Identification and Characterization of the Role of the Circulating Metabolite 3-Carboxy-4-Methyl-5-Propyl-2-Furanpropanoic Acid (CMPF) in Diabetes

by

Kacey June Prentice

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Physiology
University of Toronto

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ABSTRACT

Both gestational diabetes (GDM) and type 2 diabetes (T2D) are caused by a failure of the pancreatic beta cell to compensate for increased insulin demand. The underlying cause of this failure, particularly the rapid transition from prediabetes to diabetes, and between GDM and T2D is largely unknown. To identify circulating factors that may play a causal role in beta cell dysfunction we performed global metabolomics profiling on the plasma of GDM and T2D patients compared to normal glucose tolerant controls. We identified the furan fatty acid metabolite 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) as being highly elevated in diabetic plasma. In vivo and in vitro assays demonstrated that CMPF induces glucose intolerance by impairing glucose-stimulated insulin secretion (GSIS), suggesting CMPF may play a causal role in diabetes. Analysis of human patients showed that rapid elevation in CMPF is associated with a significantly increased risk of diabetes development, consistent with elevated CMPF being the “tipping point” in the progression from prediabetes to overt diabetes. The mechanism underlying this dysfunction involves increasing reactive oxygen species (ROS), resulting in altered gene expression, inhibition of beta cell transcription factor activity, as well as uncoupling of glucose metabolism to ATP production, thus reducing insulin biosynthesis and
secretion. Additionally, CMPF induces a state of preferential fatty acid utilization, which persists for months following treatment. This suggests that CMPF exposure during GDM may be responsible for the extremely high rate of progression to T2D postpartum. While detrimental for beta cell function, this ability of CMPF to drive fatty acid oxidation has beneficial effects for hepatic insulin sensitivity due to the prevention of steatosis. CMPF induces beta-oxidation, simulating a “fasting-like” state and promoting expression of FGF21, which is required to impair triglyceride synthesis and promote beta-oxidation long-term. Overall, CMPF is significantly elevated in human diabetes and has direct effects on beta cell function and insulin sensitivity in rodent models.
“You have brains in your head. You have feet in your shoes. You can steer yourself in any direction you choose.” ~Dr. Seuss
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<tbody>
<tr>
<td>ACC</td>
<td>Acetyl-CoA Carboxylase</td>
</tr>
<tr>
<td>ADA</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced Glycation End-Products</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BCAA</td>
<td>Branched-Chain Amino Acid</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CDA</td>
<td>Canadian Diabetes Association</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic Kidney Disease</td>
</tr>
<tr>
<td>CLAMs</td>
<td>Comprehensive Lab Animal Monitoring System</td>
</tr>
<tr>
<td>CMPF</td>
<td>3-Carboxy-4-Methyl-5-Propyl-2-Furanpropanoic Acid</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
</tr>
<tr>
<td>DIO</td>
<td>Diet-Induced Obesity</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbant Assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FFA</td>
<td>Free Fatty Acid</td>
</tr>
<tr>
<td>FGF21KO</td>
<td>Fibroblast Growth Factor 21 Knockout</td>
</tr>
<tr>
<td>GCT</td>
<td>Glucose Challenge Test</td>
</tr>
<tr>
<td>GDM</td>
<td>Gestational Diabetes Mellitus</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-Protein Coupled Receptor</td>
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<tr>
<td>GSIS</td>
<td>Glucose-Stimulated Insulin Secretion</td>
</tr>
<tr>
<td>GTT</td>
<td>Glucose Tolerance Test</td>
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<tr>
<td>GWAS</td>
<td>Genome-Wide Association Study</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Glycated Hemoglobin</td>
</tr>
<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
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<tr>
<td>HFD</td>
<td>High Fat Diet</td>
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<tr>
<td>HOMA-B</td>
<td>Homeostatic Model Assessment of Beta-cell Function</td>
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<tr>
<td>IFG</td>
<td>Impaired Fasting Glucose</td>
</tr>
<tr>
<td>IGT</td>
<td>Impaired Glucose Tolerance</td>
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<tr>
<td>IP</td>
<td>Intraperitoneal</td>
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<tr>
<td>IR</td>
<td>Insulin Resistance</td>
</tr>
<tr>
<td>ISSI-2</td>
<td>Insulin-Secretion Sensitivity Index-2</td>
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<tr>
<td>ITT</td>
<td>Insulin Tolerance Test</td>
</tr>
<tr>
<td>KATP</td>
<td>ATP-Sensitive Potassium Channel</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
</tr>
<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
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<tr>
<td>LIRKO</td>
<td>Liver-specific Insulin Receptor Knockout</td>
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<tr>
<td>MetS</td>
<td>Metabolic Syndrome</td>
</tr>
<tr>
<td>MIN6</td>
<td>Mouse Insulinoma Cell Line 6</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial Membrane Potential</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
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<tr>
<td>NAFLD</td>
<td>Non-Alcoholic Fatty Liver Disease</td>
</tr>
<tr>
<td>NASH</td>
<td>Non-Alcoholic Steatohepatitis</td>
</tr>
<tr>
<td>NDDG</td>
<td>National Diabetes Data Group</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-Esterified Fatty Acid</td>
</tr>
<tr>
<td>NGT</td>
<td>Normal Glucose Tolerant</td>
</tr>
<tr>
<td>NIDDM</td>
<td>Non-Insulin Dependent Diabetes Mellitus</td>
</tr>
<tr>
<td>ob/ob</td>
<td>Leptin Knockout Mouse</td>
</tr>
<tr>
<td>OCR</td>
<td>Oxygen Consumption Rate</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral Glucose Tolerance Test</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PUFA</td>
<td>Poly-Unsaturated Fatty Acid</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
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<tr>
<td>RER</td>
<td>Respiratory Exchange Ratio</td>
</tr>
<tr>
<td>RIP-CRE</td>
<td>Rat Insulin Promoter-Driven Cre</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>SHDS II</td>
<td>Shanghai Diabetes Study II</td>
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<tr>
<td>SRM-MS</td>
<td>Selected Reaction Monitoring Mass Spectrometry</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
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<tr>
<td>T1DM</td>
<td>Type 1 Diabetes Mellitus</td>
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<tr>
<td>T2DM</td>
<td>Type 2 Diabetes Mellitus</td>
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<tr>
<td>TCA</td>
<td>Tricarboxylic Acid</td>
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<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
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<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans Golgi Network</td>
</tr>
<tr>
<td>UCP2BKO</td>
<td>Uncoupling Protein 2 Beta Cell-Specific Knockout</td>
</tr>
<tr>
<td>VDCC</td>
<td>Voltage-Dependent Calcium Channel</td>
</tr>
<tr>
<td>WGO</td>
<td>World Gastroenterology Organization</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</tbody>
</table>
Chapter 1- Introduction
1.1 OVERVIEW OF DIABETES MELLITUS

The Canadian Diabetes Association predicts that 1 in 3 Canadians will have diabetes or prediabetes by the year 2020 at an annual health care cost of $16 billion (Canadian Diabetes Association Clinical Practice Guidelines Expert et al., 2013). These numbers represent an increase of 10% of the population and $5 billion dollars in annual healthcare costs compared to 2011. In the United States the statistics are even more alarming, as it is estimated that more than 50% of Americans, an increase of more than 15% of the population compared to 2011, could be diabetic or prediabetic by 2020 at a cost of $3.35 trillion over the next decade (UnitedHealth Group’s Centre for Health Reform and Modernization). While the proportion of the population afflicted by type 1 diabetes mellitus (T1DM), caused by an autoimmune destruction of the pancreatic beta cells, is increasing at a modest rate, rising obesity rates, sedentary lifestyles and an aging population will continue to drive massive increases in the most prevalent form of diabetes, Type 2 (T2DM). These factors are also contributing to increasing rates of a third form of diabetes, gestational diabetes (GDM), which now afflicts up to 14% of pregnancies and contributes to increased risk of future development of T2DM (Buchanan and Xiang, 2005b).

1.1.1 Classifications of Diabetes Mellitus

1.1.1.a Type 1 Diabetes Mellitus

T1DM, also referred to as juvenile diabetes or insulin-dependent diabetes mellitus (IDDM) accounts for approximately 5-10% of diabetes cases (van Belle et al., 2011). T1DM is caused by an autoimmune destruction of the pancreatic beta cells that typically occurs in childhood or adolescence, but can occur at any time. The complete absence of circulating insulin results in an inability of insulin-sensitive tissues take up glucose from the bloodstream, leading to hyperglycemia. Before the discovery of insulin in 1921, individuals with T1DM had extremely short lifespans characterized by dramatic weight loss, frequent urination, extreme thirst, lethargy, ketoacidosis, and loss of vision. While progress has been made on identifying ways to better regulate blood glucose levels in T1DM patients, including the development of long-lasting insulin analogs, continuous glucose monitoring, and insulin pumps, individuals with T1DM remain dependent on lifelong insulin supplementation and have an average life expectancy ~15 years shorter
than normal glucose tolerant individuals due to an assortment of complications (Livingstone et al., 2015).

1.1.1.b Type 2 Diabetes Mellitus

T2DM, also known as non-insulin-dependent diabetes mellitus (NIDDM), accounts for more than 90% of all cases of diabetes. The rapidly growing diabetes epidemic has prompted extensive research into the underlying causes of this disease. Decades of research have revealed that T2DM is extremely complex, and likely caused by a combination of genetic and environmental factors. Numerous population, family, and twin-based studies have defined the heritability of T2DM in a very broad range of 20-80% (Almgren et al., 2011; Mathias et al., 2009). While overall individuals with first-degree relatives having T2DM have a 3-fold increased risk for T2DM themselves, it is important to note that a large proportion of the heritability of diabetes is accounted for by the heritability of obesity, one of the strongest risk factors for T2DM development (Wheeler and Barroso, 2011a). Thus environmental components including diet and physical activity may play just as much, if not a more significant role in diabetes risk than genetics.

Unlike T1DM, which is caused by a complete absence of insulin production, T2DM is caused by relative insulin insufficiency due to reduced sensitivity of peripheral tissues (Olokoba et al., 2012). Insulin resistance is induced by factors including increased adiposity, aging, ectopic lipid accumulation, and inflammation, and suppresses insulin-stimulated glucose uptake into skeletal muscle, liver and adipose tissue (Samuel and Shulman, 2012; Wilcox, 2005). In the face of increased insulin resistance, the pancreatic beta cells increase insulin biosynthesis and secretion to meet demand. However, if the beta cells fail to adequately compensate due to numerous factors, tissues are unable to uptake glucose adequately and hyperglycemia ensues. This evolution of diabetes development occurs in stages from normal glucose tolerance (NGT), through prediabetes and eventually to overt T2DM (Weir and Bonner-Weir, 2004). Chronic hyperglycemia of both prediabetes and T2DM underlies the significant morbidity and mortality of T2DM particularly due to micro- and macrovascular complications including neuropathy, nephropathy, vision problems, delayed wound healing, cardiovascular disease, and strokes (Forbes and Cooper, 2013; Rask-Madsen and King, 2013). Combined, T2DM and its associated complications
are the seventh leading cause of death in North America, accounting for more than 1.5 million deaths annually worldwide (ADA, WHO). While significant advancements have been made in treating T2DM and its associated complications such as the development of insulin sensitizers and insulin secretagogues, the impact of intensive treatment therapies have only had a minor effect on diabetes-associated mortality rates (Action to Control Cardiovascular Risk in Diabetes Study et al., 2008). Thus, future investigation into preventative therapies is essential for alleviating the impact of T2DM.

1.1.1.c Gestational Diabetes Mellitus

Importantly, the occurrence of the third main form of diabetes, Gestational Diabetes Mellitus (GDM) is also increasing (Ferrara, 2007). GDM currently afflicts 3-14% of pregnant women and is a condition of transient glucose intolerance during pregnancy, most frequently beginning in the late second or early third trimester when there is significant insulin resistance (Buchanan and Xiang, 2005a). Similar to T2DM, the underlying cause of GDM is an inability of the pancreatic beta cells to sufficiently compensate for increased insulin demand, resulting in glucose intolerance. Importantly, GDM is rapidly resolved upon parturition with the alleviation of insulin resistance and is therefore not associated with the same vascular complications as T2DM. However, women with GDM are at an extremely increased risk of developing T2DM in the future. Numerous studies have revealed that women with GDM have a 20-50% risk of developing T2DM within the first 5 years postpartum, with up to 70% of women transitioning within 10 years (Bellamy et al., 2009; Kim et al., 2002). It is unknown if pregnancy acts as a stress test for the beta cells, revealing an underlying predisposition to diabetes development, or if the diabetic condition imparts a dysfunction increasing future susceptibility.

