2.5 Gluconeogenesis

Glucose production was performed as described previously (Sakai et al., 2012) with some modifications. Briefly, primary hepatocytes were serum starved in 0% FBS media overnight after which they were pre-treated for 2-hours with glucose free DMEM without phenol red (GIBCO Cat#A1443001) and supplemented with 20mM lactate, 2mM sodium pyruvate, 2mM L-glutamine and 15mM HEPES. The primary hepatocytes were then washed with warm PBS and treated for an additional 4-hours with glucose free DMEM containing either CMPF or 100nM glucagon.
(BACHEM Cat# H-4012). Following treatment, the media was collected and the concentration of glucose was measured using the colorimetric Glucose (GO) assay kit purchased from Sigma (Cat#GAGO20-1KT) that oxidizes glucose to gluconic acid and hydrogen peroxide which then reacts with o-dianisidine and sulfuric acid to form a coloured product. The assay was performed according to the manufacturers protocol. Readings were normalized to protein content measured from the cellular lysate through a Bradford Assay.

### 3.2.6 Triglyceride Accumulation

Triglyceride accumulation was assessed within the isolated hepatocytes using a triglyceride quantification colorimetric assay kit purchased from BioVision (Cat#K622-100). To generate a triglyceride lysate, primary hepatocytes were initially treated for 24-hours with fat supplemented media, composed of a 1:1 ratio of palmitic acid (SIGMA Cat#P9767-10G) and oleic acid (SIGMA Cat#O1008) conjugated for 4-hours with BSA. Following 24-hour fat supplementation the isolated hepatocytes were treated for an additional 24-hours with fat supplemented media and 200µM CMPF. Following treatment, the primary hepatocytes were washed thoroughly, trypsinized with 0.25% Trypsin (GIBCO Cat#25200-056), and spun at 3000 rpm for 5 min to generate a cell pellet. The cell pellet was homogenized in 5% NP-40 (Biobasic Cat#NDB0385) and heated twice for 5 min at 90°C to generate a triglyceride lysate.

### 3.2.7 FGF21 Secretion

The concentration of FGF21 secreted into the media following 20-hour CMPF treatment was measured using an FGF21 enzyme-linked immunosorbent assay (ELISA) purchased from R&D Systems (Cat#MF2100). Briefly, undiluted media was added onto a purchased FGF21 ELISA plate pre-coated with a monoclonal antibody that is specific for the mouse FGF21 protein. Any FGF21 present within the media will bind to the immobilized antibody on the plate. Following incubation, a second enzyme linked polyclonal antibody specific for mouse FGF21 was added in combination with a substrate solution that results in the production of a blue product that turns yellow upon addition of a stop solution. The intensity of colour for each well was measured based on the values of a standard curve and was indicative of the concentration of FGF21 in each well.
3.2.8 CMPF *In-Vivo* Injection

For CMPF injection, an appropriate volume of CMPF was calculated based on the weight of the mouse to create a final injection volume of 6mg/kg body weight. Injection volumes of vehicle or CMPF were aliquoted and injected along with 100ul of sterile saline using an insulin syringe (VWR Cat#324703).

3.2.9 LC-MS/MS for CMPF Accumulation

To assess the concentration of CMPF and glucuronidated CMPF in the circulation, blood samples were collected and spun at 6000 rpm for 10 min in order to extract the serum. CMPF standards and samples were then spiked with an equal amount of CMPF-d₅ internal standard. For the standard, a surrogate matrix was used composed of 4% BSA in PBS. All serum samples and standards (each 20µl) were diluted with 480µl of ultrapure water. For the hepatic cell pellets, primary hepatocytes were trypsinized with 0.25% Trypsin (GIBCO Cat#25200-056), and spun at 3000 rpm for 5 min to generate a cell pellet. The pellet was re-suspended in 1ml of 1:1 water: ethanol and sonicated for 2 min. From the sample, 200ul (or approx. 1 million cells) was taken for extraction. The cell pellet samples were then diluted with 300ul of ultrapure water. To each standard and sample (same for serum or cell pellet), 80% phosphoric acid was added and vortexed briefly. Once all samples and standards had been acidified they were thoroughly mixed, chilled and separated into an aqueous and organic phase. The upper EtOAc layer was collected and dried under a gentle flow of nitrogen gas. All residues were reconstituted in 200ul of acetonitrile and analyzed by LC-MS/MS. All samples were analyzed via LC-MS/MS using either an Agilent 1200 HPLC with a Q-Trap 5500 mass spectrometer (AB Sciex) or an Agilent 1290 with a Q-Trap 5500 mass spectrometer (AB Sciex) and performed by the Analytical Facility for Bioactive Molecules (The Hospital for Sick Children, Toronto Ontario).

3.2.10 Lysate Preparation and Western Blots

To simulate a HFD *in-vitro*, primary hepatocytes were treated for 24-hours with fat supplemented media composed of a 1:1 ratio of palmitic acid (SIGMA Cat#P9767-10G) and oleic acid (SIGMA Cat#O1008), conjugated for 4-hours to fatty acid free BSA. Following fat-preload, primary hepatocytes were treated with 200µM CMPF in the presence of fat supplementation. To assess
insulin signaling *in-vitro*, treated hepatocytes were stimulated with either 10nM or 100nM insulin (Eli Lilly Humulin R 100units/ml DIN 00586714) for 10 min prior to lysate collection. To assess AMPK/ACC signaling, primary hepatocytes were treated with 200µM CMPF or 1mM AICAR for 5, 10, 20 or 30 min prior to lysate collection. Primary hepatocytes were lysed on ice using a 10X Lysis Buffer (Cell Signaling, Cat#98035) diluted to 1X using ultrapure water (Invitrogen Cat#10977-015) and containing 10mM NaF and a protease inhibitor cocktail tablet (Roche, Cat#11836170001). A cell scraper (Greiner Cat#541070) was used to ensure all cells were removed and the lysate was homogenized with an insulin syringe (VWR Cat#324703). Following centrifugation at 10 000rpm for 10 min at 4°C, the supernatant was collected and stored at -80°C until use. Protein content within the supernatant was assessed using a Bradford Assay to allow an equal concentration of protein per sample to be run. Each sample was combined with NuPAGE reducing agent (Invitrogen Cat#NP0009) and sample buffer (Invitrogen Cat#NP0007) and heated for 5 min prior to being loaded onto the gel. Insulin lysates were run on a 10% SDS-PAGE gel (Bio-Rad Cat#456-1036) and AMPK/ACC lysates were run on a 4-15% SDS-PAGE gel (Bio-Rad Cat#456-1086) at 50V for 30 min, 75V for 30 min and 100V for the remaining time of approximately 45 min. Protein within the gel was transferred onto a PDVF membrane (Bio-Rad Cat#1704156) using a Turbo Blotter (BioRad, Canada). The membrane was probed with primary and secondary rabbit antibodies as listed in Table 2 purchased from Cell Signaling and diluted 1:1000 for primary and 1:10000 for secondary. Membranes were imaged using a Kodak Imager 4000pro (Carestream, USA) and ECL detection reagent (GE Healthcare Cat#RPN2232).

**Table 2: Western Blot Primary and Secondary Antibodies**

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Catalogue Number (Cell Signaling)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Beta Actin</td>
<td>4967S</td>
</tr>
<tr>
<td>Anti-Alpha Actinin</td>
<td>3134S</td>
</tr>
<tr>
<td>Anti-pAKT (Ser473)</td>
<td>9271S</td>
</tr>
<tr>
<td>Anti-Total AKT</td>
<td>9272S</td>
</tr>
<tr>
<td>Anti-pAMPKα (Thr172)</td>
<td>2535S</td>
</tr>
<tr>
<td>Anti-Total AMPKα</td>
<td>2532S</td>
</tr>
<tr>
<td>Anti- pACC (Ser79)</td>
<td>11818s</td>
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<tr>
<td>Anti- Total ACC</td>
<td>3676S</td>
</tr>
<tr>
<td>Rabbit Secondary</td>
<td>7074S</td>
</tr>
</tbody>
</table>
3.2.11 Gene Expression

Gene expression within the liver and isolated hepatocytes was assessed using both quantitative real-time PCR (qPCR) and standard PCR. Briefly, total RNA was extracted from isolated hepatocytes or ground liver tissue using the Qiagen RNeasy Plus mini kit (Cat#74134). Reverse transcription was performed on total RNA, using M-MLV reverse transcriptase (SIGMA Cat#M1302-40KU), Oligo dT (Invitrogen Cat#18418012) and a dNTP cocktail (Invitrogen Cat#10297-018) according to the manufactures protocols. Quantitative real-time PCR was performed on the primary hepatocyte cDNA utilizing the Viia 7 Real-Time PCR System (Life-Technology, Canada). Data is expressed as a fold change over control based on ΔΔCT values. Standard PCR was performed on liver and primary hepatocyte cDNA in the presence of RedTag (Sigma, Cat #R2648-20RXN) according to the manufacturers protocol. After which, samples were run through a 1.2% agarose gel containing Redsafe (iNtRONCat#21141) for approximately 15min and imaged on a Kodax Imager 4000pro (Carestream, USA). Densitometry was performed using ImageJ software and expressed as a percentage of GAPDH expression. All primers were designed using Primer3 and BLAST software (NCBI) and are listed in Table 3 and 4.

### Table 3: Quantitative Real-Time PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mBeta-Actin</td>
<td>CTGAATGGCCCCAGGTCTGA</td>
<td>CCCTGGCTGCCTCAACAC</td>
</tr>
<tr>
<td>mFGF21</td>
<td>CCCCAAGACCAAGCAGGATT</td>
<td>AGATTCAGGAAGAGTCAGGACG</td>
</tr>
</tbody>
</table>

### Table 4: Standard PCR Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mOAT1</td>
<td>GCAGCCTATGCACCCAACCTA</td>
<td>GGCAAGAGCAGTAGGGCAAAACC</td>
</tr>
<tr>
<td>mOAT2</td>
<td>TCTGCACCATGGCTTCTAACC</td>
<td>CAGTCCCGGTATCGTCTCTGCTC</td>
</tr>
<tr>
<td>mOAT3</td>
<td>CACCTCAGCCAAAGGTCAATG</td>
<td>CAGTAGAGTCATGGTCCCCAAG</td>
</tr>
<tr>
<td>mURAT1</td>
<td>TCCTGGCTGGAATCTCTGGA</td>
<td>ACCTCCATGGGTAACCTGTC</td>
</tr>
<tr>
<td>mOAT5</td>
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<td>GCCAGAACCAGAAATGAGAGA</td>
</tr>
<tr>
<td>mGAPDH</td>
<td>GGTCGGTGTGAACCGGATTT</td>
<td>GTGGATGCAGGGATGATGTT</td>
</tr>
</tbody>
</table>
3.2.12 Statistical Analysis

Statistical significance was assessed through a Student T-test or two-way ANOVA for repeated measures followed by a Bonferroni analysis when applicable. For all assessments, a P<0.05 was considered significant. Unless otherwise stated, all data is expressed as MEAN ±SEM.