The insulin resistance induced during pregnancy is a physiological adaptation to preserve glucose for the developing fetus. This increase in insulin resistance is attributed to release of insulin-desensitizing hormones from the placenta, maternal weight gain, inflammation, and inactivity (Barbour et al., 2002; Buchanan and Xiang, 2005a). By the end of pregnancy, the extent of insulin resistance is approximately equal to that observed in T2D (Buchanan and Xiang, 2005a). Placental hormones combined with insulin resistance have a lipolytic effect, stimulating fat breakdown and resulting in elevated circulating FFA levels.
Elevating plasma FFAs inhibits total body glucose uptake and oxidation (Sivan et al., 1998) and promotes a switch in metabolism from glucose to fatty acid oxidation, thus maximizing the amount of glucose present for the developing fetus (Bomba-Opon et al., 2006). To overcome the extreme insulin resistance, the pancreatic \(\beta\) cells compensate by increasing insulin secretion, resulting in only minor changes in blood glucose levels (Buchanan and Xiang, 2005a). This compensation is mediated by an increase in \(\beta\) cell mass as well as an increase in \(\beta\) cell function (Sorenson and Brelje, 1997). In the development of GDM it is unclear where the defect in beta cell compensation occurs. This is largely due to the lack of primary human tissue available for study from this period. Some evidence suggests that in humans, the defect is primarily functional as opposed to a defect in mass expansion, however this has yet to be conclusively determined.

1.1.2. Diagnosis of Diabetes Mellitus

The first record of diabetes dates back to 1552 BCE where the ancient Egyptians detailed remedies for the “passing of too much urine” (Ahmed, 2002). This description was common throughout the ancient world, where clinical texts have been found detailing the “melting down of the flesh and limbs into urine”. Remarkably, these symptoms of frequent urination and dramatic weight loss remain the most common first symptoms leading to a diagnosis of T1DM. Despite the differential nature of T1DM compared to T2DM and GDM, the methods of diagnosis have the same underlying premise, relying on a combination of phenotypic characteristics and quantification of glucose tolerance (Inzucchi, 2012).

1.1.2.a Diagnosis of T1DM

Before the discovery of insulin in 1921, a diagnosis of T1DM was a death sentence. Physicians identified diabetic patients based on the characteristic polyuria, extreme thirst, and hunger despite extreme weight loss (Roche et al., 2005). Indeed, it was from these symptoms that it was determined that diabetes was a carbohydrate disorder, as it was noted that the urine of diabetic patients attracted insects due to its sweet smell and taste (Ahmed, 2002). Currently, these three symptoms, in addition to hyperglycemia which can now be quantified readily in a single microliter of blood, remain the classical presentation of
T1DM, and will signal further testing for T1DM and the initiation of insulin therapy (Roche et al., 2005; van Belle et al., 2011).

As T1DM is caused by an autoimmune destruction of the pancreatic beta cells, the gold standard test for diagnosis is quantification of insulin production (Jones and Hattersley, 2013). In the insulin biosynthetic process, proinsulin is translated and undergoes folding dependent on the formation of two disulphite bridges (Qiao et al., 2006). This protein folding enables the cleavage of C-Peptide from the insulin molecule, producing C-Peptide and mature insulin that are secreted in equal molar ratios. Quantification of circulating C-Peptide provides an indication of beta cell mass and insulin production. T1DM typically presents when more than approximately 80-90% of the beta cell mass has been lost, making the condition irreversible. At the time of diagnosis, autoantibodies against the beta cells can be detected in circulation, further confirming the autoimmune destruction of this cell type and solidifying a diagnosis of T1D (Cnop et al., 2005; Roche et al., 2005; van Belle et al., 2011).

1.1.2.b Diagnosis of T2DM

Unlike T1DM, patients with T2DM do not typically present with advanced disease symptoms, but are monitored for disease progression over a period of months to years. T2DM risk is assessed based on the presence of several non-quantitative risk factors. These include, but are not limited to age, race, gender, BMI, and family history of diabetes (Fletcher et al., 2002). Combined, these factors account for up to 75% of diseases risk alone. If diabetes risk is determined to be significant based on the aforementioned parameters, the development of prediabetes or overt diabetes is assessed through monitoring of blood glucose levels in both the fasting and random fed states, as well as quantification of glycated hemoglobin (HbA1c) (American Diabetes, 2015). HbA1c is the most valuable tool for monitoring glucose tolerance status, as it reflects the chronic glycemic state over the previous three months (Bennett et al., 2007). Indications of impaired glucose tolerance through any of these parameters, based on standardized criteria, may prompt the completion of a 75g glucose bolus oral glucose tolerance test (OGTT) to provide an absolute quantification of glucose handling and insulin secretion dynamics. From these tests a diagnosis of T2DM can be made.
Various organizations define the threshold values for diagnosis of impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and overt T2DM. These diagnostic thresholds for 3 organizations, the World Heath Organization (WHO) (Adamu, 2011), Canadian Diabetes Association (CDA) (Canadian Diabetes Association Clinical Practice Guidelines Expert et al., 2013), and American Diabetes Association (ADA) (American Diabetes, 2015), are outlined in Table 1. Interestingly, the diagnostic thresholds for T2DM vary based on geographical location, indicative of the diverse nature of T2DM. In China, for example, diagnostic thresholds for HbA1c have been suggested to be lower due to increased complications associated with elevated HbA1c in that population (Bao et al., 2010). In India, threshold values for impaired fasting blood glucose are >6.1mM whereas the ADA defines this threshold as >7.0mM (Gupta et al., 2003). Overall, healthcare providers must use their discretion when making a diagnosis of T2DM.

<table>
<thead>
<tr>
<th>T2DM</th>
<th>World Health Organization</th>
<th>Canadian Diabetes Association</th>
<th>American Diabetes Association</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2011</td>
<td>2013</td>
<td>2015</td>
</tr>
<tr>
<td></td>
<td>FPG ≥ 7.0mM</td>
<td>FPG ≥ 7.0mM</td>
<td>FPG ≥ 7.0mM</td>
</tr>
<tr>
<td>or</td>
<td>or</td>
<td>or</td>
<td>or</td>
</tr>
<tr>
<td></td>
<td>2hPG ≥ 11.1mM</td>
<td>2hPG ≥ 11.1mM</td>
<td>2hPG ≥ 11.1mM</td>
</tr>
<tr>
<td>or</td>
<td>or</td>
<td>or</td>
<td>or</td>
</tr>
<tr>
<td></td>
<td>HbA1c ≥ 6.5%</td>
<td>HbA1c ≥ 6.5%</td>
<td>HbA1c ≥ 6.5%</td>
</tr>
<tr>
<td>or</td>
<td>or</td>
<td>or</td>
<td>or</td>
</tr>
<tr>
<td></td>
<td>Random Blood Glucose ≥ 11.1mM</td>
<td>Random Blood Glucose ≥ 11.1mM</td>
<td>Random Blood Glucose ≥ 11.1mM</td>
</tr>
<tr>
<td>Prediabetes</td>
<td>FPG 6.1-6.9mM</td>
<td>FPG 6.1-6.9mM</td>
<td>FPG 5.6-6.9mM</td>
</tr>
<tr>
<td>or</td>
<td>or</td>
<td>or</td>
<td>or</td>
</tr>
<tr>
<td></td>
<td>2hPG 7.8-11.0mM</td>
<td>2hPG 7.8-11.0mM</td>
<td>2hPG 7.8-11.0mM</td>
</tr>
<tr>
<td>or</td>
<td>or</td>
<td>or</td>
<td>or</td>
</tr>
<tr>
<td></td>
<td>HbA1c 6.0-6.4%</td>
<td>HbA1c 6.0-6.4%</td>
<td>HbA1c 5.7-6.4%</td>
</tr>
</tbody>
</table>

Prediabetes includes both impaired glucose tolerant (IGT) and impaired fasting glucose (IFG). FPG is Fasting Plasma Glucose. 2hPG is 2 hour Plasma Glucose during OGTT.
1.1.2.c Diagnosis of GDM

Despite clear similarities to T2DM, the diagnosis of GDM is complicated by the acute nature of the disease. Elevated glycated haemoglobin (HbA1c), for example, is indicative of chronic hyperglycemic exposure over the previous 3 months and is a common diagnostic tool used for T2D (Farmer, 2012). In GDM however, hyperglycemia is not present for long enough to cause significant differences in HbA1c. Glucose tolerance tests, including the glucose challenge test (GCT) and OGTT, are the gold standard for diagnosing GDM (Karagiannis et al., 2010). In North America, all pregnant women are advised to undergo a glucose challenge test (GCT) between 24 and 28 weeks gestation to test for GDM. This test involves the patient fasting for 12 hours before consuming a 50g bolus of glucose (Retnakaran et al., 2009). Blood glucose is measured after one hour, and if it is above 7.8mM, women are followed up with a 75g glucose OGTT for the diagnosis of GDM. GDM is confirmed if more than 2 glucose values are above the National Diabetes Data Group (NDDG) diagnostic criteria during the OGTT (Retnakaran et al., 2010). However, diagnostic criteria are constantly evolving and vary significantly by region to an even greater extent than they do for T2DM, suggesting that many GDM cases may be undiagnosed (Karagiannis et al., 2010). Examples of these diagnostic criteria are outlined in Table 2. Indeed, with the introduction of new diagnostic criteria in 2014 it is estimated that up to 18% of pregnant women in the United States will now have a diagnosis of GDM during pregnancy (DeSisto et al., 2014; Ferrara, 2007). This may be due to more restricted guidelines, or related to increasing maternal age and prepregnancy BMI.

### Table 2. Diagnostic Criteria for Gestational Diabetes According to Various Organizations

<table>
<thead>
<tr>
<th>World Health Organization</th>
<th>Canadian Diabetes Association</th>
<th>American Diabetes Association</th>
<th>National Diabetes Data Group (NDDG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OGTT</td>
<td>75g</td>
<td>75g</td>
<td>100g</td>
</tr>
<tr>
<td>FPG</td>
<td>≥ 5.1mM</td>
<td>≥ 5.3mM</td>
<td>≥ 5.3mM</td>
</tr>
<tr>
<td>1hPG</td>
<td>≥ 10.0mM</td>
<td>≥ 10.6mM</td>
<td>≥ 10.0mM</td>
</tr>
<tr>
<td>2hPG</td>
<td>≥ 8.5mM</td>
<td>≥ 8.9mM</td>
<td>≥ 8.6mM</td>
</tr>
<tr>
<td>3hPG</td>
<td></td>
<td></td>
<td>≥ 7.8mM</td>
</tr>
<tr>
<td>Threshold</td>
<td>One Value Above</td>
<td>Two Values Above</td>
<td>Two Values Above</td>
</tr>
</tbody>
</table>

FPG is Fasting Plasma Glucose. 1hPG is 1 hour Plasma Glucose during OGTT, and so forth
1.1.3 Complications of Diabetes Mellitus

Due to their common underlying pathology, both T1DM and T2DM are associated with the development of severe complications. The number one complication of diabetes is vascular disease, with diabetic patients experiencing both micro- and macro vascular complications that contribute to the significant morbidity and mortality of diabetes (Rask-Madsen and King, 2013). Patients are at an extremely elevated risk of cardiovascular disease, stroke, nephropathy, neuropathy, altered wound healing, and retinopathy to name a few. These complications are primarily attributed to chronic hyperglycemia, though alterations to the circulating lipid profile, and insulin resistance likely also significantly contribute to endothelial cell dysfunction (Avogaro et al., 2011; Sena et al., 2013). While medications are prescribed to manage the insulin resistance and hyperglycemia of diabetes, few directly target endothelial cell function, thus this complication is slowed but the deterioration of vascular function persists.

1.1.3.a Managing Morbidity and Mortality of T1DM and T2DM

Even with good glycemic control as indicated by continuous glucose monitoring and normalized HbA1c levels, patients still experience significant morbidity and mortality, suggesting that there are factors of diabetes independent of glycemia that contribute to these complications (Action to Control Cardiovascular Risk in Diabetes Study et al., 2008). A recent follow-up study of the Diabetes Control and Complications Trial (DCCT) examining the outcome of T1DM patients an average of 27 years following a 7 year period of intensive therapy revealed no improvement in mortality rates when correcting for current medication regimens. In fact, mortality was modestly increased in the intensive therapy group in the first 10 years following intensive therapy intervention (Writing Group for the et al., 2015). This finding is not unique to T1DM patients, as multiple trials in T2DM individuals have demonstrated similar results. The Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial, which examined the effect of intensive diabetes therapy on CVD outcomes in a T2DM population, revealed a significant increase in patient mortality with intensive therapy in spite of significantly improved HbA1c levels to the point that the trial was ended early (Action to Control Cardiovascular Risk in Diabetes Study et al., 2008). A third trial conducted in the United States, the Veteran Affaris Trial II, revealed that intensive therapy in T2D was associated
with increased markers of thrombosis, suggesting further detriment to vascular function (Duckworth et al., 2009). It has been suggested that intensive diabetes therapy may have the most significant benefit in critically ill patients where dysglycemia may confound other illnesses. In a large-scale meta analysis of over 37 randomized trials examining the benefit of intensive T2DM therapy in critically ill patients it was revealed that this is not the case. Consistent with the findings in the general population, intensive therapy was found to have no survival benefit. In fact, intensive therapy was found to associate with increased risk of hypoglycemia compared to standard care (Wiener et al., 2008). Combined, these studies strongly suggest that the complications of diabetes are largely independent of glycemic control. The inability to prevent the associated complications of diabetes stresses the need to prevent diabetes development significantly before onset.

1.1.3.b Complications of GDM

Despite the acute nature of GDM it is associated with numerous short- and long-term complications for both mother and fetus. In the short term, GDM increases the risk of preeclampsia, large for gestational age babies leading to pre-term births, delivery complications, and birth injuries, fetal respiratory distress, jaundice, and fetal hypoglycemia (Mitanchez, 2010). More chronic complications include a higher risk of congenital malformations, metabolic syndrome, cardiovascular disease, and perhaps the largest burden to the healthcare system, a very high rate of progression to T2D (Bellamy et al., 2009; Kim et al., 2002). Studies have determined that 25-50% of women with a history of GDM will develop T2DM within the first 5 years post-partum, with reports of up to 70% of women developing T2D within 10 years (Bellamy et al., 2009; Kim et al., 2002). Strong evidence is also emerging that fetal exposure to hyperinsulinemia and hyperglycemia associated with GDM puts the child at a significantly increased risk of obesity, metabolic syndrome and T2D later in life (Reece, 2010). Once diagnosed with GDM, treatment consists of dietary management, blood glucose monitoring and administration of insulin if blood glucose concentrations cannot be controlled by diet alone (Alwan et al., 2009).
1.2 METABOLIC SYNDROME IN THE PATHOGENESIS OF DIABETES

All forms of diabetes mellitus are fundamentally disorders of insulin action. In T1DM, hyperglycemia is induced by the absence of insulin due to a loss of the insulin producing beta cells. The pathology of T2DM and GDM, however, is somewhat more complex. These forms of diabetes are caused by a combined insulin secretory defect, along with a desensitization of the peripheral tissues to the activity of insulin. Thus, these two components represent important avenues of research for increasing our understanding of and developing treatments for T2DM and GDM.

Development of insulin resistance is widely believed to be the initiating factor in the development of diabetes. Up to 35% of the population has a condition termed Metabolic Syndrome (MetS), a cluster of disorders that affect energy utilization and storage that increases in prevalence with obesity and aging (Kaur, 2014). MetS is characterized by the presence of at least 3 medical conditions including abdominal obesity, high blood pressure, elevated fasting blood glucose, and/or dyslipidemia with elevated plasma triglycerides, low density lipoprotein (LDL) and reduced high density lipoprotein (HDL) (Despres and Lemieux, 2006; Eckel et al., 2005). This cluster of disorders are almost always associated with an insulin resistant state and thus significantly increase the risk of cardiovascular disease and diabetes. In fact, patients meeting these parameters also meet the diagnostic criteria of prediabetes (Kaur, 2014).

Insulin resistance, and thus MetS, contributes to the pathogenesis of diabetes in a number of ways. Reduced responsiveness of peripheral tissues including liver, adipose, and skeletal muscle to insulin results in hyperglycemia due to an impaired ability to uptake glucose from the blood (Pessin and Saltiel, 2000). This hyperglycemia is further exacerbated by reduced ability of insulin to suppress hepatic glucose production, thus resulting in more glucose being produced and entering circulation. In addition to hyperglycemia, insulin resistance prevents the antilipolytic activity of insulin in the liver and adipose tissue, promoting the generation and release of free fatty acids and contributing to dyslipidemia (Boden and Shulman, 2002; Wilcox, 2005). Combined, these factors are believed contribute to beta cell dysfunction and death through a process termed glucolipotoxicity, reducing insulin secretion and activity even further (Poitout et al., 2010; Poitout and Robertson, 2008).
1.2.1 Overview of Insulin Action

Insulin primarily acts in the peripheral tissues including the liver, skeletal muscle, and adipose tissue to regulate energy utilization and storage (Wilcox, 2005). In the fed state, insulin is released from the pancreatic beta cell to act on these tissues and promote glucose uptake and the synthesis of lipids, proteins and carbohydrates. Additionally, it acts to suppress the de novo production of energy substrates that are required for function in the fasting state. Secretion of glucagon, the counter-regulatory hormone of insulin that is produced by the pancreatic alpha cells and acts to promote gluconeogenesis (Habegger et al., 2010), is also suppressed by insulin secretion (Cooperberg and Cryer, 2010).

1.2.1.a Insulin-Stimulated Glucose Uptake

Insulin primarily stimulates glucose uptake into two main depots; skeletal muscle and adipose tissue where it can be oxidized for energy generation or stored for future utilization (Leney and Tavare, 2009). In these tissues, insulin acts through the insulin receptor (IR) to activate a downstream signaling cascade, ultimately inducing glucose uptake through potentiation of the translocation of the glucose transporter GLUT4 to the plasma membrane.

The insulin receptor is a receptor tyrosine kinase that acts to phosphorylate itself and multiple ligands upon insulin stimulation. The canonical insulin signaling pathway (Figure 1) is mediated through phosphorylation of insulin receptor substrate 1 (IRS-1), leading to activation of phosphatidylinositol (PI) 3-kinase (PI3K) and its localization at the membrane (Taniguchi et al., 2006). This activated enzyme generates a lipid product phosphatidylinositol 3,4,5-triphosphate (PIP₃), which accumulates to recruit the serine/threonine kinase PDK1. It is ultimately this kinase that phosphorylates and activates several critical downstream mediators including Akt2. Combined activity of these proteins regulate GLUT4 translocation from cytosolic vesicles to the plasma membrane, potentiating glucose uptake into muscle and adipose (Chang et al., 2004), as well as mediate suppression of lipolysis and glucose production from the adipose and liver (Choi et al., 2010).
Activation and membrane localization of Akt2 in particular is required for the translocation and activity of GLUT4 (Leney and Tavare, 2009). This has been well established through multiple interventions including modulation of Akt2 expression and activity. Overexpression of dominant negative forms of Akt2 (Cong et al., 1997; Wang et al., 1999), knockdown/knockout experiments (Katome et al., 2003), neutralizing antibodies against Akt2 (Hill et al., 1999), and small molecule inhibitors (Gonzalez and McGraw, 2006; Green et al., 2008) demonstrate a lack of GLUT4 translocation and insulin-stimulated glucose disposal. Inhibition of glucose uptake in Akt2 knockout mice is so severe, in fact, that mice develop insulin resistance and overt diabetes (Cho et al., 2001; Dummler et al., 2006; Garofalo et al., 2003). Expression of constitutively active forms of Akt2 are able to stimulate GLUT4 translocation and glucose uptake in an insulin-independent fashion, indicating that Akt2 is both necessary and sufficient for insulin-mediated glucose disposal.

**Figure 1. Insulin Signaling Pathway Downstream of Insulin Receptor Activation**
1.2.1.b Inhibition of Glucagon Secretion

Within the pancreatic islets of Langerhans there are multiple endocrine cell types. The majority of the islet is comprised of insulin-producing beta cells (reports vary between 60-80%), while the remainder of cells are divided between glucagon-producing alpha cells (~10%), somatostatin-producing delta cells (~5%), and to a much smaller extent pancreatic polypeptide producing PP cells and ghrelin-producing epsilon cells, which exist only in the fetal pancreas (Kim et al., 2009). The distribution of these cell types vary by species, with murine islets characterized by a core of beta cells surround by an alpha cell shell, and human islets having all cell types distributed throughout the islet (Pisania et al., 2010). In both cases, the endocrine cells are in close proximity with one another, as well as with endothelial cells to facilitate constant monitoring of circulating nutrients and to release hormones into circulation.

While the endocrine hormones have profound effects on distal tissues, they also act in a paracrine fashion to regulate the activity of other endocrine cells types. Somatostatin, for instance, acts as a potent inhibitor of both insulin and glucagon secretion (Weckbecker et al., 2003). In fact, when performing pancreatic clamp studies, somatostatin is infused to eliminate the contribution of the pancreas to the circulating hormone profile (Wu and Ho, 1987). Insulin and glucagon act as counter-regulatory hormones to regulate systemic glucose homeostasis. While insulin is a potent stimulator of glucose uptake and synthesis of lipids, proteins, and carbohydrates for storage in the fed state, glucagon has an opposing effect, and is secreted in the fasting state to potentiate gluconeogenesis and lipolysis to increase circulating free fatty acids and glucose when exogenous nutrients are absent (Habegger et al., 2010). The rapid release of insulin upon refeeding acts to shut down glucagon secretion, such that excess nutrients are not being produced when they are no longer required.

This mechanism of insulin-mediated suppression of glucagon secretion is not well understood. It is unclear if it is insulin itself that has suppressive activity, or whether there may be a role for factors co-secreted with insulin in altering the activity of alpha cells. The primary evidence for direct activity of insulin through the insulin receptor to mediate an inhibition of glucagon secretion comes from treatment of the alpha cell line In-R1-G9
cells with wortmannin, an inhibitor of PI3K. Blockage of PI3K eliminates the ability of insulin treatment to suppress glucagon secretion over time in both static and perifusion secretion assays (Kaneko et al., 1999). This study, however, utilized a cell line and recombinant insulin, which ameliorates many of the physiologically occurring signals that occur upon refeeding. In vivo, there is evidence that insulin acts through the insulin receptor to activate Akt within the alpha cell, facilitating the internalization and silencing of the GABA$_A$ receptor. GABA, which is released from the beta cells under low glucose conditions, binds its receptor to stimulate glucagon release (Xu et al., 2006). Thus insulin may be required to facilitate alternate mechanisms of suppression. The final mechanistic proposal is that factors co-secreted with insulin have the inhibitory effect on glucagon release. The primary candidate for this role is zinc, which is crystallized with insulin within secretory granules and secreted in high concentrations along side insulin (Wijesekara et al., 2009). Studies utilizing STZ-treated rats that lack insulin-producing beta cells show that under hypoglycemic conditions, reducing circulating zinc levels potentiates glucagon secretion independent of changes in circulating insulin (Zhou et al., 2007). This finding, however, remains controversial and many believe that direct insulin action is the primary regulator of glucagon secretion.

1.2.1.c Suppression of Hepatic Glucose Production

A hallmark of T2DM and GDM is a failure of insulin to suppress hepatic glucose production resulting in exacerbated hyperglycemia. During fasting, glucagon secretion stimulates gluconeogenesis from the liver to prevent hypoglycemia. Upon refeeding, insulin acts directly on the liver, as well as indirectly through the other tissue types and through inhibition of glucagon secretion to suppress hepatic glucose production (Lin and Accili, 2011).

The direct effect of insulin to suppress hepatic glucose production is clearly exemplified in a model of liver-specific deletion of the insulin receptor (the liver insulin receptor knockout (LIRKO) mouse). LIRKO mice are glucose intolerant and resistant to the effect of insulin, demonstrating enhanced hepatic glucose production even under hyperinsulinemic conditions (Fisher and Kahn, 2003; Michael et al., 2000). Insulin regulates hepatic glucose production through the canonical insulin-signaling pathway,
resulting in activation of Akt2. Activated Akt2 acts to phosphorylate an essential hepatic transcription factor, FOXO1 (Figure 1), resulting in the sequestering of FOXO1 within the cytosol and preventing its transcriptional activity (I et al., 2015). FoxO proteins are major targets of insulin action, regulating gluconeogenic and glycolytic gene expression, and thus nutrient metabolism in the liver (Eijkelenboom and Burgering, 2013). Inhibition of FOXO1 in particular reduces the transcription of rate-limiting enzymes in the gluconeogenic pathway including G6Pc and PCK1. Genetic ablation of FOXO1 is sufficient to prevent hepatic glucose production even in the absence of the insulin receptor or with inhibition of Akt activity directly (I et al., 2015). Therefore, insulin regulation of hepatic glucose production is mediated by FOXO1 activity.