3.3 Results

3.3.1 Direct Effect of CMPF on Liver Metabolism In-vitro

The liver is critical in the control of nutrient levels within the body and functions as a master regulator of energy homeostasis, partaking in all aspects of glucose and lipid metabolism (Rui, 2014). When administered through intraperitoneal injection prior to a HFD, CMPF appears to beneficially influence lipid metabolism within the liver resulting in a decrease to steatosis development (Prentice et al., Unpublished). In addition, mass spectrometry on the livers of mice following CMPF injection indicate that CMPF is able to enter and accumulate within the liver. This data, combined with the metabolic switch that CMPF induces within the beta cell to enhance lipid metabolism suggests that CMPF is acting on the liver to alter lipid metabolism (Liu et al., 2016). Further investigation is required as to whether this effect is a direct result of CMPF altering hepatic function, or an indirect action of CMPF affecting metabolism within the body. With the use of isolated primary hepatocytes, we first aim to confirm that CMPF is acting directly on the liver to alter energy metabolism.

3.3.1a Validation of Hepatic Lipid Metabolism

To determine a direct effect of CMPF on the liver, primary murine hepatocytes were isolated from untreated wild-type mice using a two-step collagenase perfusion method (W.-C. Li, K. L. Ralphs, & D. Tosh, 2010). As primary hepatocytes are very fragile, optimization of the isolation procedure is critical to ensure proper functionality and health of the isolated cells. Through manipulation of the perfusion time in addition to the composition of the digestion buffer we have succeeded in the consistent isolation of healthy hepatocytes (Dentin et al., 2007; Severgnini et al., 2012). Hepatocyte health following isolation and seeding is primarily determined through visual assessment. Four-hours following initial seeding, a healthy
hepatocyte will adhere to the bottom of the plate and will contain a cuboidal shape with either one or two distinct nuclei (Fig 15A). Following overnight culture, the cuboidal shape will be lost as the healthy hepatocytes spread (Fig 15B).

**Figure 15: Visual Representation of Hepatocytes in Culture.** (A) Four hours following initial seeding, healthy hepatocytes attach to the plate and have a cuboidal shape with one or two distinct nuclei. (B) Following overnight culture, healthy hepatocytes spread out onto the plate losing their cuboidal shape.

If adherence and spreading does not occur this is a guarantee that the hepatocytes will not respond appropriately. To assess the functionality of our isolated hepatocytes we began with an assessment of their ability to uptake, utilize, produce, and store triglycerides, as the hepatocyte’s ability to perform these functions allows it to be central in the regulation of lipid metabolism. To assess the hepatocytes ability to uptake fatty acids from their environment, isolated hepatocytes were treated with radiolabelled palmitate (Wilmsen et al., 2003). When hepatocytes were exposed to radiolabeled palmitate for increasing periods of time, there was a time dependent increase in intracellular palmitate concentration, demonstrating efficient fatty acid uptake (Fig 16A). For future experimentation measuring fatty acid uptake, an incubation time of 3 min was utilized, as this is sufficient time to detect differences in uptake.

To assess the hepatocytes ability to utilize fats as an energy substrate, fatty acid oxidation was measured using radiolabeled oleic acid (Bland, 2016). Fatty acid oxidation is primarily controlled through the activity of ACC, a protein that when active produces acetyl-coA which inhibits CPT1 and the uptake of fatty acids into the mitochondria thereby preventing fatty acid oxidation from occurring (Ben Djoudi Ouadda et al., 2009; Mihaylova & Shaw, 2011). To stimulate fatty acid oxidation, primary hepatocytes were treated for 24-hours with TOFA, an allosteric inhibitor of ACC (McCune & Harris, 1979). Following treatment with the ACC inhibitor TOFA, our primary hepatocytes responded appropriately with a 40% increase in fatty acid oxidation (Fig 16B). Next, radiolabeled acetate was used to assess the primary
hepatocytes ability to generate de novo triglycerides through lipogenesis (Akie & Cooper, 2015). Primary hepatocytes were treated for 24-hours with insulin, a hormone that is released during periods of food intake to promote nutrient storage through the stimulation of lipogenesis (Rui, 2014). In the absence of insulin, primary hepatocytes had a 25% decrease in lipogenesis, demonstrating maintenance of their lipogenic ability when cultured with insulin (Fig 16C). Finally, to assess our primary hepatocytes ability to store triglycerides, hepatocytes were treated for 24-hours with fat supplemented media composed of a combination of both saturated (palmitic acid) and unsaturated (oleic acid) fatty acids. Primary hepatocytes that received fat supplemented media visually displayed higher fat content, as seen by the presence of many small, opaque, circular droplets within their cytoplasm when compared to non-fat supplemented controls (Fig 16 E and F). Quantification of triglyceride concentration within these hepatocytes showed a significant elevation in triglyceride accumulation following fat supplementation (Fig 16D). These results indicate that the isolated primary hepatocytes are functional and have retained their ability to uptake, utilize, produce, and store lipids.

Figure 16: Validation of Functional Lipid Metabolism in Isolated Hepatocytes. (A) Fatty acid uptake over time in isolated hepatocytes treated with C\textsuperscript{14} radiolabeled palmitate (n=2). (B) Oxidation of C\textsuperscript{14} radiolabeled oleic acid in isolated hepatocytes treated with the ACC inhibitor TOFA and expressed as a fold change over vehicle control (n=8). (C) Production of radiolabeled triglycerides from C\textsuperscript{14} radiolabeled acetate in isolated hepatocytes treated overnight with or without 100nM insulin (n=3). (D) Quantification of triglyceride accumulation in isolated hepatocytes treated for 24-hours with FAT supplemented media composed of a 1:1 ratio of oleic acid (300µM) and palmitic acid (300µM) (n=6). Representative image of primary hepatocytes following (E) 24-hour treatment in non-FAT supplemented media and (F) 24-hour treatment in FAT supplemented media in which there is visual accumulation of lipid droplets within the hepatic cytoplasm. *P<0.05, **P<0.01, ****P<0.0001 All error bars SEM.
3.3.1 b Effect of CMPF on Hepatic Lipid Metabolism

The liver is the primary regulator of lipid metabolism within the body and holds the ability to uptake, utilize, store, secrete, and produce fatty acids as required (Fig.17). Circulating fatty acids contained within lipoprotein particles are lipolyzed and taken up into the liver where they are either stored as triglycerides to be utilized during a period of nutrient deprivation, or they are broken down within the hepatocyte though beta-oxidation to produce ATP (Rui, 2014). In addition, the hepatocyte has the ability to undergo lipogenesis, whereby triglycerides are synthesised from acetyl-coA, an intermediate component of glucose metabolism, allowing for the storage of excess energy (Jump, 2011; Rui, 2014). Triglycerides that are produced within the liver, may be stored within the hepatic cytoplasm but as the hepatocyte typically has a limited capacity for triglyceride storage, the vast majority of them are packaged and released into the circulation in the form of very-low density lipoprotein particles (VLDL) (Feingold & Grunfeld, 2015). To determine a direct effect of CMPF on hepatic function, we first assessed their ability to uptake, utilize, produce, and store fatty acids following 24-hour CMPF treatment.

![Figure 17: Lipid metabolism within the liver](image)

Schematic diagram outlining the fate and production of free fatty acids taken up into the liver. Dietary fatty acids taken up into the liver are either oxidized in the mitochondria to produce ATP or they are converted into triglycerides. Triglycerides are also produced from excess glucose through lipogenesis. Hepatic triglycerides are either stored within the hepatic cytoplasm or packaged into VLDL particles and released into the circulation.
Following exposure to radiolabelled palmitate, primary hepatocytes treated with CMPF displayed no observable difference to their rate of fatty acid uptake (Fig 18).

Next, to assess CMPF’s effect on fatty acid utilization within the hepatocytes, primary hepatocytes were incubated with radiolabelled oleic. As fatty acid oxidation is primarily controlled through the activity of ACC, the ACC inhibitor TOFA was used as a positive control and resulted in a 40% increase in fatty acid oxidation (Fig 19A and B). Similar to the increase in fat utilization observed within the beta cell, treatment with CMPF induced a significant 10% increase in hepatic fatty acid oxidation at a high 200µM CMPF concentration (Fig 19B). This high CMPF concentration is comparable to the in-vivo injection volume mentioned previously.

**Figure 18: CMPF does not alter hepatic fatty acid uptake.** Uptake of C¹⁴ radiolabelled palmitate over 3 min in primary hepatocytes following 24-hour treatment with 200µM CMPF (n=6). Data was normalized to protein concentration and is expressed as a fold change over vehicle control. *P<0.05. All error bars SEM.

**Figure 19: CMPF stimulates hepatic fatty acid oxidation.** (A) When active, ACC produces malonyl-CoA from acetyl-coA to inhibit the uptake of fatty acids into the mitochondria, thereby inhibiting fatty acid oxidation. (B) Fatty acid oxidation per hour in isolated hepatocytes treated for 24-hours with 200µM CMPF or the ACC inhibitor TOFA (100uM) (n=5). *P<0.05, **P< 0.01, ****P<0.0001. All error bars SEM.
In addition to controlling the rate of fatty acid oxidation, the activity of ACC also controls the production of triglycerides through lipogenesis, allowing these opposing pathways to occur in contrast to each other (Mihaylova & Shaw, 2011). When active, ACC converts malonyl-coA to acetyl-coA which is then incorporated into long chain fatty acids and combined with glycerol to generate triglycerides (Fig 20A). In the presence of the ACC inhibitor TOFA, there was a significant decrease in lipogenesis within the isolated hepatocytes (Fig 20B). This decrease in lipogenesis in response to ACC inhibition was further confirmed following treatment with two additional allosteric ACC inhibitors; S-2E and PF-05175157 (Fig 20B) (Griffith et al., 2014; Koichi Ohmori et al., 2003; K. Ohmori et al., 2004). It is important to note, that this inhibition to lipogenesis following ACC inhibition occurs even in the presence of insulin stimulation. During a period of nutrient excess when insulin levels within the circulation are high, insulin stimulates lipogenesis to occur in order to produce energy stores that can be used during periods of fasting (Cherrington et al., 2007). As such, all treatment groups additionally contained 100nM insulin in order to ensure maintenance of their lipogenic capability in culture. In the absence of insulin stimulation, the primary hepatocytes displayed a 25% decrease in lipogenesis (Fig 20B). In light of CMPF’s ability to enhance fatty acid oxidation, it was expected that CMPF treatment would inhibit lipogenesis. Interestingly, following 24-hour treatment, a high 200µM dose of CMPF significantly increased the rate of lipogenesis by 10% (Fig 20B).