There is increasing evidence that insulin acts indirectly through the adipocyte and brain to regulate hepatic glucose production. Within the adipocyte, insulin suppresses lipolysis, resulting in a decrease in circulating non-esterified fatty acid (NEFA) and glycerol levels (Choi et al., 2010; Sindelar et al., 1997). In the fasting states, NEFAs are oxidized within the liver to promote gluconeogenesis through the production of ATP, NADH, and acetyl-CoA, all of which activate pyruvate carboxylase a key enzyme in the gluconeogenesis pathway. With the reduction of NEFAs upon insulin stimulation, this pathway becomes inactivated, thus suppressing glucose production. Furthermore, insulin acts on the adipocyte to stimulate the production and secretion of adipokines adiponectin and leptin, which act to regulate insulin resistance through inhibition of hepatic glucose production (Kwon and Pessin, 2013). Thus, insulin may act through adipocytes to indirectly regulate hepatic glucose production.

Several recent investigations have demonstrated that insulin may be a potent regulator of hepatic glucose production through hypothalamic signaling. Infusion of insulin into the hypothalamus directly ameliorates glucose production independently of the systemic action of other hormones, including glucagon (Pocai et al., 2005). Inhibition of insulin signaling, through manipulation of insulin receptor or its downstream targets can prevent this activity, indicating a specificity of this effect. The mechanism of action mediating this effect is less clear, though evidence is increasing for the presence of a hypothalamic-liver axis where stimulation of the hypothalamus initiates signaling through the vagus nerve (Buettner and Camacho, 2008). This activates hepatic STAT3 and suppresses
gluconeogenic genes PEPCK and G6Pase to decrease hepatic glucose production (Girard, 2006). The levels of insulin required and the timing of this effect remain to be elucidated. Overall, insulin acts through multiple mechanisms to control hepatic glucose production.

1.2.1.d Suppression of Lipolysis

In the fasting state, when glucose supply is limited, the production of fatty acids from adipose and liver provides a high-energy fuel source for all metabolic tissues to ensure survival (Jensen et al., 1987). Upon refeeding, however, this process of lipolysis is potently inhibited through the activity of insulin (Choi et al., 2010). Perhaps unsurprisingly, insulin acts through the insulin receptor directly to mediate this process. In addition to activating Akt to stimulate Glut4 translocation and glucose uptake, this activated Akt also acts to regulate cytosolic levels of cyclic-AMP (cAMP), which in turn regulates the activity of essential lipolytic enzyme hormone sensitive lipase (HSL), and perilipin, a lipid-droplet membrane associated protein required for lipid mobilization. Furthermore, insulin activation and cAMP modulation act to activate the master regulatory kinase AMP-activated protein kinase (AMPK), which further suppresses lipolytic activity (Omar et al., 2009).

In the fasting state, glucagon acts though the glucagon receptor, a G-protein coupled receptor (GPCR) to increase cAMP (Hussain et al., 2000). This increase activates protein kinase A (PKA), which phosphorylates and activates both HSL and perilipin. These proteins form a functional complex that facilitate the conversion of diacylglycerol into monoacylglycerol, liberating fatty acids and eventually glycerol into circulation (Duncan et al., 2007). In the presence of insulin, activated Akt phosphorylates and activates phosphodiesterase 3B (PDE), an enzyme responsible for the degradation of cAMP into 5’ AMP. This conversion modulates lipolysis both through reducing cAMP and thus PKA activity, as well as activating AMPK. Activated AMPK directly inhibits HSL activity through phosphorylation of an inhibitory residue at Ser565 (Qiao et al., 2011). This inhibitory activity of AMPK is a potent regulator of lipolysis, as pharmacological activation of AMPK with AICAR is sufficient to block lipogenesis in the presence of glucagon in the adipocyte cell line 3T3-L1 (Hong et al., 2014). Thus, insulin is a potent regulator of lipolysis in adipocytes.
1.2.2. Causes of Insulin Resistance

The activity of insulin is clearly essential for the regulation of whole body metabolic homeostasis. The development of insulin resistance, therefore, is a severe condition with profound consequences including diabetes, cardiovascular disease and significant morbidity and mortality. Interestingly, the pathology of insulin resistance is common among diverse tissue types and is associated with nutrient overload, inflammation, and oxidative stress (Kwon and Pessin, 2013; Shoelson et al., 2006; Wilcox, 2005). The prevalence of insulin resistance increases with BMI and with aging, two conditions that are characterized by the presence of these key insulin desensitizing factors.

1.2.2.a Nutrient Overload

Under conditions of nutrient excess, the adipocyte, the site of lipid storage, becomes overwhelmed leading to excess circulating nutrients, primarily lipid metabolites (Boren et al., 2013). These metabolites are taken up into metabolic tissues such as liver and muscle, where they can be used as energy substrates. However, when the nutrient abundance overwhelms the oxidative capacity of these tissues, the metabolites are stored for future use (Angulo, 2002). This ectopic lipid accumulation and nutrient overload strongly contribute to desensitization of these tissues to insulin through action on the insulin receptor.

When ectopic lipid accumulation occurs in the liver, it is known as steatosis. This disorder is classified as its own disease and can be subdivided into 3 categories: simple fatty liver disease (also referred to as non-alcoholic fatty liver disease (NAFLD)), non-alcoholic steatohepatitis (NASH), and alcoholic fatty liver disease (Angulo, 2002; Bayard et al., 2006; Birkenfeld and Shulman, 2014). NAFLD is closely associated with being overweight or obese, and is defined as when fat comprises at least 5-10% of the liver mass. The World Gastroenterology Organization (WGO) estimates that 30% of the global population has NAFLD, with up to 90% of those with obesity or diabetes having this condition (Chalasani et al., 2012). While NAFLD is benign from the standpoint of hepatic function, in up to 25% of cases it progresses into NASH. NASH is characterized by fatty liver with associated inflammation, and is associated with elevated rates of
morbidity and mortality due to cirrhosis, liver failure, and hepatocellular carcinoma (HCC) (WGO Guidelines, (Farrell and Larter, 2006)). Alcoholic fatty liver disease, on the other hand, is caused by excessive long-term alcohol consumption. It is associated with increased inflammation leading to liver scarring and cirrhosis, and is not associated with BMI.

NAFLD alone is strongly associated with the insulin resistance characteristic of obesity and diabetes (Angulo, 2002). In fact, insulin-sensitive and insulin-resistant obese subjects can be stratified based on muscle and liver lipid accumulation alone (Stefan et al., 2008). When NAFLD occurs in lean subjects, associated with lipid storage disorders, individuals also develop severe insulin resistance and a significantly elevated risk of developing T2DM (Bugianesi et al., 2010). The mechanism underlying NAFLD-induced insulin resistance is associated with increased diacylglycerol (DAG) accumulation, which induces activation of PKCε and results in inhibition of insulin receptor signaling (Birkenfeld and Shulman, 2014). This inactivation of the tyrosine kinase activity of the insulin receptor prevents activation of Akt and thus induces loss of insulin action to suppress gluconeogenesis and lipolysis. This pathway has been elucidated in numerous rodent models of high-fat diet induced NAFLD (Alves et al., 2011; Birkenfeld and Shulman, 2014; Erion et al., 2009; Jornayvaz et al., 2010; Samuel et al., 2007). Interestingly, this pathway is activated after only 3 days on a high fat diet, before alterations in body weight, systemic inflammation, or systemic insulin resistance occur, speaking to the importance of hepatic insulin resistance in regulation of whole body metabolism (Samuel et al., 2007).

1.2.2.b Inflammation and Oxidative Stress

As the adipose tissue expands with increasing weight gain, reductions in blood supply leads to hypoxia, the initiation of adipocyte inflammation and ultimately cell death (Berg and Scherer, 2005; Gustafson et al., 2007). Under these stressed conditions, the adipocytes produce and secrete numerous cytokines and other bioactive molecules including TNFa, leptin, adipnectin, interleukin-6 (IL-6), and resistin (Fain, 2006). Secretion of these factors recruits macrophages to the dying adipocytes. Infiltrating macrophages surround dying adipocytes forming “crown-like structures”, which act as
niche networks for further production and secretion of cytokines. The pro-inflammatory factors act locally within the adipose depots, and enter into circulation where they can act on distal tissues.

Initial studies linking inflammation to insulin resistance determined that increasing circulating TNFa alone was sufficient to induce systemic insulin resistance in rodent models (Hotamisligil et al., 1993). Further studies into the role of cytokines in insulin resistance have demonstrated that cytokines including TNFa and interleukin-1b (IL-1b) act through cell-surface TLR receptors to activate JNK, p38 MAPK, and IKKb/NF-kB pathways in insulin sensitive tissues (Hirosumi et al., 2002; Nguyen et al., 2005). Activation of JNK and MAPK results in phosphorylation of IRS-1 at Ser302 and S307, which act as inhibitory marks and prevent phosphorylation by the insulin receptor. Further evidence suggests that activation of these kinases results in serine phosphorylation of the insulin receptor directly, blocking its activation to induce insulin resistance. Activation of NF-kB downstream of IKKb also induces insulin resistance indirectly through transcriptional regulation (Shoelson et al., 2006).

Nutrient overload, inflammation, and the process of aging in general are all associated with increased production and decreased clearance of reactive oxygen species (ROS), resulting in oxidative stress. Chronic oxidative stress activates a number of the same stress-response pathways as chronic inflammation, including JNK, p38 MAPK, and NF-kB (Evans et al., 2005; Henriksen et al., 2011; Park et al., 2009). Independent activation of these pathways through ROS has been demonstrated in a number of in vitro studies utilizing chronic exposure of isolated muscle to hydrogen peroxide (Archuleta et al., 2009). Interestingly, this treatment was associated with IRS degradation, further exacerbating insulin resistance above inhibitory phosphorylation associated with inflammation.

A number of therapeutics have been proposed to treat insulin resistance that reduce inflammation and oxidative stress. Use of anti-inflammatory agents that target the NF-Kb pathway including salicylates and the NF-kB-specific agent Celastrol have been shown to reduce systemic insulin resistance, even in genetic induced obese models (Kim et al., 2013; Netea et al., 2001). Clinical trials of high-dose salicylate treatment showed
improvements in in vivo glucose and lipid homeostasis in T2DM patients, supporting the use of this approach for the treatment of diabetes. Conversely, approaches aimed at targeting ROS directly have proven less successful. Antioxidants are widely available for the prevention of oxidative stress, including potent dietary sources including polyunsaturated fatty acids (PUFAs) (Richard et al., 2008). Interestingly, however, clinical trials using potent antioxidant agents have been largely unsuccessful (Golbidi et al., 2011). Several large-scale trials have demonstrated no significant impact of antioxidant treatment on glucose homeostasis, suggesting that direct targeting of the molecular pathways, such as NF-kB by salicylates, is required for therapeutic effect.
1.3 BETA CELL FUNCTION IN THE PATHOGENESIS OF DIABETES

Ultimately it is the inability of the β cell to respond to changing metabolic demands that leads to the development of T2DM and GDM. This is clearly exemplified by the large percentage of so-called “metabolically healthy” obese individuals with normal glucose tolerance in spite of substantial fat mass and insulin resistance. In these individuals, the pancreatic beta cells have increased insulin secretion to accommodate for increased demand, thus preventing DM development.

1.3.1 Beta Cell Nutrient-Sensing and Insulin Secretion

1.3.1.a Glucose-Stimulated Insulin Secretion

The pancreatic β cell has a unique ability to sense glucose and other nutrients and secrete insulin appropriately in response. Glucose-stimulated insulin secretion (GSIS) occurs in two phases: the triggering phase (1st), which is in direct response to increased blood glucose (Figure 1), and the amplification phase (2nd), which potentiates secretion until blood glucose levels return to normal (Gerich, 2002) (Figure 2). In the 1st phase, glucose enters into the β cell through glucose transporters and is metabolized primarily in mitochondria. Increased flux through the electron transport chain facilitates generation of ATP, resulting in an increase in the cytosolic ATP:ADP ratio. An increase in ATP stimulates closure of ATP-sensitive potassium channels (K_ATP), halting efflux of K+ ions to initiate plasma membrane depolarization. Voltage-dependent calcium channels (VDCC) sense this change in potential, stimulating their activity promoting Ca^{2+} influx to trigger the release of insulin (MacDonald et al., 2005; Schuit et al., 2001).

1.3.1.b The Incretin Effect

Glucose-stimulated insulin secretion, however, is not sufficient to maintain blood glucose levels in the narrow range required. Several signals both internal and external to the β cell act to amplify insulin secretion in the 1st phase, and to potentiate secretion in the 2nd phase. The most prominent potentiation of insulin secretion occurs through the so-called “incretin effect” (Kazafeos, 2011). As nutrients enter the gut following feeding, they are
sensed by enteroendocrine K- and L-cells which represent approximately 1% of all the cells in the duodenum, ileum, and jejunum (Ezcurra et al., 2013). These cells are stimulated to release incretin hormones Gastric Inhibitory Peptide (GIP) and Glucagon-Like Peptide-1 (GLP-1), respectively, which act to modulate the whole-body response to nutrient consumption. In the beta cell GIP and GLP-1 act through cell-surface G-protein coupled receptors (GPCRs) to increase cAMP, altering cytosolic Ca2+ concentrations and potentiating the glucose-stimulated insulin secretion response (Mudaliar and Henry, 2010). Incretin hormones also have extra-pancreatic effects including regulation of gastric emptying and satiety, to regulate the increase in blood glucose levels (Drucker, 2006). Due to the glucose-dependence of the incretin effect, several medications now target the GLP-1 receptor (GLP-1R) or extend the half-life of the incretin hormones in circulation as therapeutics to increase insulin secretion with a lower risk of hypoglycemia compared to more traditional insulin secretagogues (Drucker, 2011).

**Figure 2. Schematic Diagram of Glucose-Stimulated Insulin Secretion**
1.3.1.c Non-Glucose Nutrient Signals

Aside from hormonal cues, several nutrient factors in addition to glucose regulate insulin release from beta cells. Long-chain free fatty acids (FFAs), for example, can activate membrane GPCRs, causing increased cytosolic cAMP and subsequently Ca\(^{2+}\) concentrations and leading to insulin secretion (Mancini and Poitout, 2013). In addition, FFAs can also enter the β cell to be metabolized (Nolan et al., 2006). FFAs are broken down into fatty acyl-CoA, which has been proposed to facilitate closure of K\(_{\text{ATP}}\) channels, promote granule fusion, and feed mitochondrial metabolism to generate ATP, all of which promote insulin exocytosis (Prentki and Madiraju, 2012). Similarly, amino acids such as L-alanine can also be taken up by the β cell and metabolized to generate ATP, resulting in insulin secretion (Newsholme et al., 2005), while others stimulate insulin secretion by other means (Henquin, 2011). Overall, the complexity of insulin secretion means that many steps between nutrient sensing and secretion are susceptible to failure, resulting in impaired insulin secretion and diabetes development (Barroso et al., 2003; Buschard et al., 2006; Del et al., 2005; Gloyn et al., 2004; Laukkanen et al., 2005; Smith et al., 2007; Tammaro et al., 2008; Unger, 1991; Webster et al., 2008; Zhao et al., 2005; Zhao et al., 2008).

1.3.2 Evolution of Beta Cell Function

Fluctuations in insulin demand naturally occur throughout the human lifespan. Difference in diet and activity level, fluctuations in weight, aging, and pregnancy all significantly impact insulin sensitivity. The beta cell must therefore be able to compensate for periodic increases in demand in order to maintain tight regulation over glycemia. Under persistent conditions of insulin resistance, as occurs in the pathological progression to diabetes, beta cell compensation becomes insufficient, leading to adaptation, decompensation, and ultimately failure.

1.3.2.a Compensation in T2DM

Upon the induction of insulin resistance the pancreatic beta cells compensate through increases in overall levels of insulin secretion in both fasted and fed states, as well as an
amplified response to an acute glucose load (Weir and Bonner-Weir, 2007). These increases are mediated through multiple mechanisms. Rodent models and autopsy studies of human tissue donors suggest that much of the compensation is mediated by increases in beta cell mass (Bouwens and Rooman, 2005). This is likely due to increases in both beta cell number, as well as beta cell hypertrophy. Monitoring of islet cell proliferation in mice fed a high fat diet to induce obesity using BrdU treatment, a uracil analog that incorporates into newly-synthesized DNA, reveal a significant increase in beta cell proliferation under insulin resistance conditions (Golson et al., 2010; Stamateris et al., 2013). Furthermore, in genetic models of obesity and insulin resistance such as the leptin knockout mouse (ob/ob), islet size is dramatically increased compared to lean littermate controls (Bock et al., 2003). Finally, examination of beta cell mass in pancreata isolated from human donors have revealed a correlation between increased BMI and beta cell mass when patients maintain normal glucose tolerance (Saisho et al., 2013). Therefore, expansion of beta cell mass is a key compensatory response to maintain euglycemia under insulin resistant conditions.

In addition to increased beta cell mass, it has been proposed that beta cell function also increases in response to insulin resistance. Total insulin content of pancreatic islets increases, suggesting increased insulin biosynthesis (Weir and Bonner-Weir, 2007). This is further supported by increased expression of chaperone proteins critical for proper insulin processing and secretion (Lipson et al., 2006). In addition, some studies have suggested that beta cells amplify their response to incretin hormones and other glucose and non-glucose nutrient signals, including fatty acids and amino acids. This amplification of insulin secretion prevents spikes in blood glucose levels. Indeed, an amplified response to the incretin hormone GLP-1 likely acts to mediate other compensatory responses including anti-apoptotic and proliferative effects to permit increases in beta cell mass (Prentki and Nolan, 2006).

### 1.3.2.b Compensation in GDM

In pregnancy insulin resistance typically remains unchanged until 24-26 weeks gestation, at which point there is a rapid and dramatic increase that persists until delivery. Indeed, by late pregnancy the extent of insulin resistance is approximately equal to that observed
in T2DM (Buchanan and Xiang, 2005a). To overcome this, the pancreatic β cells dramatically increase insulin secretion extremely rapidly to produce only minor changes in blood glucose levels (Buchanan and Xiang, 2005a). Similar to T2DM, this compensation is mediated by increases in both β cell mass and function (Sorenson and Brelje, 1997). Various factors unique to pregnancy are thought to be responsible for these rapid increases. Placental hormones prolactin and placental lactogen, as well as the tryptophan metabolite serotonin (Kim et al., 2010) have been strongly associated with inducing beta cell mass expansion. Rodent models of serotonin depletion have been used to model GDM due to the severe deficiency in beta cell mass expansion. Additionally, placental lactogen has also been associated with increasing β cell function, mediating a decrease in the threshold glucose concentration required for glucose-stimulated insulin secretion from 5.7mM to 3.3mM in rats, thus preventing hyperglycemia (Parsons et al., 1992). Interestingly, these changes are also rapidly reversible, with beta cell mass and function returning to normal shortly following delivery.

1.3.3 Beta Cell Decompensation

While beta cell compensation can persist for the entire life course for some overweight and obese insulin resistant individuals, chronic exposure to worsening insulin resistance exhausts the beta cell compensatory capacity in subjects who go on to develop prediabetes and overt diabetes. In these individuals, blood glucose levels begin to rise in the fasting state, indicative of an impairment in insulin secretion (Weir and Bonner-Weir, 2004). Indirect measures of β cell function such as the Insulin-Secretion Sensitivity Index-2 (ISSI-2) score, or homeostatic model assessment of β-cell function (HOMA-B) in human patients reveal that the decline in beta cell function correlates with increasing blood glucose levels (Festa et al., 2013). This is also exemplified in women with GDM who have significantly diminished β cell function compared to normal glucose tolerant (NGT) controls (Retnakaran et al., 2010; Xiang et al., 2010).

1.3.3.a Loss of First Phase Insulin Secretion

The first defect that occurs in beta cell function is the loss of 1st phase insulin secretion (Gerich, 2002). This has been noted in numerous animal models, as well as in human
subjects undergoing oral glucose tolerance testing. Remarkably, the loss in 1st phase insulin secretion occurs within an extremely narrow range of fasting plasma glucose levels. If fasting plasma glucose levels remain below 5.6mmol/l, subjects exhibit normal glucose-stimulated insulin secretory responses (Brunzell et al., 1976; Weir and Bonner-Weir, 2004). However, once fasting blood glucose levels raise above this point, acute glucose-stimulated insulin secretion becomes dramatically impaired, and become almost absent once fasting glucose levels reach 6.4mmol/l. Importantly, this loss of function remains specific to the 1st phase, with maintenance of 2nd phase and non-glucose insulin stimulus responses until later stages of disease progression.

In spite of relatively minor changes in glucose tolerance in this early decompensation phase, the beta cell exhibits significant changes in morphology and gene expression. The most obvious morphological change is the observed beta cell hypertrophy. While the compensatory period is primarily mediated by beta cell proliferation, the hypertrophy of the decompensation period is likely caused by a persistence of growth-stimulating signals (such as hyperglycemia) combined with an inhibition of proliferation (Bouwens and Rooman, 2005; Cnop et al., 2005). Furthermore, the beta cells exhibit changes in gene expression related to elevated glucose levels and inflammatory signaling (Laybutt et al., 2001). These are primarily in genes regulating glucose shunting (alternate glucose utilization pathways aside from glycolysis), antioxidants, and apoptotic genes (Kaneto et al., 2001; Laybutt et al., 2002). These alterations in expression set the stage for further declines in beta cell function and development of overt diabetes, with ~11% of prediabetic individuals progressing to T2DM per year.

1.3.4 Beta Cell Failure

The transition from the prediabetic period of beta cell decompensation to overt beta cell failure and diabetes occurs surprisingly quickly. This is particularly apparent in the pathogenesis of GDM in which development of insulin resistance only preceeds a diabetes diagnosis by a few weeks. This suggests that there likely exists a “tipping point” of beta cell dysfunction where compensation is no longer feasible. The underlying cause of this tipping point remains controversial (Matveyenko and Butler, 2008). Some argue that it is due to inadequate beta cell
mass. This may be due to chronic beta cell apoptosis induced by glucolipotoxicity, or potentially ER stress that also activates an apoptotic response (Donath and Halban, 2004). Alternately, there is evidence that beta cell mass may be maintained in many diabetic individuals, suggesting that some stress factor alters beta cell functionality to prevent insulin secretion and resulting in diabetes development (Meier and Bonadonna, 2013; Rahier et al., 2008).

1.3.4.a Glucolipotoxicity

Free fatty acids (FFAs) are elevated in both GDM and T2D, due to increased adipose tissue mass, reduced FFA clearance rates, and insulin resistance, which prevents the anti-lipolytic activity of insulin, resulting in inappropriate lipolysis and further increases in circulating FFA levels (Boden and Shulman, 2002). In T2D, it has been demonstrated that chronic exposure to these conditions result in glucolipotoxicity, a condition of inappropriate accumulation of unoxidized FFAs in non-adipose tissues including muscle, liver and the beta cell, in combination with hyperglycemia (Poitout and Robertson, 2008). Beta cells are particularly vulnerable to glucolipotoxicity due to their limited ability to store triacylglycerol, which results in the accumulation of toxic metabolites including ceramides and sphingomyelins (Fontes et al., 2010; Poitout et al., 2010). These lipid species impair mitochondrial function, particularly glucose oxidation, in addition to increasing reactive oxygen species (ROS) production and inducing oxidative stress (Barlow and Affourtit, 2013). Again, the beta cell is particularly vulnerable to this assault due to a limited antioxidant capacity. Oxidative stress not only induces apoptosis, but also decreases insulin transcription due to altered transcription factor binding. ROS impairs autocrine insulin signaling, causing nuclear exclusion of the critical beta cell transcription factor PDX1 and preventing transcription of insulin and insulin processing genes, glucose transporter, and glucokinase, the rate-limiting enzyme in glycolysis (Kawamori et al., 2003). Overall, elevations in plasma FFAs cause decreased glucose metabolism and reduced insulin gene transcription, resulting in impaired insulin biosynthesis and dysregulated insulin secretion. (Fontes et al., 2010; Poitout et al., 2010; Poitout and Robertson, 2008).
1.3.2.b  ER Stress

In order for compensation to occur, the beta cell must significantly increase insulin biosynthesis and secretion to meet increased demand. This increased synthesis places significant stress on the endoplasmic reticulum (ER), which is responsible for the synthesis and folding of secreted proteins (Fonseca et al., 2009; Fonseca et al., 2007). As demand increases, there is increased production of misprocessed proteins, which must be re-folded or signaled for degradation. As the number of misfolded proteins increases, a feedback mechanism involving the ER luminal domain of several proteins (IRE-1, PERK and ATF6) become activated, triggering the unfolded protein response (UPR) to increase production of chaperones and protein processing enzymes to aid in appropriate folding. If activation of the UPR is insufficient to overcome the ER stress, the cell attenuates translation and slows mRNA degradation to decrease protein load, while simultaneously increasing expression of proteins critical for autophagy to promote clearance of misprocessed proteins (Osorio, 2014). This decrease in translation and protein production decreases insulin supply, causing a worsening in glucose tolerance and further inducing hyperglycemia and glucoxipotoxicity. The final stage of ER stress involves hyperactivation of ATF6, which acts to suppress insulin gene expression and induce apoptosis (Seo et al., 2008).
1.4 UTILIZING –OMICS APPROACHES TO UNDERSTAND DIABETES

While obesity and the associated insulin resistance is one of the strongest risk factors for the development of diabetes only about 30% of overweight and obese individuals have the disease. It remains unclear why only a subset of obese, insulin resistant individuals experience beta cell failure and progress to diabetes. With the development of high-throughput “-omics” platforms, it is now possible to perform unbiased global screening in large and diverse populations to identify potential risk factors and/or causal agents in the development of diabetes. Use of genomics and proteomics enable the examination of the genetic contribution to diabetes development, while metabolomics reveals the intersection of genetics and environment. Beyond identification of causal agents, these platforms hold promise in enabling the prediction of future disease risk allowing for intervention before disease onset to prevent complications.