![Figure 20: CMPF stimulates hepatic lipogenesis.](image)

(A) The enzyme ACC when active catalyzes the rate limiting reaction in the production of triglycerides. (B) Lipogenesis per hour in isolated primary hepatocytes following 24-hour treatment with 200µM CMPF or the ACC inhibitors: TOFA (100µM), S-2E (10µM), PF-05175157 (10µM). All treatments occur in the presence of 100nM insulin (n=3). *P<0.05, **P<0.01, ****P<0.0001. All error bars SEM.
Within a diabetic individual, it is possible for concentrations of CMPF to reach values of 200µM but in healthy individuals the values of CMPF are typically much lower. Basel levels of CMPF within the circulation are often closer to 5µM but can reach values up to 80-100µM within individuals that consume high quantities of fish (Prentice et al., Unpublished; Zheng et al., 2016). Given the wide range of CMPF concentrations that can exist within the circulation, a dose response of CMPF on fatty acid oxidation and lipogenesis was performed. Interestingly, very low concentrations of 5µM CMPF appear to exert an opposite effect to high dose CMPF, resulting in a significant decrease to both fatty acid oxidation and lipogenesis (Fig 2 1A and B). This finding indicates that at low concentrations CMPF is likely acting through a separate mechanism within the cell. Future studies are warranted to further examine the low dose effect of CMPF.

Figure 21: Low dose CMPF exerts an opposite effect. (A) Oxidation of C\textsuperscript{14} radiolabelled oleic normalized to protein concentration and expressed as a fold change over vehicle control (n=5) and (B) Production of triglycerides from C\textsuperscript{14} radiolabelled acetate measured through radioactive counts per minute and expressed as a fold change over control (n=3) in isolated hepatocytes treated for 24-hours with increasing concentrations of CMPF and the ACC inhibitor TOFA (100µM). *P<0.05, **P<0.01, ****P<0.0001. All error bars SEM.

The combined end product of fatty acid oxidation, and lipogenesis is the concentration of triglycerides that accumulates within the hepatic cytoplasm. When treated for 24-hours with fat supplemented media composed of both saturated; palmitic acid, and unsaturated; oleic acid, primary hepatocytes accumulated significantly more triglycerides than their non-fat supplemented controls (Fig 22 B and C).
Figure 22: CMPF does not alter hepatic triglyceride accumulation *in-vitro.* (A) Treatment paradigm for measuring triglyceride accumulation. Primary hepatocytes are treated for 24-hours with either control or FAT supplemented media followed by an additional 24-hour treatment with or without 200 µM CMPF. FAT supplementation is composed of a 1:1 ratio of oleic acid and palmitic acid. Quantification of triglyceride accumulation normalized to protein content following a FAT preload of (B) 600µM FAT supplementation; 300µM palmitic acid and 300µM oleic acid (n=5) and (C) 400 µM FAT preload; 200µM palmitic acid and 200µM oleic acid) (n=3). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. All error bars SEM.

Following an additional 24-hour treatment with CMPF, there was no significant difference in triglyceride accumulation between fat-preloaded controls and fat-preloaded hepatocytes treated with CMPF (Fig 22A and B). It is possible that the concentration of fat within the media was too large and overpowering CMPF’s ability to significantly decrease fat accumulation. As such, the same experiment was performed at a lower combined fat concentration of 400µM. Similar to the high concentration treatment, there was a significant increase in triglyceride accumulation following fat supplementation but there was no difference observed between pre-loaded control and pre-loaded CMPF treated hepatocytes (Fig 22C). Although these results do not coincide with the decrease in hepatic steatosis seen *in-vivo,* it is possible that the colorimetric triglyceride assay used is not sensitive enough to properly detect what may only be a minor decrease within the 24-hour period. Alternatively, it is also possible that the significant decrease in hepatic triglyceride accumulation seen *in-vivo* is the result of an indirect action of CMPF on the liver, either through the elevation of a circulating factor such as FGF21 or through the action of CMPF on a separate metabolic tissue such as the adipose tissue or CNS that in turn affects hepatic function. Therefore, future studies are required possibly with the use of cellular staining such as Oil Red O to further examine if there are minute changes in hepatic triglyceride accumulation following CMPF treatment *in-vitro.*
Therefore, we have demonstrated that CMPF does directly impact lipid metabolism within the liver, resulting in an enhancement to both fatty acid oxidation and lipogenesis at high CMPF concentrations, with no apparent change to triglyceride accumulation over 24-hours.

### 3.3.1c Validation of Hepatic Glucose Metabolism

Similar to lipid metabolism, the hepatocyte plays a critical role in maintaining glucose homeostasis, primarily through the regulation of glucose storage and production. Within the liver, glucose uptake occurs primarily through the use of the GLUT2 transporter, which allows for the constitutive uptake of glucose from the circulation (Rui, 2014). Due to this constitutive uptake, glucose transport into the hepatocyte cannot be regulated by typical controls such as insulin. This occurs in contrast to cell types such as the adipocyte or myocyte that contain the GLUT4 transporter, in which increased insulin within the circulation enhances transporter translocation to the plasma membrane, increasing uptake of glucose into the cell (Saltiel & Kahn, 2001). To ensure that glucose uptake can be sufficiently measured, radiolabelled 2-deoxy glucose was used to assess glucose uptake in L6 wild-type muscle cells following insulin stimulation. As expected, treatment with insulin in L6WT muscle cells enhanced glucose uptake by 70% demonstrating functionality of our glucose uptake assay (Fig 23A).

To assess our hepatocytes ability to undergo gluconeogenesis, glucose production was measured using a colorimetric assay to detect the levels of glucose within the media. Glucagon, a hormone that is secreted by the alpha cell in response to low blood glucose levels and stimulates gluconeogenesis, led to a 30% increase in glucose production within our isolated hepatocytes, indicating that the isolated hepatocytes have retained their ability to produce glucose during times of energy deprivation (Rui, 2014) (Fig 23B).

![Figure 23: Validation of Glucose Functional Assays.](image-url) (A) Uptake of C\textsuperscript{14} radiolabeled 2-deoxy glucose over 5 min in L6WT muscle cells following 15min stimulation with 100nM insulin (n=4). (B) Glucose concentration in the media of isolated hepatocytes following 4-hour treatment with 100nM glucagon (n=3). *P<0.05, ***P<0.01. All error bars SEM.
3.3.1d Effect of CMPF on Hepatic Glucose Metabolism

Within the β-cell, CMPF induced a metabolic switch to a preference for fatty acid oxidation over glucose utilization. As we have seen an enhancement to lipid metabolism within the hepatocyte following CMPF treatment we were next interested in the effect of CMPF on glucose metabolism, particularly glucose uptake and production. Following 24-hour treatment, CMPF did not alter glucose uptake apart from a significant decrease at very low 5µM concentrations (Fig 24A). During periods of nutrient deprivation when blood glucose levels are low, glucagon is released from the alpha cells within the pancreas and signals to the hepatocyte to release glucose into the circulation, either from glycogen stores or from the de-novo production of glucose through gluconeogenesis (Ramnanan et al., 2011). Following a 2-hour fast to deplete internal glycogen stores, 4-hour treatment with glucagon stimulated hepatic glucose production by 30% but there was no significant difference seen in gluconeogenesis following 24-hour CMPF treatment (Fig 24B).

Figure 24: High dose CMPF does not alter hepatic glucose uptake or glucose production. (A) Uptake of C\textsuperscript{14} radiolabelled 2-deoxy glucose over 5 min in isolated hepatocytes treated for 24-hours with increasing concentrations of CMPF (n=4). Data is normalized to protein concentration and expressed as a fold change over control. (B) Concentration of glucose produced and secreted from the primary hepatocytes into the media following treatment with increasing concentrations of CMPF and 100nM glucagon (n=3). ***P<0.001, ****P<0.0001. All error bars SEM.

Therefore, 24-hour CMPF treatment does not directly alter glucose uptake or production within the liver at high concentrations. However, there is a significant inhibition of glucose uptake seen at low doses of CMPF.
3.3.2 Alterations in Cellular Signaling Following CMPF Treatment

When functional pathways are altered within a cell, it often occurs in combination with changes to cellular signalling. Given that CMPF directly enhances lipid metabolism in-vitro, we were next interested in potential alterations that CMPF may be inducing at the signaling level. When CMPF was injected prior to a HFD in-vivo, there was a decrease in intrahepatic lipid accumulation and improved whole body insulin sensitivity (Prentice et al., Unpublished). Due to the relationship between excessive triglyceride accumulation and impaired insulin signaling we are interested in the effect that CMPF has on insulin signaling following an in-vitro high fat stress (Jump, 2011).

In addition, due to the increase to both fatty acid oxidation and lipogenesis seen in-vitro, we are particularly interested in CMPF’s ability to activate the AMPK/ACC pathway as it is the major regulatory pathway of both fatty acid utilization and production (Mihaylova & Shaw, 2011). Finally, injection of CMPF in-vivo leads to enhanced levels of circulating FGF21, a protein that is primarily produced within the liver and is known to beneficially influence fatty acid oxidation to decrease hepatic steatosis (Ge et al., 2012). Therefore, we are also interested in whether this increase to FGF21 in-vivo is directly due to CMPF action within the liver.

3.3.2a Validation of Hepatic Cell Signaling

When insulin binds to the insulin receptor on the surface of the primary hepatocyte it initiates an intracellular signaling cascade that results in the phosphorylation of AKT (Taha & Klip, 1999). When stimulated with insulin for 5 min prior to lysate formation, insulin enhanced AKT phosphorylation in a dose dependent manner (Fig 25). This indicates that insulin signaling is intact within our primary hepatocytes.

![Figure 25: Insulin stimulation enhances AKT phosphorylation.](image-url)
The AMPK-ACC pathway is an essential regulator of fatty acid oxidation and fatty acid synthesis within the liver. When phosphorylated, AMPK becomes activated leading to the phosphorylation and subsequent inactivation of ACC in order to promote fatty acid oxidation and inhibit fatty acid synthesis (Mihaylova & Shaw, 2011). To ensure functional signaling between AMPK and ACC, primary hepatocytes were stimulated with AICAR; a cell permeable activator of AMPK activity. Primary hepatocytes stimulated with AICAR showed an increase in phosphorylation of both AMPK and ACC (Fig 26).

3.3.2b Insulin Signaling

An increase in intracellular triglyceride accumulation is strongly associated with an impairment to insulin signaling (Kumashiro et al., 2011). Typically, the binding of insulin to the insulin receptor on the cell surface will initiate an intracellular signalling cascade that includes the phosphorylation of AKT (Taha & Klip, 1999). To determine if CMPF is able to directly enhance hepatic insulin signalling, primary hepatocytes were treated with fat supplemented media composed of both saturated: palmitic acid; and unsaturated: oleic acid, at a combined concentration of 600µM for 24-hours followed by an additional 24-hour treatment in the presence of CMPF (Fig 27A). An increase in intracellular triglyceride accumulation, increases the concentration of DAG and ceramide, both of which should impair phosphorylation of the IRS proteins preventing activation of the intracellular signaling cascade (Kumashiro et al., 2011). As expected, fat supplementation impaired phosphorylation of AKT, demonstrating sufficient induction of insulin resistance (Fig 27 B, C and D). Following an additional 24-hour treatment with CMPF in the presence of fat supplementation, CMPF trended towards enhanced AKT phosphorylation compared to fat supplemented controls, indicating that CMPF directly improves lipid impaired insulin sensitivity (Fig 27 B,
C and D). This improvement to insulin sensitivity is likely a result of CMPF causing a slight decrease in triglyceride accumulation.