1.4.1 Genomics

Following the success of the human genome project, several studies have explored the possibility of a common underlying genetic cause of T2DM through genome-wide association studies (GWAS). Diverse populations representing multiple ethnicities have been screened to identify genetic loci related to diabetes risk. Surprisingly, given the strong heritability of T2DM, GWAS have been largely unsuccessful in identifying genetic causes of diabetes. While dozens of genes have been identified as linked to diabetes risk, no data to date supports the use of genetic screening for diabetes risk, as genomics only account for ~10% of T2DM cases (Lyssenko and Laakso, 2013; Sun et al., 2014). The greatest risk factor allele, an SNP in TCF7L2, only increases susceptibility by ~17%, and is only found in an estimated 2-7% of the population (Luo et al., 2009).

1.4.1.a Genetics of Beta Cell Dysfunction

To identify potential risk loci associated with T2D, a large number of GWAS studies have been performed in various populations (Wheeler and Barros, 2011a). These studies have identified 44 independent loci that show genome-wide significant association with T2D. Of the 44, only two genes, PPARg and KCNJ11 show robust association with T2D.
susceptibility in all populations (Wheeler and Barroso, 2011a). These two genes represent the two main components of T2DM, with PPARg regulating lipid accumulation and insulin resistance, and KCNJ11, a K_{ATP} channel, regulating insulin secretion. Interestingly, the vast majority of genes identified as associated with T2DM regulate beta cell function and insulin secretion (Florez, 2008).

1.4.1.b Genomics of GDM

Interestingly, no studies have been conducted to specifically investigate the heritability of GDM using familial clustering (Kwak et al., 2012). This is likely due to the difficulty in performing prospective studies in anticipation of pregnancy and retrospective studies post-partum (Kwak et al., 2012; Watanabe et al., 2007). However, due to the strong relationship between GDM and T2D it is likely that these diseases have a common genetic origin. The heritability estimate of T2D is quite high ($h^2=0.62$), and women with GDM have a significantly greater parental history of T2D compared to NGT controls (13.2% vs. 30.1%, $p<0.001$) (Kwak et al., 2012). A small-scale GWAS study conducted in a Korean population to identify risk factors for GDM revealed that genetic variants associated with T2D were also enriched in GDM subjects, further supporting a similar genetic background between these diseases (Kwak et al., 2012). Although GWAS are instrumental in identifying novel disease genes, loci identified using this method only account for approximately 10% of familial clustering of T2D in European populations (Wheeler and Barroso, 2011b), suggesting environmental factors are likely critical in the pathogenesis of T2D.

1.4.2 Proteomics

Differences in genetic sequence may or may not have an impact on protein expression and functionality. Therefore, examination of protein abundance is a step closer to phenotypic function and therefore may be more causally related to diabetes development. Proteomics can be used to examine the proteins expressed in specific tissue types, as well as in circulation, in a quantitative or semi-quantitative manner (Altelaar et al., 2013). Given the prominent role of beta cell dysfunction in the development of diabetes, it is likely that there is differential protein
abundance in the beta cell during the pathological progression of disease. While this provides valuable insight into the causes of diabetes, beta cell proteomics would be impossible to quantify in human patients at risk for diabetes development. Thus, quantification of circulating proteins may be used to both predict diabetes development, but may also play a causal role in diabetes development as mediating signals between tissues.

1.4.2.a Tissue Proteomics

Proteomic studies investigating islets from T2D and normal glucose tolerant donors have confirmed previous reports regarding the progression of diabetes development. A 2009 study by Nyblom and colleagues (Nyblom et al., 2009) investigating the proteome of islets from human T2D and control donors showed that inflammatory response, caspase-mediated apoptosis, islet regeneration, and proliferation pathways were all activated in T2D islets compared to controls. This is consistent with the pathways activated in insulin resistance and beta cell failure in murine models of diabetes development. In mice, proteomics has been used to uncover novel proteins that may be involved in regulating islet function in T2D. It was in a proteomics screen comparing islets from diabetic ob/ob mice to lean controls that it was discovered that carboxypeptidase E (CPE) upregulation was associated with improved islet function (Sanchez et al., 2002). Further investigation revealed that CPE is decreased in models of T2D and is linked to β cell dysfunction and cell death associated with lipotoxicity (Jeffrey et al., 2008). These types of studies show the potential of proteomics to enhance our understanding of beta cell dysfunction in T2D. However, most proteomics studies are focused on overt diabetes, which may miss key proteins linked to diabetes onset (Liu et al., 2009; Maahs et al., 2010), or are performed in obese models where it is difficult to discern biomarkers of obesity from those used to predict diabetes (Han et al., 2011).

1.4.2.b Plasma Proteomics

The field of plasma proteomics is rapidly advancing with advances being made in sensitivity of detection. The primary challenge with plasma proteomics is that only 50 proteins represent more than 90% of the spectral counts from total plasma proteins (Tu et al., 2010). Thus when total plasma proteins are quantified, peptides from this small subset
of proteins overwhelm the system, interfering with the signal from the vast majority of the lower abundance proteins. Many studies have used a depletion protocol to remove the most abundant proteins and allow detection of the remaining ones. However, many lower abundant proteins have strong binding affinity for albumin, the most highly abundant plasma protein and are thus also removed from the analysis (Granger et al., 2005). Newer techniques allow for the quantification of thousands of proteins, including those of extremely low abundance, without the use of depletion columns (Harel et al., 2015). Therefore, significant advances in plasma proteomic techniques are likely imminent.

A limited number of existing studies have generated promising data in spite of the technical limitations of this approach. Consistent with dyslipidemia in the diabetic condition, several studies have identified altered abundance of apolipoproteins, key proteins for the transportation of lipids in the plasma. Apolipoprotein-A1 has been found to be increased in the plasma of Chinese T2DM patients (Liu et al., 2009), while in an independent study Apolipoprotein H was decreased in T2DM patients who had an elevated 1st phase insulin secretion compared to controls (Sundsten et al., 2008). A third study found increases in Apolipoprotein C1, as well as angiotensinogen, which correlates with diabetes and hypertension and may be a factor in the increase risk of heart disease that comes with a diabetes diagnosis (Dayarathna et al., 2008). While several changes have been identified, they are all consistent with known complications of T2DM, and thus have unfortunately not provided significant insight into causes of T2DM development.

1.4.2.c Proteomics of GDM

There is a need to diagnose GDM early in order to prevent associated complications; therefore, many studies have focused on the identification of early biomarkers. There is strong support for the idea that factors found in the plasma during early pregnancy will have strong predictive powers for the future risk of GDM development, however the acute nature of the disease and the ethical complications associated with fasting during pregnancy, make studies looking for predictive biomarkers difficult (Savvidou et al., 2010). Previous studies have used limited panel screens investigating hormones and select proteins to determine their predictive power. Hyperinsulinemia, decreased
adiponectin and decreased sex-hormone binding globulin (SHBG) in the plasma have shown the strongest predictive power when measured at 10-11 weeks gestation for development of GDM at week 28 (Buchanan and Xiang, 2005b; Georgiou et al., 2008; Nanda et al., 2011; Retnakaran et al., 2005; Williams et al., 2004). However, expression of these proteins was variable and differences between GDM and controls were small (Georgiou et al., 2008; Nanda et al., 2011). Additionally, differences in expression of adiponectin and SHBG only moderately increased the rate of accurate prediction of future GDM development from 61.6% based on maternal factors such as BMI, previous GDM, family history of diabetes and ethnicity, to 74.1% when combined with these maternal factors. Expression of adiponectin and SHBG can also be decreased by hyperinsulinemia, suggesting these factors may be markers of GDM rather than causative of it (Miehle et al., 2012; Sun et al., 2012). Retinol-binding protein 4 (RBP4) has also been shown to be significantly elevated in GDM compared to NGT pregnant controls (Choi et al., 2008; Klein et al., 2010). However, studies attempting to link elevated RBP4 to prediction of GDM development or to insulin resistance in general have shown no significant correlation (Abetew et al., 2013). Therefore, while many studies have investigated circulating protein markers that could be predictive and/or causative of GDM, none have proven any significant relationship.

1.4.3 Metabolomics

Metabolomics is a rapidly advancing field that provides a snapshot of physiological processes through quantitative investigation of the byproducts of metabolism (Oliver et al., 1998; Tweeddale et al., 1998). Over the last decade, studies utilizing metabolomics for the investigation of biological systems, disease processes, and biomarkers have increased exponentially with publications in high impact journals (Patti et al., 2012).

1.4.3.a The Advantage of Metabolomics

While traditional “-omics” approaches such as proteomics and genomics are powerful tools for the prediction and characterization of a disease, metabolomics represents the collection of final downstream products of the interaction between genes and influences
like environment, health, pharmaceutical interventions, and the activity of protein pathways (Friedrich, 2012). Metabolomics enables the detection of short- and long-term physiological or pathological changes in cells, tissues or body fluids, making this the ideal technique for investigating multifactorial diseases such as diabetes. As both GDM and T2DM are characterized by alterations in nutrient abundance, which can have direct effects on β cell function and survival, it is not surprising that metabolomics profiling has revealed several significant differences between diabetic and NGT patients. There have been a few studies that have linked changes in metabolites to obesity (Newgard et al., 2009), glucose tolerance (Wopereis et al., 2009), and T2DM (Adams et al., 2009; Fiehn et al., 2010; Suhre et al., 2010).

Unlike mass spectrometry-based proteomic approaches, metabolite identity cannot be predicted based on fragmentation patterns (Shen et al., 2014). Thus, a significant limitation to metabolomics is in only being able to identify known compounds. This technology is rapidly advancing, however, with databases expanding from hundreds of metabolites to thousands within the last decade. Additionally, the use of stable-isotope labeled metabolites enables absolute quantification of metabolite abundance. This remains a significant limitation of proteomics approaches, as discussed above.

1.4.3.b Metabolomics of Diabetes

Several metabolomics studies have been performed on various populations of obese, insulin resistant and/or diabetic individuals compared to matched controls (Bain et al., 2009; Friedrich, 2012; Suhre et al., 2010; Wang-Sattler et al., 2012). Overall, many of the altered metabolites reflect the inactivity of insulin including elevated levels of free fatty acids, due to the loss of the antilipolytic activity of insulin, as well as increases in carbohydrate species due to impaired insulin-stimulated uptake. Furthermore, several studies have associated levels of specific fatty acid species with diabetes development. Of particular interest is a striking elevation of poly-unsaturated fatty acids (PUFAs), which are essential fatty acids with distinct antioxidant properties (Prentice et al., 2014; Suhre et al., 2010). Their elevation suggests a potential increase in membrane breakdown, a potential consequence of increased inflammation associated with obesity and diabetes. Interestingly, one of the earliest alterations in the metabolite profile of insulin resistant
and prediabetic individuals is a significant increase in the branched chain amino acids (BCAAs) leucine, isoleucine and valine (Newgard et al., 2009; Wang et al., 2011). This robust, and highly reproducible result suggests that increases in BCAAs may in fact play a causal role in the induction of insulin resistance due to the extremely strong correlation. A study using dietary supplementation of BCAAs suggests that this may in fact be the case. Supplementing a high fat diet with BCAAs exacerbated obesity-induced insulin resistance (Newgard et al., 2009). A similar study has been performed to explore the relationship between another metabolite increased in diabetes, 2-amino adipic acid (2-AAA), and glucose intolerance (Wang et al., 2013). After finding a significant increase in 2-AAA levels in the plasma of diabetic patients in northern Europe, researchers examined the effect of the metabolite on glucose-stimulated insulin secretion from isolated islets. Consistent with a potential causal role in diabetes development, treatment with 2-AAA impaired insulin secretion in vitro and glucose tolerance in mice treated chronically with the metabolite. Overall, metabolites differentially regulated in the progression of diabetes may play a causal role in diabetes development in addition to being potential biomarkers of disease development.