Figure 27: CMPF improves lipid impaired insulin sensitivity in primary hepatocytes. (A) Schematic of treatment paradigm used to induce insulin resistance in isolated hepatocytes prior to CMPF treatment. Primary hepatocytes are treated with FAT supplementation composed of 300μM palmitic acid and 300μM oleic acid for 24-hours followed by an additional 24-hour treatment with or without 200μM CMPF. Prior to lysate formation, hepatocytes are stimulated with either 10 or 100nM insulin for 5 min. (B) Representative western blot and quantification of Ser473 AKT phosphorylation following (C) 10nM (n=4) and (D) 100nM insulin stimulation (n=4). *P<0.05 All error bars SEM.

Although the triglyceride accumulation assay mentioned previously indicated no alteration to triglyceride accumulation following CMPF treatment, a measurement of protein phosphorylation is much more sensitive and likely better able to detect minute changes compared to a colorimetric assay. Therefore, it is possible that CMPF induces a minor decrease in triglyceride accumulation over a 24-hour period leading to improved insulin signaling following a fat pre-load.
3.3.2c AMPK/ACC Phosphorylation

The rate of both fatty acid oxidation and lipogenesis is primarily controlled through the phosphorylation of the AMPK/ACC pathway, in which AMPK phosphorylation results in the phosphorylation and inactivation of ACC thereby increasing fatty acid oxidation and decreasing fatty acid synthesis (Mihaylova & Shaw, 2011). Due to CMPF’s ability to stimulate fatty acid oxidation within the hepatocyte, we were next interested in the effect that short term CMPF treatment has on the phosphorylation of both AMPK and ACC. Following treatment with CMPF, there was no alteration to the phosphorylation of either AMPK or ACC (Fig 28D and G). In addition, the total quantity of AMPK and ACC protein within the treated hepatocytes was unchanged (Fig 28C and F). This indicates that CMPF is not activating fatty acid oxidation through upstream AMPK/ACC signaling and may instead be interacting directly with ACC to inhibit its activity.

Figure 28: CMPF does not activate phosphorylation of the AMPK/ACC pathway. (A) Representative western blot and quantification of (B) Total AMPK and (C) Phosphorylated AMPK (n=4) (D) Representative western blot and quantification of (E) Total ACC and (F) Phosphorylated ACC (n=4) following treatment with 200µM CMPF in isolated hepatocytes. All error bars SEM.
3.3.2d FGF21 Expression and Secretion

Circulating levels of FGF21 are primarily produced within the liver and are known to beneficially influence whole body energy metabolism, enhancing energy expenditure and leading to an increase in hepatic fatty acid oxidation in order to reduce hepatic steatosis (Ge et al., 2012). Given the significant increase in circulating FGF21 seen in-vivo we wanted to confirm that CMPF directly stimulates FGF21 expression and secretion. Following 24-hour treatment with CMPF, both the expression and secretion of FGF21 was significantly induced within the isolated hepatocytes demonstrating direct stimulation (Fig 2A and B).

Figure 29: CMPF stimulates hepatic FGF21 expression and secretion. (A) FGF21 mRNA expression from isolated hepatocytes treated for 24-hours with increasing concentrations of CMPF. Data is expressed as a relative fold change calculated from $\Delta^{\Delta \text{Ct}}$ (n=7). (B) Concentration of FGF21 secreted into the media from isolated hepatocytes following 24-hour treatment with increasing concentrations of CMPF expressed as a fold change over control (n=7). *P<0.05, **P<0.01. All error bars SEM.

3.3.3 Entry of CMPF into the Hepatocyte

In order for CMPF to have a direct effect on hepatic function, CMPF must either enter the hepatocyte to elicit an effect or it must interact with a receptor on the cell surface to initiate an intracellular signaling cascade. Given that CMPF is found at high concentrations within non-perfused whole liver tissue 1-hour following intraperitoneal injection, we next aimed to confirm whether CMPF can directly enter the isolated hepatocyte and to elucidate the mechanism that may be facilitating its entry.
3.3.3a CMPF Enters the Hepatocyte

To confirm entry of CMPF into the hepatocyte, mass spectrometry was performed on isolated hepatocytes (See Fig 14 for elution profile of CMPF). Following 24-hour treatment, CMPF significantly accumulated within the hepatocyte compared to vehicle treated controls (Fig 30).

![Figure 30: CMPF accumulates in primary hepatocytes. Mass spectrometry of CMPF accumulation following 24-hour treatment in primary hepatocytes (n=3). *P<0.05. All error bars SEM.]

3.3.3b CMPF passes Through the Liver to be Glucuronidated

Glucuronidation, is a process that primarily occurs within the liver, in which a substance is made more soluble by the addition of glucuronic acid so that it may be more easily cleared from the circulation (Regan et al., 2010). Following Lovaza supplementation in humans, there was not only a significant accumulation of CMPF within the circulation but also of glucuronidated CMPF (Prentice et al., Unpublished). To further confirm that CMPF must pass through the liver in order to be glucuronidated, CMPF was administered into mice using three separate modes, either by oral gavage, subcutaneous injection or intraperitoneal injection (Fig 31A). Utilizing three separate modes of CMPF administration varies the time required for CMPF to reach the liver and therefore potentially delays the appearance of glucuronidated CMPF within the circulation. Intraperitoneal injections travel directly through the hepatic portal vein to the liver, oral gavage must first be absorbed within the digestive tract and subcutaneous injections must be taken up into the circulation and travel through the body before reaching the liver (Turner, Brabb, Pekow, & Vasbinder, 2011). Following CMPF administration, the concentration of CMPF within the circulation was highest following intraperitoneal administration (Fig 31B). This is likely a result of there being incomplete absorption into the circulation during oral gavage and subcutaneous injection. As expected,
glucuronidated CMPF appeared within the circulation much earlier following intraperitoneal injection than both oral gavage and subcutaneous injection (Fig 31C). Subcutaneous injection which has the longest route to reach the liver, presented with glucuronidated CMPF last. This data suggests that if CMPF must travel farther within the body prior to reaching the liver then glucuronidated CMPF will take longer to appear within the circulation, suggesting that CMPF must first enter and pass through the liver in order to be glucuronidated.

**Figure 31: CMPF is glucuronidated within the liver.** (A) Schematic of CMPF administration representing the time required for CMPF to reach the liver within each mode. Subcutaneous injections will reach the liver last as they must first pass throughout the body. Intraperitoneal injections will travel directly through the hepatic portal vein and therefore take the least amount of time. Oral gavage must first be absorbed in the digestive tract prior to reaching the portal vein and will therefore have an intermediate delivery time. (B) Concentration of CMPF in the circulation over time and (C) Concentration of glucuronidated CMPF calculated as the fold change in glucuronidated CMPF compared to baseline. (n=2/ administration method). *P<0.05, **P<0.01, ***P<0.001. All error bars SEM.

### 3.3.3c Organic Anion Transporter Expression in the Liver

CMPF is present at basal levels within non-diabetic individuals but it is typically cleared quite effectively from the circulation by the kidney. In individuals with uremia, in which there is impairment to a group of organic anion transporters (OAT) within the kidney, CMPF becomes elevated within the circulation indicating that a member of the OAT family may be responsible for CMPF transport (Sassa et al., 2000). The organic anion family comprises a group of ten highly similar multispecific transmembrane proteins that facilitate movement of a wide range
of organic anion substrates (Anzai et al., 2006). These organic anion substrates require a hydrophobic backbone in combination with a negative charge in their structure and include di-carboxylates like CMPF (Anzai et al., 2006). The members of the OAT family are primarily responsible for facilitating transport into the cell and differ most amongst each other through their various tissue distribution. Within the kidney, the primary isoform responsible for CMPF clearance is OAT3 but it is not the only isoform that is present (Sun, Wu, van Poelje, & Erion, 2001). Within the mouse kidney, in addition to OAT3, the organic anion transporters OAT1, OAT2, OAT5, OAT9 (homologous to OAT7 in the human), OAT10 and URAT1 are additionally expressed (Fig 32).

Looking at OAT expression through standard PCR within whole murine liver, OAT3 is not expressed but OAT2 shows consistent levels of expression (Fig 33 A and C). In addition, OAT9 appears to be expressed, although its expression is much more variable between mice. Within primary hepatocytes, OAT2 continues to be consistently expressed (Fig 33 B and D). This occurs in contrast to OAT9 which is no longer expressed within the isolated hepatocytes, likely a result of damage during hepatocyte isolation. In addition, the literature suggests that OAT2 has much broader substrate specificity compared to OAT9, with OAT9 preferring to transport sulfate conjugates (Henjakovic, Hagos, Krick, Burckhardt, & Burckhardt, 2015; Shen et al., 2015; Shin et al., 2007). These results indicate that OAT2 is the likely transporter responsible for facilitating CMPF transport into the hepatocyte.

**Figure 32: Organic anion expression within the kidney.** Standard PCR expression of the organic anion transporter family in whole kidney tissue expressed as a percentage of GAPDH (n=6). All error bars SEM.
Figure 3: Organic anion expression within the liver. Standard PCR of OAT family expression in (A) Whole liver tissue (n=3) and (B) Primary hepatocyte (n=12). Quantification of OAT family expression in (C) Whole liver tissue (n=3) and (D) Primary hepatocyte (n=12) expressed as a percentage of GAPDH. For each PCR blot, whole kidney tissue is used as a positive control, and water is used as a negative control. All error bars SEM.

3.3.3d Organic Anion Transporter Inhibition with Probenecid

In an attempt to confirm that CMPF utilizes OAT2 to enter and accumulate within the hepatocyte, primary hepatocytes were treated with probenecid, a general OAT family inhibitor (Takeda et al., 2001). Interestingly, probenecid treatment enhanced CMPF accumulation within isolated hepatocytes (Fig 34).

Figure 34: Probenecid enhances CMPF accumulation in primary hepatocytes. Mass spectrometry of CMPF accumulation in primary hepatocytes following treatment with 200µM CMPF and 1mM probenecid (n=3). *P<0.05. All error bars SEM.
The literature suggests that although probenecid potently inhibits OAT isoforms like OAT3 and OAT1, it is much less effective in the inhibition of OAT2 (Henjakovic et al., 2015). Therefore, accumulation of CMPF within the primary hepatocytes following probenecid treatment likely still occurs as a result of probenecid being only a very weak OAT2 inhibitor. In addition, probenecid is a potent inhibitor of the MRP transporter family, responsible for the efflux of xenobiotic and glucuronidated compounds from the liver, inhibition of which may possibly explain an apparent enhancement to CMPF accumulation following probenecid treatment (Borst, Evers, Kool, & Wijnholds, 2000; Weiss, Theile, Ketabi-Kiyavanash, Lindenmaier, & Haefeli, 2007). Further studies are warranted, potentially with a knockdown of OAT2 in the isolated hepatocytes in order to confirm if OAT2 is responsible for CMPF entry.