1.4.3.c Using Metabolites to Predict Diabetes

Applying metabolomics to predict and diagnose diabetes has recently emerged on the commercial market. The metabolomic service Metabolon Inc. has developed two distinct assays for the detection of insulin resistance (IR) and the prediction of impaired glucose tolerance (IGT) in human subjects (Cobb et al., 2013; Tripathy et al., 2015). Based on global metabolomics profiling of several large human populations, they identified a panel of 5 metabolites that were consistently altered in insulin resistance. This panel, called Quantose IR, is able to detect insulin resistance to a comparable extent as a hyperinsulinemic euglycemic clamp study in human patients, which is the current gold standard (Cobb et al., 2013). This is a major advancement over the clamp technique, as it is highly sensitive, absolutely quantitative, and uses only one small blood sample of <1ml. These are significant improvements as compared to the clamp technique that requires overnight hospitalization, infusion with radiolabeled tracers, and constant blood sampling over multiple hours (Tam et al., 2012).
Most individuals with insulin resistance have no abnormalities in glucose metabolism, making detection of insulin resistance relatively inconsequential for the prediction of diabetes. An advantage of the metabolomics platform is that diagnostic panels can be readily modified to change the metabolites of interest for better prediction of other disease states. Using the Quantose IR panel as a starting point, Metabolon modified the panel by adding and additional 4 metabolites to improve the assay for detecting IGT (Tripathy et al., 2015). The recently introduced Quantose IGT panel is highly sensitive for detecting impaired glucose tolerance, again compared to the clinical gold standard oral glucose tolerance test which is time consuming and highly unpleasant for patients. Thus the use of metabolomics for the prediction and diagnosis of diabetes and insulin resistant states is an emerging field that has great promise. Identification of new and more sensitive biomarkers will increase the applicability of these assays to diverse populations and improve healthcare at reduced cost and with shorter hospital visits for patients.
1.5 3-CARBOXY-4-METHYL-5-PROPYL-2-FURANPROPANOIC ACID

In our initial global metabolomics profiling of diabetic human plasma we identified several differentially abundant metabolites as compared to normal glucose tolerant controls, consistent with previous reports. Our study, however, identified one novel metabolite as being highly significantly increased in both GDM and T2DM. 3-Carboxy-4-Methyl-5-Propyl-2-Furanpropanoic acid (CMPF) was increased 5-10 fold in GDM and T2DM populations of mixed age, ethnicity, gender, and geographical location (Prentice et al., 2014). CMPF had never been associated with diabetes development before our identification; however, it was previously identified as a potential uremic toxin due to its extreme elevation in the plasma of uremic patients (Miyamoto et al., 2012; Sassa et al., 2000).

1.5.1 CMPF Generation

CMPF is thought to be the end product of furan fatty acid (furan FA) metabolism. Furan fatty acids are characterized by a furan ring with a fatty acid side chain of 9-13 carbons in length, a 3-5 carbon alkyl group, and one or two methyl groups (Spiteller, 2005). These species exist as two moieties, the pentyl alkyl moiety and the propyl alkyl moiety, the latter of which includes CMPF and its parent species (Nolan, 2014; Spiteller, 2005). It is largely unknown if these lipid species can be produced endogenously by mammals, and furthermore, the enzymes responsible for the generation of CMPF are currently unknown. However, it is apparent that furan FAs can be incorporated into phospholipids, cholesterol esters, and triglycerides in the mammalian body (Spiteller, 2005). Liberation of these fatty acids due to membrane turnover or breakdown, or altered lipid processing, may therefore cause an increase in CMPF generation. Alternately, furan FAs are largely thought to be diet derived. While pentyl alkyl furan FAs are relatively abundant in food, predominantly fruits and vegetables as well as fish to some extent, the propyl alkyl moiety that gives rise to CMPF are not as abundant, making its origin less clear. These lipids have been shown to be produced algae and accumulate in fish (Hannemann et al., 1989). Indeed, circulating levels of CMPF correlate with fish consumption in a recent global metabolomics study of a large US population (Guertin et al., 2013). However, levels seen with dietary differences are significantly lower than would be required for the concentrations of CMPF observed in human circulation under pathogenic conditions (Nolan, 2014). Further, high-temperature cooking of PUFAs produces furanoid compounds that could be converted into
CMPF, potentially representing a further source, however this remains to be determined (Berdeaux et al., 2012). Intriguingly, CMPF is produced by some species of bacteria, giving rise to the idea that CMPF may be generated by the gut microbiome (Nolan, 2014).

1.5.2 CMPF Clearance

While it is possible that the elevation in circulating levels of CMPF is due to increased generation or increased consumption of furan FAs in the diet, it may also be the case that CMPF becomes elevated due to impaired clearance. The strongest evidence for this is the fact that CMPF was originally identified as a potential uremic toxin due to its extreme elevation in the serum of individuals with chronic kidney disease (CKD) and uremia (Niwa et al., 1988). These individuals have a significant loss of the transport proteins responsible for clearance of solutes from the blood, leading to an elevation in numerous metabolites, including CMPF. Numerous studies have demonstrated a 10-fold or more increase in circulating CMPF levels in patients with CKD undergoing dialysis (Itoh et al., 2012; Niwa et al., 1994; Sassa et al., 2000).

1.5.2.a Organic Anion Transporters

Due to this relationship between kidney dysfunction and elevation in CMPF, the majority of studies on CMPF have investigated its role in the kidney. Fairly recently the specific transporters of CMPF were identified as members of the organic anion transport (OAT) family (Deguchi et al., 2006; Deguchi et al., 2004). The OATs function as organic anion exchangers, transporting one anion molecule into the cell and simultaneously transporting one endogenous dicarboxylic acid out of the cell (Sekine et al., 2006). The presence of endogenous dicarboxylic acids within the cell is therefore critical for OAT function, thus they are expressed along with a sodium-dicarboxylate co-transporter, NaDC3 (SLC33A3), which functions to transport dicarboxylic acids back into the cells (Stellmer et al., 2007). Utilizing overexpression models and quantifying uptake of isotopically labeled CMPF it was determined OAT1 (SLC22A6) and OAT3 (SLC22A8), are responsible for the transport of CMPF from the plasma to the proximal tubule cells of the basolateral membrane of the kidney (Deguchi et al., 2005). It is important to note that these transporters are not specific for CMPF, and are responsible for the transport and
excretion of numerous other compounds including hippurate (HA), indoleacetate (IA), indolelactate (IL), indoxyl sulfate (IS) and uric acid. Studies using OAT-specific inhibitors p-aminohippurate (PAH) and benzylpenicillin (PCG) have demonstrated that by blocking both OAT1 and OAT3 the secretion of CMPF can be completely inhibited in vivo. Using concentrations of PCG that selectively inhibit OAT3, 65% of CMPF secretion was prevented, indicating that this is the primary kidney transporter of CMPF.

1.5.2.b Organic Anion Transporters and Diabetes

Multiple studies have been conducted investigating changes in gene expression in various tissue types associated with T2D. The kidney has been of particular interest due to the prevalence of diabetic nephropathy as a severe complication of both T1DM and T2DM. Interestingly, the expression of OAT3 is significantly reduced in the kidneys in rodent models of T2DM (Mishra et al., 2004). In the db/db mouse model of obesity and T2DM, the OAT3 transporter has significantly reduced expression by microarray. Another study investigating kidney gene expression in diabetic FVB/NJ mice at 2, 4 and 8 months of age show a decrease in OAT3 expression that correlates with age, with 4 and 8 month old mice having significantly lower OAT3 at 1.47 and 2.06 fold respectively (both significant at P<0.0001) (Chang et al., 2012). This finding suggests that CMPF levels may be elevated in the plasma of diabetic patients due to a down-regulation of expression of its transporter in the kidney, thus limiting its ability to be secreted in the urine.

1.5.3 CMPF as a Uremic Toxin

Characterization of CMPF has largely been with respect to its role as a uremic toxin due to its elevation in uremia and CKD. CMPF is thought to contribute to uremia through inhibition of drug transport, impaired hepatic metabolism, thyroid dysfunction, as well as through inducing oxidative stress and metabolic dysfunction in the proximal tubule cells of the kidney.

1.5.3.a Albumin Binding of CMPF

In circulation, CMPF is highly albumin bound, with an association constant of $K_a=1.3 \times 10^7$/M that allows it to out-compete binding of other ligands, including all other uremic
toxins that have been investigated (Sakai et al., 1995). This has important consequences for the effective transport of drugs that are highly albumin bound. A study investigating the binding of salicylate, the primary ingredient of aspirin and other pain-relieving medications, showed significant inhibition in the presence of increasing concentrations of CMPF (Niwa et al., 1988). Furthermore, it has been suggested that CMPF displaces binding of endogenous albumin ligands including bilirubin and free fatty acids such as oleate (Lim et al., 1993b; Tsutsumi et al., 2000). Indeed, increasing concentrations of CMPF to 0.4mmol/l, as observed in uremic patients, caused a 35-40% increase in the unbound fraction of $^{14}$C oleate (Lim et al., 1993b). Displacement of these substrates to such a large degree can have significant metabolic effects. In particular, elevation of these compounds can impair transport of thyroid hormone $T_4$ into hepatocytes, lowering the circulating levels of $T_3$, a characteristic of uremia (Lim et al., 1993a; Lim et al., 1995). This impaired transport leads to elevated $T_4$ in circulation, causing further displacement of other families of drugs, as well as thyroid dysfunction due to the perceived hormonal imbalance (Lim et al., 1993).

### 1.5.3.b Induction of Oxidative Stress

One of the key characteristics of uremic toxins is the ability to stimulate reactive oxygen species generation and induce oxidative stress, which contributes to the progression of renal failure (Motojima et al., 2003). Indoxyl sulfate for example, which is also transported through OAT1 and OAT3, has been shown to induce cytokine production through the generation of ROS within proximal tubule cells, contributing to inflammation and cell death (Shimoishi et al., 2007). While indoxyl sulfate is metabolized to induce ROS production, CMPF CMPF acts as a pro-oxidant, interacting with existing superoxide anion radicals and peroxy radicals, produced through mitochondrial metabolism and lipid peroxidation respectively, to form a CMPF radical adduct. This CMPF species subsequently interacts with oxygen molecules and iron to increase ROS through the fenton reaction (Miyamoto et al., 2012). The existing ROS species required for the formation of CMPF radicals are likely formed by other uremic toxins, which have been associated with increased cell death and the progression of renal dysfunction. CMPF alone has not been associated with the induction of cell death or apoptosis in any tissue type.
1.6 HYPOTHESIS

Ultimately it is the inability of the beta cell to respond to changing metabolic demands that leads to the development of both GDM and T2D. The beta cell failure associated with GDM and the progression from GDM to T2D is not well understood and is controversial (Retnakaran et al., 2010). Therefore the overall goal of this research is to examine the factors that may be responsible for the beta cell failure observed in GDM and the progression to T2D. The hypothesis addressed within this thesis is that circulating factors differentially expressed in GDM compared to normal glucose tolerant pregnancies are causally important in the beta cell failure associated with GDM development and progression to T2D. Furthermore, because these factors are differentially abundant in circulation, they may lead to the development of novel early detection methods and new preventative treatment options.

1.7 SPECIFIC AIMS

1.7.1 Aim 1: Use global metabolomics profiling to identify circulating factors associated with GDM and T2DM

Fasting plasma samples from women newly diagnosed with GDM was compared to normal glucose tolerant matched controls. An independent global metabolomics screen was subsequently performed on a population of male patients with T2DM compared to normal glucose tolerant controls. Overall, there were many similar alterations in the two conditions, indicating similarities between the conditions, but also many differences, which may reflect medications, duration of diabetes, and population differences.

1.7.2 Aim 2: Assay Metabolite Effect on Beta Cell Function

From the global metabolomic assays, the furan fatty acid metabolite CMPF was selected for further characterization based on its having the greatest fold change in GDM and also being significantly altered in T2DM. A series of in vivo and in vitro assays were performed investigating the effect of physiologically relevant levels of CMPF on beta cell function under lean and metabolically-stressed conditions, as well as the long term effect of CMPF exposure to
investigate the link between GDM and future T2DM. Glucose tolerance and insulin secretion was evaluated in vivo. Isolated tissues were evaluated by immunohistochemistry. In vitro, islet glucose-stimulated insulin secretion was evaluated. Further mechanistic studies involved functional tests of the stimulus-secretion pathway, quantitative PCR, transcriptome analysis by microarray, immunofluorescent and electron microscope imaging, and western blotting. Pharmacological and genetic inhibition of the CMPF transporter OAT3 was used to elucidate mechanism and represent novel drug targets.