3.4 Discussion

Since its initial discovery in diabetic individuals, an elevation in circulating levels of CMPF has been believed to be harmful to the health of an individual, resulting in beta cell dysfunction and the progression of diabetes (Prentice et al., 2014). Recently our lab, in parallel to other recent publications, has discovered that CMPF is additionally elevated within the circulation, albeit to a lower extent, following the consumption of Lovaza; a prescription fish oil supplementation (Prentice et al., Unpublished; Zheng et al., 2016). Fish oil supplementation is widely accepted as a therapeutic treatment due to its cardio protective effects and its ability to effectively reduce hypertriglyceridemia (Eslick et al., 2009). Many believe that the beneficial components of fish oil are the omega-3 polyunsaturated fatty acids EPA and DHA. However, investigation in the field is still underway to determine if fish oil contains additional active components that are responsible for its therapeutic effects. When administered to mice, our lab has found the fish oil metabolite CMPF to reduce both serum and hepatic triglycerides, and improve whole body insulin sensitivity (Prentice et al., Unpublished). These results indicate that CMPF beneficially enhances lipid metabolism within the body and may be an active metabolite of fish oil consumption. With this study, the main goal was to confirm that the in-vivo benefits to hepatic function following CMPF administration are a direct result of CMPF action within the hepatocyte rather than an indirect effect of CMPF within the body. With the use of isolated hepatocytes, we have demonstrated that CMPF indeed accumulates within the hepatocyte where it directly enhances hepatic function.
resulting in an increase to both hepatic fatty acid oxidation and lipogenesis and a trend towards improved insulin signaling. In addition, CMPF stimulates both the expression and secretion of FGF21 from the hepatocyte.

With the use of mass spectrometry, we have demonstrated that CMPF is able to enter the hepatocyte, both through its accumulation following 24-hour treatment in-vitro and through its requirement to pass through the liver prior to glucuronidation in-vivo. Although OAT2 has been suggested within the literature to be able to transport di-carboxylates like CMPF and it is clear that OAT2 is expressed both in an intact liver as well as in isolated hepatocytes, further investigation is required to conclude whether OAT2 is facilitating CMPF transport (Shen et al., 2015). Extensive research has occurred on the specifics of both OAT1 and OAT3 but knowledge on OAT2 remains limited. As such, there are no pharmacological inhibitors currently known to selectively inhibit OAT2, and general OAT family inhibitors such as probenecid provide only weak inhibition (Henjakovic et al., 2015). In addition, the liver is responsible for detoxification and filtering of the blood (Rui, 2014). Due to this role, the liver contains an abundance of transporters that may be additionally inhibited with the use of pharmacological inhibition, confounding any results. Therefore, future work to determine if OAT2 is facilitating CMPF transport requires mass spectrometry of CMPF accumulation following siRNA knockdown of OAT2 within either the isolated hepatocytes, or within the more stable liver HepG2 cell line that similarly expresses OAT2.

Within the pancreatic beta cell, CMPF treatment impaired glucose utilization while enhancing fatty acid oxidation (Liu et al., 2016; Prentice et al., 2014). As glucose is an essential energy source for many organs in the body such as the brain, the liver does not utilize glucose as its primary energy source unless it is in abundance (Houten & Wanders, 2010). It is for this reason that we chose to only assess CMPFs effect on glucose uptake and production, of which we noted no change following high concentration CMPF treatment, in order to focus on the effects of CMPF on lipid metabolism.

Once CMPF has entered and accumulated within the hepatocyte it enhances the oxidation of free fatty acids. Along with the increase in fatty acid oxidation following CMPF treatment there was an unexpected increase that additionally occurred to lipogenesis. Typically, the pathways of fatty acid utilization and production occur in contrast to each other through regulation of the
AMPK/ACC pathway, in which ACC1 is localized within the cytoplasm and responsible for regulation of lipogenesis, and ACC2 is localized to the mitochondrial membrane to control the rate of fatty acid oxidation (Mihaylova & Shaw, 2011). Importantly, CMPF treatment did not alter the phosphorylation of either AMPK or ACC indicating that CMPF is not activating either pathway through an upstream mechanism but may instead be interacting directly with ACC to inhibit its activity. If CMPF is a direct inhibitor of ACC, it is possible that it contains specificity for ACC2 over ACC1 within the cell resulting in activation of both fatty acid oxidation and lipogenesis. Future work to assess the interaction between CMPF and ACC is required either with the use of an activity assay utilizing isolated ACC proteins treated with CMPF or through analysis of protein-protein interactions using a biochemical assay such as the interaction between fluorescently labelled proteins.

Although specific inhibition of ACC isoforms may explain the increase to both fatty acid oxidation and lipogenesis, it is additionally possible that an increase to both is a result of an alteration in either lipid secretion from the hepatocyte or an increase in cholesterol production as a result of the limitations that exist within the lipogenesis assay. As the majority of lipids that are produced within the liver through lipogenesis are packaged together with cholesterol and lipoproteins into very-low density lipoprotein particles and secreted into the circulation to be taken up and stored within the adipose tissue, it is possible that CMPF is altering VLDL secretion making it appear as though there is enhanced lipid production (Cox & Garcia-Palmieri, 1990). The lipogenesis assay mentioned in section 3.3.1b measures the concentration of newly synthesized lipids contained within the cellular lysate but does not take into account the concentration of newly synthesized lipids that are secreted from the hepatocyte. To assess the rate of VLDL secretion, we attempted to measure the concentration of lipids secreted into the media following lipogenesis. The rate limiting step in the packaging and production of lipoprotein particles is the production and availability of the primary organizational protein, Apolipoprotein B (ApoB) (Feingold & Grunfeld, 2015). High concentrations of oleic acid have been reported to enhance production of ApoB resulting in enhanced lipoprotein secretion (Lewis, 1997; Moberly, Cole, Alpers, & Schonfeld, 1990). Alternatively, insulin stimulation is believed to inhibit the secretion of VLDL particles (Cherrington et al., 2007). Following 24-hour treatment, the intracellular concentration of newly synthesized lipids was decreased following oleic acid treatment and increased in response to insulin treatment, as would be expected from enhanced and inhibited VLDL secretion respectively.
(Appendix A Figure 1A). However, we were unable to detect levels of radiation within the media that were sufficiently higher than the blank, likely a result of the amphiphilic lipoprotein particles being soluble in polar solvents and separating into the aqueous fraction during lipid extraction, making us unable to conclude whether CMPF was altering secretion of newly synthesized triglycerides (Appendix A Figure 1B). Future experimentation is required to elucidate whether CMPF is altering the secretion of VLDL particles from the liver. As ApoB is the primary structural lipoprotein required for VLDL formation, the secretion of VLDL particles from the hepatocyte could be assessed through the measurement of ApoB expression within the media utilizing an ApoB ELISA kit. Although the VLDL particles generated within the liver are primarily composed of newly synthesized lipids, they must also contain cholesterol and cholesterol esters (Cox & Garcia-Palmieri, 1990; Khan et al., 1989). As an indirect measure of the production and secretion of VLDL particles, future work aims to measure the concentration of cholesterol and cholesterol esters that are secreted into the hepatic media with the use of a colorimetric cholesterol/cholesterol ester assay kit. In addition, it would be wise to examine the concentration of cholesterol and cholesterol esters within the cellular lysate, as the lipogenic assay is limited in its ability to distinguish between the production of triglycerides and the production of cholesterol, both of which originate from acetyl-coA (Khan et al., 1989). Therefore, measuring the concentration of cholesterol within the cytoplasm following CMPF treatment, in addition to measuring the activity and expression of key lipogenic transcription factors such as SREBP and ChREBP, would allow us to determine if the enhancement we are observing in lipogenesis following CMPF treatment is really due to an increased production of triglycerides.

Within all of the in-vitro assays performed in this study, the primary limitation lies in the ability to isolate and maintain healthy hepatocytes. As the isolated hepatocytes can only be maintained in culture for a maximum of 48-hours before they begin to de-differentiate and lose their hepatic function, the maximum CMPF treatment time that could be utilized was 24-hours (W.-C. Li, K. Ralphs, & D. Tosh, 2010; Shulman & Nahmias, 2013). This limited our ability to assess certain cellular changes such as triglyceride accumulation that may have been more pronounced following a longer treatment period. In addition, as the isolated hepatocytes are very sensitive and fragile, optimization of the isolation procedure was critical as the length of time from beginning isolation to culture of the cells, in addition to the individual times required for perfusion or digestion, could severely decrease cell viability. We succeeded in optimizing conditions that consistently produced
a high percentage of viable cells but even then, their responses within the various functional assays were variable and although significant they were not extreme. For example, we achieved an only 30% increase in oxidation following treatment with our positive control TOFA. In addition, *in-vitro* assays are limited in their sensitivity. Although very specific and the most effective way to measure metabolism *in-vitro*, the use of radiolabelling in particular is not always the most sensitive. This is demonstrated in the measurement of fatty acid oxidation in which the rate of oxidation is determined through a measurement of radiolabelled CO2. By measuring the CO2 produced through the oxidation of radiolabelled oleic, one can confidently conclude that any detected radiation is a result of fatty acid oxidation but as the experiment is performed there are many opportunities for radiolabelled CO2 to be lost thereby decreasing the sensitivity of the assay. Therefore, although the responses noted *in-vitro* appear to be minimal in comparison to the *in-vivo* effects, this could be a result of the limitations that come from *in-vitro* experimentation and are still sufficient to demonstrate a direct effect.

The most notable difference observed between CMPF administration prior to a HFD *in-vivo* compared to CMPF treatment *in-vitro*, is a failure of CMPF to significantly reduce hepatic triglyceride concentration *in-vitro* when measured utilizing a triglyceride colorimetric assay. This result though occurs in contrast to the improved insulin sensitivity that is observed following CMPF treatment in the presence of a fat preload. As the fat preload was utilized to induce insulin resistance within the isolated hepatocytes, a trend towards restoration of insulin signaling would indicate that fat accumulation within those hepatocytes has decreased. This disparity between results could in part be due to a lack of sensitivity within the colorimetric assay itself. It is likely that following only 24-hour treatment with CMPF any decrease in triglyceride accumulation would be quite minimal. As the assay measures colour change in relation to the concentration of glycerol, very small changes may not be detectable. This is in contrast to western blotting and the measurement of protein phosphorylation which is a much more sensitive, although indirect method, to suggest that CMPF does decrease triglyceride accumulation *in-vitro* but that the degree of decrease over a 24-hour period is minimal. Finally, the results from our isotope studies were also quite modest with only a maximal increase of 13% observed to fatty acid oxidation so from this minimal effect we could not expect to see dramatic differences in fat accumulation over a 24-hour period *in-vitro*. Further experimentation with the use of oil red O staining in order to visually quantify the lipid droplets in the hepatic cytoplasm, or with the use of thin layer chromatography
followed by spectrophotometry to separate and quantify the levels of triglycerides, would be wise in order to confirm if there is a minor change in triglyceride accumulation following CMPF treatment.

Finally, treatment of primary hepatocytes induced a significant increase in both the expression and secretion of FGF21. Although it is established that increased levels of circulating FGF21 within the body enhance fatty acid oxidation within the hepatocyte there is controversy within the field as to whether FGF21 has the ability to act in an autocrine fashion (Kharitonenkov & Larsen, 2011; Z. Lin et al., 2013). Therefore, the results of this study so far are limited in their ability to distinguish whether CMPF is in fact interacting with ACC to enhance fatty acid oxidation or if CMPF is simply enhancing FGF21 which acts back on the hepatocyte to increase fatty acid oxidation. Further investigation is required with the use of FGF21 knockout mice to determine if CMPFs increase to fatty acid oxidation can occur in the absence of FGF21.

Therefore, in chapter 3 we have concluded that CMPF treatment \textit{in-vitro} leads to an enhancement in hepatic lipid metabolism resulting in an increase to both fatty acid oxidation and lipogenesis, likely through a direct inhibition of ACC2. In addition, CMPF results in a minor decrease of hepatic triglyceride accumulation, demonstrated through an improvement to lipid impaired insulin sensitivity. Finally, CMPF directly stimulates both the expression and secretion of FGF21, elevation of which \textit{in-vivo} is likely responsible for the significant alteration in whole body lipid metabolism. Within the next chapter we will investigate the mechanism that allows CMPF to stimulate expression of FGF21.
Chapter 4: Underlying Mechanism Mediating CMPF’s Effect in the Liver

4.1 Introduction

An increase in FGF21 within the circulation is highly beneficial in the regulation of glucose and lipid metabolism (Fisher & Maratos-Flier, 2016). When injected into diabetic db/db mice, FGF21 has been demonstrated to reduce body weight, hyperlipidemia, hyperglycemia and hepatic steatosis, in combination with an enhancement to insulin sensitivity (Fisher & Maratos-Flier, 2016; Ge et al., 2012; Maratos-Flier, 2017). As a result of these beneficial effects, research into the regulation of FGF21 has spiked dramatically over the past few years in order to promote FGF21 stimulation as a potential therapeutic for obese individuals. Regulation of FGF21 occurs through the enhanced activity of transcription factors, which include PPARγ, retinoic acid receptor β (RARβ), sirtuin 1, CREBH, PPARγ coactivator 1a and retinoic acid receptor related orphan receptor α, but the major regulator of FGF21 activation is through the transcription factor PPARα (Erickson & Moreau, 2016). PPARα is a nuclear receptor that is primarily activated through ligand binding of a small lipophilic molecule such as a free fatty acid (Schmidt et al., 1999; Varga, Czimmerer, & Nagy, 2011). Once a ligand is bound, activated PPARα enhances the expression of genes such as FGF21 that are involved in lipid metabolism including those to regulate fatty acid uptake, fatty acid oxidation, and fatty acid production (Fisher & Maratos-Flier, 2016).

CMPF directly stimulates fatty acid oxidation within the hepatocyte in the absence of any AMPK or ACC phosphorylation. As the AMPK/ACC pathway is the primary regulator of fatty acid oxidation, an absence in upstream signaling following CMPF treatment indicates that CMPF may be directly inhibiting ACC activity. To assess this direct inhibition, our lab performed a biochemical assessment assay measuring the activity of isolated acetyl-coA carboxylase enzymes following CMPF treatment. To perform this experiment, human ACC1 and ACC2 enzymes were produced by baclovirus and incubated in a 96 well plate for 90 min in the presence of a reaction mixture containing CMPF, as well as acetate, and $^{14}$C radiolabelled sodium bicarbonate (Prentice et al, Unpublished). Following incubation, the reaction mixture was transferred to a Millipore GF/C filter plate and allowed to dry overnight. Active ACC will utilize the isotopically labelled
carbon during its enzymatic reaction to produce radiolabelled malonyl-coA, therefore allowing the radioactive count to be an indicator of enzymatic activity (Fig 35). Following incubation, we noted that there was a decrease in both ACC 1 and 2 activity in the presence of CMPF indicating direct inhibition (Fig 35) (Prentice et al, Unpublished).

![ACC Inhibition](image)

**Figure 35: CMPF directly inhibits ACC activity in-vitro.** Biochemical assessment assay measuring the production of radiolabelled malonyl-coA from isolated acetyl-coA carboxylase enzymes 1 and 2 following treatment with 200µM CMPF and the potent ACC inhibitor CP-640186. (n=3). Data is expressed as the activity based on malonyl-coA production relative to the control. **P<0.01, ***P<0.001, ****P<0.0001. All error bars SEM. (Prentice et al. Unpublished)

In addition, when administered both in-vivo and in-vitro, CMPF directly stimulated the expression and secretion of FGF21 within the liver (Prentice et al., Unpublished). As CMPF is a small lipophilic molecule it holds the potential to be an activating ligand of PPARα. Due to the direct increase in both FGF21 expression and fatty acid oxidation within the liver following CMPF treatment, we are next interested in whether these two events occur separately from each other or if they are a result of one another. Within this chapter we will explore whether an inhibition of ACC affects the expression and secretion of FGF21 from the hepatocyte. To assess the relationship between ACC inhibition and an activation of FGF21 expression, primary hepatocytes will be treated with direct ACC inhibitors that both structurally resemble that of CMPF as well as those that are structurally different in order to assess the expression and secretion of FGF21, measured through quantitative real-time PCR and an FGF21 Elisa kit respectively.
4.2 Materials and Methods

4.2.1 Preparation of Reagents

CMPF was purchased from Cayman Chemicals (Cat# 10007133) and dissolved to a stock concentration of 100mM using 70% Ethanol. The ACC inhibitors, TOFA (Cat# T6575), S-2E (Cat# S6445) and PF-05175157 (Cat# PZ0299) were purchased from Sigma Aldrich and dissolved in DMSO to a final concentration of 20mM, 100mM and 100mM respectively. CMPF and ACC inhibitors were all stored at 4°C. AICAR (Cat# A9978) was purchased from Sigma Aldrich and dissolved in ultrapure water to a stock concentration of 100mM and stored at -20°C. Prior to in-vitro treatment, CMPF was conjugated to free-fatty acid free BSA (Sigma Aldrich Cat# A8806) at a ratio of 3:1, for 4-hours at 37°C.

4.2.2 Animal Use

All mice used were male c57BL/6 mice purchased from Jackson Laboratory (Maine, USA). Following delivery, mice were allowed to acclimatize to our animal facility for 1-week prior to experimentation. Unless otherwise stated, all mice were fed a standard Rodent Diet. All animal studies were performed in accordance with the Canadian Council of Animal Care Guidelines and were approved by the Animal Care Committee at the University of Toronto.

4.2.3 Primary Hepatocyte Isolation and Culture

Primary hepatocytes were isolated from male 7-9-week-old c57BL/6 mice using a two step-collagenase perfusion method, as described previously (Dentin et al 2007, and Zhang et al. 2012). Briefly, blood flow through the thoracic vena cava was tied off and the abdominal vena cava was cannulated using a 25G butterfly needle (VWR Cat#CABD367341). A small incision was made within the hepatic portal vein to allow liquid flow through the liver. The liver was perfused for 5 min with perfusion buffer (GIBCO Cat# 17701-038), followed by an additional 3-4 min perfusion with digestion buffer composed of 0.25mg/ml collagenase IV (SIGMA Cat#C5138-1G) dissolved in low glucose DMEM (GIBCO Cat#11885-084). During the perfusion, both the perfusion and digestion buffer were warmed and maintained at a temperature of 42°C. Following digestion, the liver was excised, broken apart and filtered through a 70-micron cell filter (FALCON Cat#352350)
using ice cold high glucose DMEM (GIBCO Cat#11995-065) with p/s (GIBCO Cat#15140-122) and 10% FBS (SIGMA Cat#F1051-500ML). The filtered cells were centrifuged at 100g for 3 min at 4°C to generate a cell pellet of live isolated hepatocytes that were re-suspended and counted using Trypan Blue (GIBCO Cat# 15250-061) to allow an equal seeding density of 500 000 cells/well. Primary hepatocytes were allowed to adhere to the plate in the presence of 10% FBS for 4-hours, after which the media was changed to warm high glucose DMEM with p/s and 0% FBS along with any additional treatment reagents.

4.2.4 FGF21 Secretion

The concentration of FGF21 secreted into the media following 20-hour CMPF treatment was measured using an FGF21 enzyme-linked immunosorbent assay (ELISA) purchased from R&D Systems (Cat#MF2100). Briefly, undiluted media was added onto a purchased FGF21 ELISA plate pre-coated with a monoclonal antibody that is specific for the mouse FGF21 protein. Any FGF21 present within the media will bind to the immobilized antibody on the plate. Following incubation, a second enzyme linked polyclonal antibody specific for mouse FGF21 was added in combination with a substrate solution that results in the production of a blue product that turns yellow upon addition of a stop solution. The intensity of colour for each well was measured based on the values of a standard curve and is indicative of the concentration of FGF21 in each well.

4.2.5 Gene Expression

Gene expression within isolated hepatocytes was assessed using both quantitative real-time PCR (qPCR) and standard PCR. Briefly, total RNA was extracted from isolated hepatocytes using the Qiagen RNeasy Plus mini kit (Cat#74134). Reverse transcription was performed on total RNA, using M-MLV reverse transcriptase (SIGMA Cat#M1302-40KU), Oligo dT (Invitrogen Cat#18418012) and a dNTP cocktail (Invitrogen Cat#10297-018) according to the manufactures protocols. Quantitative real-time PCR was performed on the primary hepatocyte cDNA utilizing a Viia 7 Real Time PCR System (Life Technology, Canada). Data is expressed as a fold change over control based on ΔΔCT values. All primers were designed using Primer3 and BLAST software (NCBI) and are listed in Table 3 located in Chapter 3.
4.2.6 Statistical Analysis

Statistical significance was assessed through a Student T-test or two-way ANOVA for repeated measures followed by a Bonferroni analysis when applicable. For all assessments, a P<0.05 was considered significant. Unless otherwise stated, all data is expressed as MEAN ±SEM.

4.3 Results

4.3.1 ACC Inhibition Stimulates FGF21 Expression and Secretion

Physiologically, the short-term regulation of fatty acid oxidation is primarily controlled through the AMPK/ACC pathway, in which AMPK phosphorylation results in the phosphorylation and inactivation of ACC thereby increasing fatty acid uptake into the mitochondria to be oxidized (Mihaylova & Shaw, 2011). Due to CMPF’s ability to directly stimulate fatty acid oxidation within the isolated hepatocyte, we examined the effect that short term CMPF treatment has on the phosphorylation of both AMPK and ACC. As CMPF was not found to alter the phosphorylation or total protein expression of either AMPK or ACC while simultaneously activating oxidation, this indicated that CMPF is not stimulating fatty acid oxidation through activation of upstream signaling but may instead be acting through a direct inhibition of ACC.

4.3.1a CMPF Structurally Resembles the ACC Inhibitor TOFA

TOFA or 5-(tetradeoxy)-2-furoic acid, is a potent chemical inhibitor that is known to interact directly with ACC to both competitively and reversibly inhibit its function (Halvorson & McCune, 1984; McCune & Harris, 1979). Interestingly, when the structure of CMPF was compared to the structure of TOFA there were many similarities, including the presence of both a furan ring and carboxyl side chain (Fig 36). These similarities in structure indicate that CMPF may be acting similarly to TOFA as a structural inhibitor of ACC activity.
4.3.1b Direct ACC Inhibition

Due to the structural similarities that exist between CMPF and TOFA, we were next interested in whether TOFA has a similar effect to CMPF on the expression and secretion of FGF21. Following 24-hour treatment, TOFA significantly increased both the expression and secretion of FGF21 from the isolated hepatocytes similar to CMPF (Fig 37 A and B). In addition to structurally resembling each other, both CMPF and TOFA also structurally resemble a free fatty acid and therefore could potentially be activating ligands of the transcription factor PPARα (Varga et al., 2011). To assess this confounding factor, primary hepatocytes were treated for 24-hours with two additional allosteric ACC inhibitors that are structurally different from CMPF, TOFA and free fatty acids (Fig 37 C and D) (Corton, Gillespie, Hawley, & Hardie, 1995; Griffith et al., 2014; Koichi Ohmori et al., 2003). Following treatment with both S-2E and PF-05175157 (both pharmacological, structural inhibitors of ACC) there was again a significant increase to both FGF21 expression and secretion (Fig 37 A and B). These results indicate that CMPF may be stimulating FGF21 expression and secretion from the isolated hepatocytes through inhibition of ACC rather than acting as a ligand to directly activate PPARα activity.
4.3.1c Indirect ACC inhibition

To confirm that ACC inhibition is able to stimulate FGF21 expression and to further demonstrate that the ACC inhibitors are not acting as ligands for PPARα activation, primary hepatocytes were next treated for 24-hours with AICAR, a stimulator of AMPK activity. Stimulation of AMPK activity would lead to the phosphorylation and subsequent inhibition of ACC. Treatment with increasing concentrations of AICAR lead to a dose dependent increase in FGF21 secretion (Fig 38). This result demonstrates that inhibition of ACC, whether that be direct or through phosphorylation, stimulates both the expression and secretion of FGF21 within the hepatocyte.

Figure 37: Direct ACC inhibition stimulates expression and secretion of FGF21. (A) FGF21 mRNA expression calculated as 2^{-ΔΔCT} and (B) FGF21 secreted into the media from isolated hepatocytes treated with TOFA (100µM), S-2E (10µM) and PF-05175157 (10µM) for 24-hours (n=3). Chemical structure of the ACC inhibitors (C) S-2E and (D) PF-05175157. *P<0.05, **P<0.01. All error bars SEM.

Figure 38: Inhibition of ACC through phosphorylation stimulates FGF21 secretion. Concentration of FGF21 secreted into the media from isolated hepatocytes following 24-hour treatment with AICAR (n=3). Data is expressed as a fold change over control. *P<0.05. All error bars SEM.
4.4 Discussion

The circulating protein FGF21 beneficially enhances lipid metabolism leading to a reduction in hyperlipidemia, an improvement to glucose tolerance and insulin sensitivity, and a reversal of hepatic steatosis development (Ge et al., 2012). Due to the beneficial phenotype that results from an elevation in circulating FGF21, methods to enhance FGF21 expression and secretion as a potential therapeutic to treat metabolic disturbances that arise in obese individuals are of particular interest. With this study, we introduce the novel concept that both direct and indirect inhibition of acetyl-coA carboxylase (ACC) stimulates the expression and secretion of FGF21 within an isolated hepatocyte.

The furan fatty acid metabolite CMPF, acts directly on the liver to enhance both fatty acid oxidation and FGF21 secretion. As fatty acid oxidation is enhanced in the absence of upstream AMPK/ACC signaling, and as the structure of CMPF resembles that of a known ACC inhibitor TOFA, it is likely that CMPF is inhibiting ACC directly. In addition, when treated with CMPF, isolated ACC enzymes had a decreased rate of malonyl-coA production indicating direct inhibition (see Fig 35). With the use of primary hepatocytes, we have demonstrated that not only is the enhancement to fatty acid oxidation likely due to direct ACC inhibition but that CMPF directly inhibiting ACC would also result in an increase to FGF21 expression and secretion. Although I believe the evidence stated in favor of CMPF being a direct inhibitor of ACC activity to be strong, further confirmation could be obtained with the use of fluorescence using a method such as time-resolved FRET analysis that detects protein-protein interactions (Kenworthy, 2001).

The primary limitation in our conclusion that a direct inhibition of ACC stimulates FGF21 expression lies in our inability to definitively conclude that CMPF, or the other direct ACC inhibitors, are not ligand activating PPARα in addition to their inhibition of ACC activity. Although, the elevation in FGF21 secretion noted following the indirect inhibition of ACC, through the activation of upstream AMPK activity, strongly indicates that ACC inhibition is responsible for enhanced FGF21 expression. To further explore this concept, our lab has performed a PPARα luciferase assay. Within this assay direct stimulation with CMPF failed to stimulate luciferase activity of the PPARα promoter indicating that CMPF does not have the ability to directly activate PPARα activity. Rather than CMPF or the other ACC inhibitors acting as
activating ligands themselves, it is more likely that the inhibition of ACC results in the accumulation of intracellular metabolic intermediates that have the ability to ligand activate PPARα and subsequently enhance FGF21 gene transcription.

The inhibition of ACC may lead to the downstream activation of a transcription factor such as PPARα to enhance FGF21 gene transcription but it is also possible that inhibition of ACC alters histone acetylation (Galdieri & Vancura, 2012). Through an alteration in histone acetylation, exposure to the DNA template is enhanced thereby enhancing gene expression. Histone acetylation is a process in which an acetyl group, typically from acetyl-coA, is transferred onto lysine residues of the histone core (Kuo & Allis, 1998). Acetylation of the lysine residues removes their positive charge, decreasing their attraction to the negatively charged DNA backbone and causing the chromatin to obtain a more relaxed structure. In the presence of ACC inhibition, acetyl-coA is no longer converted into malonyl-coA and therefore would be in abundance within the cell (Galdieri & Vancura, 2012). Therefore, it is possible that constant ACC inhibition alters chromatin structure, although further experimentation would be required to determine if acetylation is altered within the FGF21 promoter region.

Therefore, within chapter 4 we have concluded that CMPF, whose structure closely resembles that of the allosteric ACC inhibitor TOFA, is likely interacting directly with ACC to inhibit its function. This direct inhibition of ACC not only accounts for the increase in fatty acid oxidation but is likely a causative factor in the stimulation of FGF21 transcription and translation as treatment of the primary hepatocytes with both structural and upstream inhibitors of ACC lead to a similar increase in FGF21 expression.
Chapter 5: Discussion

5.1 Conclusion and Future Directions

Within this thesis, I have presented my work examining the effect of CMPF on lipid metabolism within the liver, through the direct treatment of isolated hepatocytes. In 2014, Prentice et al identified the furan fatty acid metabolite CMPF, to be a causative factor in the development of diabetes due to its negative influence on beta cell function. In two separate publications, both Prentice et al (2014) and Liu et al. (2016) determined that a rapid elevation of CMPF within the circulation induces a metabolic switch within the beta cell to a preferential use of fatty acid oxidation over glucose utilization. This metabolic switch within the beta cell impairs glucose sensing resulting in a subsequent impairment to insulin secretion (Prentice et al., 2014). As the beta cell relies heavily on its ability to sense glucose and respond accordingly with the secretion of insulin, an upregulation in fatty acid oxidation is quite detrimental, but if CMPF were to enhance lipid metabolism in a tissue responsible for the regulation of lipid homeostasis the effect could be quite beneficial. In addition to being elevated in diabetes, CMPF has additionally been found to be elevated in the circulation following consumption of a diet rich in fish oil (Zheng et al., 2016). This finding occurs in parallel to a recent study in our lab currently under review for publication, that has found CMPF to be the major metabolic by-product of the prescription fish oil Lovaza (Prentice et al., Unpublished). Fish oil consumption is commonly prescribed to reduce hypertriglyceridemia and has been shown to be beneficial in the reduction of hepatic steatosis (Di Minno et al., 2012; Eslick et al., 2009). Within the same study, we have found that when CMPF is injected into mice prior to a HFD, it enhances whole body lipid metabolism resulting in improved whole-body insulin sensitivity and a reduction in hepatic steatosis. These results are indicative of a double-edged effect of CMPF within the body. The primary purpose of the present study was to determine if CMPF has the ability to act directly on the liver to enhance hepatic lipid metabolism, or if the reduction to hepatic steatosis observed in-vivo is the indirect result of CMPF action elsewhere in the body. With the use of isolated hepatocytes, we have concluded that following CMPF treatment CMPF accumulates in the hepatocyte whereby it directly influences hepatic lipid metabolism through a stimulation of both fatty acid oxidation and lipogenesis (Fig 39). In addition, CMPF directly stimulates hepatic FGF21 expression and secretion (Fig 39). It is likely that both
the enhancement to fatty acid oxidation as well as the induction of FGF21 expression are a result of CMPF acting as a direct structural inhibitor of ACC. Although an enhancement to fatty acid oxidation would contribute to a reduction in hepatic triglyceride accumulation, the results observed in-vitro are minimal, making it likely that the increase in circulating FGF21, induced by CMPF, is the primary contributor to chronically influence whole body energy metabolism in-vivo and significantly reduce hepatic steatosis to improve insulin sensitivity (Fig 39).

**Figure 39: Summary of CMPF action within the hepatocyte.** Through direct ACC inhibition CMPF directly enhances both fatty acid oxidation and FGF21 expression and secretion. However, it is likely the increase in circulating FGF21 that is responsible for CMPFs profound effect to reduce hepatic steatosis in-vivo.

With the conclusion of this study we have inferred that CMPF is a direct inhibitor of the metabolic enzyme ACC. This conclusion is based on the following findings presented in Chapters 3 and 4. First, that CMPF enhances fatty acid oxidation in the absence of either AMPK or ACC phosphorylation, both of which are key in the regulation of lipid oxidation. Second, that CMPF structurally resembles that of a known ACC inhibitor TOFA. Third, treatment with CMPF decreases the activity of isolated ACC enzymes and lastly, that inhibition of ACC both structurally and through phosphorylation results in an increase in the expression and secretion of FGF21. Although I believe the evidence provided in favor of CMPF acting as a direct inhibitor of ACC to be strong, future work could focus on confirming this direct interaction and exploring its potential
consequences. In particular through assessing the interaction of CMPF with ACC in-vitro. As CMPF enhances both fatty acid oxidation and lipogenesis following 24-hour treatment, it was postulated in Chapter 3 that CMPF may be preferentially inhibiting ACC2 over ACC1 allowing for the activation of both pathways. In the introduction of Chapter 4, data was presented from a biochemical assessment assay that was performed that showed CMPF to inhibit both ACC1 and ACC2 activity when outside the cell. As this experiment was performed in an isolated setting it does not take into account CMPFs entry and subsequent interaction with hepatic cellular components. Therefore, it is still possible that CMPF preferentially interacts with ACC2 when inside the cell. Future experimentation could include the use of time-resolved FRET analysis. With this technique, a donor and an acceptor fluorophore are attached to the proteins of interest (Kenworthy, 2001). If the fluorophores come into close proximity to each other, as would occur during direct ligand binding, then excitation of the donor fluorophore would cause the acceptor fluorophore to fluoresce.

Although it is clear that CMPF leads to enhanced transcription of the metabolic protein FGF21, an additional limitation lies in the scope of this study as it is likely that FGF21 is not the only gene whose transcription is affected following CMPF treatment. To expand on this study, it would be interesting to perform a microarray within the primary hepatocytes following 24-hour treatment with CMPF with a focus on the up or downregulation of genes involved in the regulation of lipid metabolism. Potential genes of interest include those involved in the regulation of lipogenesis and fatty acid oxidation such as the nuclear sterol regulatory binding protein 1 (SREBP1), cAMP response element binding protein (CREB), acetyl-coA carboxylase 1 and 2 (ACC1 and ACC2) or peroxisome proliferator–activated receptor alpha (PPARα) (Kersten, 2014; Rui, 2014).

Hepatic steatosis associated with the development of Non-Alcoholic Fatty Liver Disease, is the most common cause of liver dysfunction within North America (Smith & Adams, 2011). The presence of NAFLD is strongly correlated with the presence of additional metabolic disturbances such as cardiovascular disease and Type 2 Diabetes. The ability to prevent the development of hepatic steatosis in obese individuals is critical to maintain hepatic insulin sensitivity in order to properly regulate the levels of glucose and lipids in the circulation. At this time, there are no therapies available to specifically target lipid metabolism within the liver indicating the need for research into this area. The work that has been presented in this study provides insight into the
double-edged effect of CMPF within the body and provides a novel method of preventing hepatic steatosis in obese individuals.

The majority of experimentation within this study utilized a pharmacological dose of CMPF at 200µM. This high dose of CMPF corresponds to the elevated levels of CMPF seen in the circulation of diabetic individuals and was used so as to keep results consistent with previous experimentation that has been performed on CMPF within our lab. In a healthy individual, the levels of CMPF are typically much lower, barely passing the 5µM range and in individuals that consume high quantities of fish oil supplementation CMPF reaches maximum levels in the range of 80-100 µM (Prentice et al., Unpublished; Zheng et al., 2016). When primary hepatocytes were treated with an intermediate concentration of 100µM there was no significant difference seen to fatty acid oxidation. It is important to note though that at high concentrations the enhancement to fatty acid oxidation in-vitro was only 13%, therefore it remains possible that in-vitro assays are not sensitive enough to detect a significant increase in oxidation at a lower dosage. For this reason, it would be wise to repeat the in-vivo experimentation using physiological doses of CMPF to determine if the enhancement to lipid metabolism and reduction in hepatic fat accumulation still occurs at intermediate doses of CMPF. Particularly in light of the findings from a preliminary study performed by an additional member of the lab, in which treatment of islets with low CMPF concentrations failed to impair beta cell function. In addition, when a low, 5µM concentration of CMPF was administered to the primary hepatocytes the effect on lipid metabolism appeared to be opposite, leading to a reduction in both fatty acid oxidation and hepatic lipogenesis. This suggests that CMPF is likely acting through multiple mechanisms within the hepatocyte. At a very low concentration of 5uM, any effect of CMPF that is observed would be very specific. This is in contrast to the inhibition of ACC that is only observed following treatment with high CMPF concentrations, indicating that it is likely weak inhibition. For this reason, the decrease in fatty acid oxidation observed following 5uM CMPF, is likely the result of an absence in ACC inhibition. Regulation of lipogenesis primarily occurs through the activity of lipogenic transcription factors such as SREBP (Rui, et al. 2014). As the activity of SREBP is known to be inhibited by fatty acids, it is possible that CMPF inhibits lipogenic gene transcription, thereby potentially explaining the inhibition of lipogenesis that is occurring at low CMPF concentrations but further investigation is warranted to determine the exact mechanism through which CMPF is acting at low concentrations.
Although the data presented within this study, in addition to the evidence provided as preliminary data in Section 1.3.4, suggests a beneficial effect of CMPF to reduce hepatic steatosis, it cannot be ignored that these beneficial effects of CMPF on the liver are occurring in combination with the negative effects of CMPF on the beta cell to induce beta cell dysfunction. In the event that a pharmacological dose of CMPF is required to significantly enhance fatty acid oxidation and reduce hepatic steatosis, a therapeutic treatment would need to be developed that maintains CMPF’s beneficial effect on the liver while minimizing its negative effects on beta cell function. For this reason, a full understanding of CMPF’s action within the hepatocyte is essential. One method that could be utilized to combat this dual effect of CMPF is structural modification (Pandeya & Dimmock, 1997). It is possible that modifications to CMPF’s side chains could alter its ability to enter various cell types or elicit certain effects thereby allowing the synthetic molecule to maintain only CMPFs beneficial functions. In addition, future work could target the entry of CMPF into the cell. Evidence strongly suggests that CMPF utilizes the family of organic anion transporters to enter various tissues of the body (Deguchi et al., 2004; Sekine et al., 2000). Isoforms of the organic anion family differ most in their tissue distribution, with OAT2 being almost exclusively expressed within the liver (Shen et al., 2015). If knockdown experiments revealed OAT2 to be the transporter responsible for facilitating CMPF entry into the hepatocyte then it is possible that treatment of CMPF in combination with a pharmacological inhibitor such as probenecid would help to eliminate its negative effects. Probenecid is an FDA approved drug currently prescribed for the treatment of gout where it primarily acts on the kidney to inhibit OAT function (Kydd, Seth, Buchbinder, Edwards, & Bombardier, 2014). As mentioned at the end of Chapter 3, although probenecid is a strong inhibitor of the primary isoforms OAT1 and OAT3, it is only a weak inhibitor of OAT2 (Shen et al., 2015). Co-treatment of CMPF with probenecid could prevent the uptake of CMPF into tissues such as the beta cell while still allowing entry into tissues such as the hepatocyte where OAT2 is almost exclusively expressed.

Within the body, the liver is the primary hub in the control of lipid homeostasis but there are many additional metabolic tissues within the body such as the adipose and the central nervous system that work closely with each other to regulate energy metabolism. The results of this study, combined with the work performed by Prentice et al and Liu et al demonstrate the ability of CMPF to enhance fatty acid oxidation in both the beta cell and the hepatocyte. These results indicate that CMPF’s enhancement of lipid metabolism is not tissue specific. CMPF is likely acting on
numerous metabolic tissues within the body. As such, future experimentation focusing on the effects of CMPF within the adipose tissue and the central nervous system are required. Just as it is important to fully understand the effect of CMPF within the liver, it is essential that we understand the role that it plays in additional metabolic tissues if we are to consider it as a potential therapeutic.

The fibroblast growth factor protein FGF21 belongs to a group of endocrine growth factors that lack a heparin binding domain allowing them to be secreted from their tissue of production, travel through the circulation, and act on peripheral tissues (Fisher & Maratos-Flier, 2016; Suzuki et al., 2008). Due to the absence of the heparin binding domain, endocrine growth factors like FGF21 bind with weak affinity to the tyrosine kinase fibroblast growth factor receptors (FGFR 1-4) and require the use of a co-receptor β-Klotho to initiate cellular signaling (Suzuki et al., 2008). Within the liver, both B-Klotho in addition to the four FGFRs have been found to be expressed and it is clear that when circulating levels of FGF21 in the body are elevated, lipid metabolism within the liver is altered but it remains controversial in the field as to whether FGF21 has the ability to act locally following secretion in order to alter lipid metabolism directly (Ge et al., 2012; Kharitonenkov & Larsen, 2011; Maratos-Flier, 2017). It has been repeatedly observed that when the levels of FGF21 are elevated in-vivo it results in the activation of hepatic fatty acid oxidation in addition to an induction in ERK1/2 signaling within the liver (Kharitonenkov & Larsen, 2011). However, researchers such as Lin et al (2013) have failed to detect any effects of FGF21 within isolated livers and primary hepatocytes. This indicates that the in-vivo effects of FGF21 within the liver are indirect and may be regulated by secondary factors such as adiponectin released from the adipose tissue following FGF21 stimulation. Lin et al (2013) found within adiponectin knockout mice that the beneficial effects of FGF21 to reduce hepatic steatosis, hyperlipidemia and liver injuries were no longer present demonstrating that adiponectin is key in the regulation of FGF21’s lipid lowering abilities. Due to the controversy that exists over FGF21 activation within the liver and due to the increase in both fatty acid oxidation and FGF21 secretion noted following CMPF treatment, future experimentation is required to determine if CMPF remains able to enhance fatty acid oxidation directly in-vitro and decrease hepatic steatosis in-vivo in the absence of FGF21 with the use of whole body FGF21 knockout mice.

In conclusion, within this thesis I have presented a thorough analysis of the effects of CMPF on both glucose and lipid metabolism within the liver. With the use of isolated hepatocytes, I have demonstrated that 24-hour treatment with CMPF directly enhances hepatic lipid metabolism
resulting in an increase in both fatty acid oxidation and lipogenesis with a trend towards improved insulin sensitivity. CMPF treatment also stimulates the expression and secretion of FGF21 from the liver, a metabolic protein which is known to enhance whole body lipid metabolism when elevated within the circulation. In addition, I present the novel idea that an increase in FGF21 expression and secretion is induced through an inhibition of the regulatory enzyme acetyl-coA carboxylase (ACC). As CMPF has previously been perceived to be a negative metabolic by-product, these results demonstrate a double-edged effect of CMPF within the body and provide insight into a potential therapeutic for the reduction of hepatic triglyceride accumulation in obese individuals.
Bibliography


Deguchi, T., Kouno, Y., Terasaki, T., Takadate, A., & Otagiri, M. (2005). Differential Contributions of rOat1 (Slc22a6) and rOat3 (Slc22a8) to the in Vivo Renal Uptake of


Varga, T., Czimmerer, Z., & Nagy, L. (2011). PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation(). *Biochimica et Biophysica Acta, 1812*(8), 1007-1022. doi:10.1016/j.bbadis.2011.02.014


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doi:10.1038/srep29522
Figure 1: Lipid secretion assay in primary hepatocytes. (A) Fold change radioactive count of intracellular lipid content in primary hepatocytes following 90 min incubation with $^{14}$C radiolabelled acetate (n=1) and (B) Radioactive count of lipid secretion (n=4) in primary hepatocytes following 90 min incubation with $^{14}$C radiolabelled acetate. Cells were treated for 24-hours with 100nM insulin and 400µM oleic.