1.7.3 Aim 3: Evaluate Metabolite Effect on Whole Body Glucose Homeostasis

The effect of CMPF on other metabolic tissues and its role in regulating insulin sensitivity was examined. Whole body metabolism was evaluated using MRI imaging and metabolic cages (CLAMs). Insulin sensitivity was evaluated by insulin tolerance test and hyperinsulinemic euglycemic clamps. Closer examination revealed a direct effect on hepatic insulin sensitivity, which was characterized by microarray and metabolomic profiling, quantitative PCR, western blotting, and use of FGF21 knockout mice. Isolated hepatocytes were used to characterize direct vs. indirect effects of CMPF.
Chapter 2- The Furan Fatty Acid Metabolite CMPF is Elevated in Diabetes and Induces Beta Cell Dysfunction

The following chapter is published in *Cell Metabolism*:


Contributions by co-authors to the figures presented are stated in the figure legends

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2.1 ABSTRACT

Gestational diabetes (GDM) results from failure of the β cells to adapt to increased metabolic demands, however, the cause of GDM and the extremely high rate of progression to type-2 diabetes (T2D) remain unknown. Using metabolomics, we show that the novel furan fatty acid metabolite CMPF is elevated in the plasma of humans with GDM. Further analysis revealed significantly elevated CMPF in the plasma of impaired glucose tolerant and T2D patients. In mice, diabetic levels of plasma CMPF induced glucose intolerance, impaired glucose-stimulated insulin secretion and decreased glucose utilization. Mechanistically, we show that CMPF acts directly on the β cell causing impaired mitochondrial function, oxidative stress, dysregulation of key transcription factors, and ultimately reduced insulin biosynthesis. Importantly, CMPF-induced β cell dysfunction could be prevented by specifically blocking its transport or through anti-oxidant treatment. Thus, CMPF provides a novel link between β cell dysfunction and GDM/T2D that could be targeted therapeutically.
2.2 INTRODUCTION

GDM currently afflicts 3-14% of pregnant women and is a condition where women with no history of glucose intolerance transiently develop diabetes during late pregnancy when there is significant insulin resistance (Buchanan and Xiang, 2005b). Acute complications of GDM include fetal macrosomia leading to delivery complications, birth injuries, congenital malformations, pre-eclampsia, and fetal hypoglycemia (Mitanchez, 2010). Longer-term, mothers who had GDM have an increased risk of metabolic syndrome, fatty liver disease, cardiovascular disease, and importantly, a very high rate of progression to T2D with 25-50% of women developing T2D within 5 years postpartum (Bellamy et al., 2009; Kim et al., 2002). Strong evidence is also emerging that fetal exposure to the hyperinsulinemia and hyperglycemia associated with GDM puts the child at a significantly increased risk of obesity, metabolic syndrome and T2D later in life (Reece, 2010). Although there is general agreement that the significant decline in β cell function is the cause of GDM, mechanistically it is not clear if it is due to insufficient β cell mass and/or altered secretory dynamics (Buchanan and Xiang, 2005b; Kim et al., 2010; Retnakaran et al., 2010).

Many studies have associated glucolipotoxicity with β cell dysfunction in T2D, suggesting that metabolites are likely to be causally related to diabetes development (Poitout et al., 2010). Differential abundance of metabolites including free fatty acids (FFAs) and amino acids are important diabetes markers, with previous studies showing elevated levels of these analytes in both GDM and T2D (Bjorntorp et al., 1969; Boden, 2002, 2003; Bomba-Opon et al., 2006; Diaz et al., 2011). To date, no study has effectively utilized metabolomic screening of plasma to identify factors that cause GDM or induce progression from GDM to T2D. In the present study, we identified a furan fatty acid metabolite, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), that is elevated in the plasma of GDM, T2D, and pre-diabetic individuals compared to matched controls with normal glucose tolerance (NGT). Under normal fasting conditions, the circulating concentration of CMPF (20-40µM) (Sassa et al., 2000) is comparable to that of palmitate (~60µM) (Quehenberger et al., 2010). Furan fatty acids are incorporated into phospholipids and cholesterol esters (Spiteller, 2005) and are catabolized into dibasic urofuran acids, including CMPF, which are excreted in the urine (Spiteller, 2005). Uremia, the only other
condition where CMPF is known to be elevated (Niwa et al., 1988), is universally associated with basal hyperinsulinemia and impaired first phase insulin secretion (Allegra et al., 1994; De Marchi et al., 1987; DeFronzo, 1978; Nakamura et al., 1985). However, CMPF has never been directly linked to glucose intolerance or diabetes. Here we show that CMPF causes β cell dysfunction at concentrations found in plasma from uremic, GDM and T2D patients through impairment of mitochondrial function and inhibition of insulin biosynthesis.
2.3 RESULTS

2.3.1 Identification of elevated CMPF in GDM

We used a mass spectrometry approach to examine 342 metabolites in fasting plasma samples from two cohorts of pregnant women with or without GDM matched for age, race, family history of diabetes and pre-pregnancy BMI (Table 3) (Lawton et al., 2008). Women with GDM were found to have a significantly altered metabolic profile compared to their respective NGT controls, reflective of their diabetes (Butte, 2000) (Figure 3a,b). Overall, the most significant changes were observed in the levels of FFAs (Bjorntorp et al., 1969; Boden and Shulman, 2002; Bomba-Opon et al., 2006), primarily poly-unsaturated fatty acids (PUFAs) and long chain fatty acids (Figure 3a,b). Interestingly, and unique to our study, the metabolite with the largest fold change in our GDM population was a furan fatty acid metabolite, CMPF (Figure 3b-d). Elevation of CMPF in GDM patients was independent of age, BMI, fasting blood glucose, fasting plasma insulin and triglyceride levels (Table 4). An ELISA specific for CMPF quantified and validated the elevated CMPF in the same cohort, showing a 7-fold upregulation in GDM (Figure 3e). Intriguingly, in a subset of the same women one year postpartum who had GDM and became impaired glucose tolerant (IGT), CMPF was even more dramatically elevated (12-fold) in compared to women who were NGT at both timepoints (Figure 3f). Therefore, CMPF is significantly upregulated in GDM and subsequent IGT, suggesting it may be linked to both GDM and the progression to T2D.
Figure 3. CMPF is elevated in GDM and T2D patients compared to NGT controls.

A) Differentially expressed metabolites in GDM patients compared to NGT controls categorized based on super-pathway (N=24, P<0.05). b) Relative abundance of a subset of lipids with differential levels in GDM compared to NGT controls (N=12/group) based on global metabolomics. c) Relative CMPF levels in two independent metabolomics screens on separate cohorts of GDM patients (N=12/group/cohorts). d) Chemical structure of CMPF. Levels of CMPF in the plasma of patients with (e) GDM (N=24) and (f) IGT in the postpartum period (N=5) compared to NGT controls. Values are mean +/- SEM. *P<0.05, **P<0.01, ***P<0.001. *Metabolomics performed by Metabolon Inc.
Table 3. Patient clinical characteristics GDM cohorts

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>GDM(n = 24)</th>
<th>NGT(n = 24)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>34.58 (3.73)</td>
<td>33.25 (4.70)</td>
<td>0.4138</td>
</tr>
<tr>
<td>Pre pregnancy BMI</td>
<td>25.21 (3.77)</td>
<td>25.52 (4.54)</td>
<td>0.8609</td>
</tr>
<tr>
<td>Fasting Glucose (mmol/l)</td>
<td>4.74 (0.58)</td>
<td>4.40 (0.37)</td>
<td>0.0345</td>
</tr>
<tr>
<td>Fasting Insulin (pmol/l)</td>
<td>87.58 (61.11)</td>
<td>73.75 (42.05)</td>
<td>0.5429</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.73 (2.47)</td>
<td>2.06 (1.24)</td>
<td>0.4517</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>3.26 (1.22)</td>
<td>2.74 (0.92)</td>
<td>0.0568</td>
</tr>
<tr>
<td>Plasma Creatine (relative units)</td>
<td>1.06 (0.25)</td>
<td>0.96 (0.25)</td>
<td>0.1315</td>
</tr>
<tr>
<td>Plasma Creatinine (relative units)</td>
<td>0.95 (0.15)</td>
<td>1.02 (0.15)</td>
<td>0.0667</td>
</tr>
<tr>
<td>CMPF (µM)</td>
<td>150.88 (21.06)</td>
<td>21.80 (6.36)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data expressed as mean (S.D.)
Table 4. Adjusted mean CMPF levels (µM) in GDM cohorts

<table>
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<tr>
<th>MODEL</th>
<th>GDM(n = 24)</th>
<th>NGT(n = 24)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>151.25</td>
<td>21.42</td>
<td>&lt;0.0001</td>
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<tr>
<td>Adjusted for Age, Pre-pregnancy BMI, Fasting Glucose</td>
<td>150.28</td>
<td>22.39</td>
<td>0.0002</td>
</tr>
<tr>
<td>Adjusted for Age, Pre-pregnancy BMI, Fasting Glucose, Fasting Insulin</td>
<td>151.06</td>
<td>21.61</td>
<td>0.0004</td>
</tr>
<tr>
<td>Adjusted for Age, Pre-pregnancy BMI, Fasting Glucose, Fasting Insulin, Triglycerides</td>
<td>152.62</td>
<td>20.53</td>
<td>0.0015</td>
</tr>
</tbody>
</table>

*Adjustments performed by Ravi Retnakaran
2.3.2 CMPF is Also Elevated in T2DM

Due to the strong similarities between GDM and T2D, we examined whether CMPF was also elevated in T2D relative to NGT controls. Global metabolomics profiling on a cohort of T2D patients compared to NGT controls revealed strong similarities to the GDM population, including a significant elevation in CMPF (Figure 4a, Table 5). In a distinct cohort of patients with mixed gender, age, race, and BMI, CMPF was significantly elevated to GDM-comparable levels in T2D patients as compared to NGT controls (Figure 4b, Table 6). The elevation of plasma CMPF in T2D patients was independent of gender, age, BMI, and race (Figure 4c-f). However, levels of CMPF were significantly higher in NGT males than females (Figure 4c). Interestingly, treatment with metformin, an anti-diabetic medication that suppresses hepatic triglyceride production (Viollet et al., 2012), had no effect on plasma CMPF levels (Figure 4g).
Figure 4. CMPF is elevated in T2D patient plasma
a) Relative abundance of a subset of lipids with differential abundance in GDM compared to NGT controls (N=12/group), or T2DM compared to NGT controls (N=25/group) based on global metabolomic analysis. Quantitative levels of CMPF in the plasma of a distinct cohort of patients with T2DM compared to NGT controls (N=27-28). Concentration of CMPF in NGT and T2D patients classified based on (h) gender, (i) age, (j) BMI, (f) race, and (g) metformin treatment status. Values mean +/- SEM. *P<0.05, **P<0.01, ***P<0.001. *CMPF ELISA performed by Lucy Jun
### Table 5. Patient clinical characteristics T2D global metabolomics cohort

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>T2DM (n=25)</th>
<th>NGT (n=25)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>48.52 (6.27)</td>
<td>40.11 (6.45)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BMI</td>
<td>25.58 (3.23)</td>
<td>25.16 (2.48)</td>
<td>0.5993</td>
</tr>
<tr>
<td>Fasting Glucose (mmol/l)</td>
<td>8.90 (2.83)</td>
<td>5.11 (0.35)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>2hr Plasma Glucose (mmol/l)</td>
<td>13.55 (4.28)</td>
<td>5.8 (0.55)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HbA1c</td>
<td>8.52 (1.88)</td>
<td>5.38 (0.33)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>2.44 (1.83)</td>
<td>1.68 (0.92)</td>
<td>0.0624</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.19 (1.22)</td>
<td>5.07 (1.04)</td>
<td>0.6913</td>
</tr>
<tr>
<td>CMPF (Relative Abundance)</td>
<td>1.78 (0.27)</td>
<td>0.88 (0.07)</td>
<td>0.0020</td>
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</table>

Data are mean (S.D.)
Table 6. Patient clinical characteristics T2D ELISA validation cohort

<table>
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<tr>
<th>VARIABLE</th>
<th>T2DM (n=27)</th>
<th>NGT (n=26)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>66.85 (10.64)</td>
<td>65.22 (13.16)</td>
<td>0.6762</td>
</tr>
<tr>
<td>BMI</td>
<td>25.8 (3.56)</td>
<td>25.61 (2.28)</td>
<td>0.8487</td>
</tr>
<tr>
<td>Gender (% Male)</td>
<td>59.25</td>
<td>65.38</td>
<td>0.6749</td>
</tr>
<tr>
<td>Metformin Treatment (%)</td>
<td>70.37</td>
<td>0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CMPF (µM)</td>
<td>147.98 (19.24)</td>
<td>45.05 (5.81)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Data are mean (S.D.)
2.3.3 CMPF impairs glucose tolerance in mice

To determine if CMPF has any relationship to glucose tolerance, we performed a series of in vivo experiments in which plasma CMPF was elevated to human diabetic-levels. CMPF was injected into mice intraperitoneally (IP) and plasma samples taken at several time points. Plasma and tissue concentrations of CMPF were quantified by ELISA as well as selected reaction monitoring mass spectrometry (SRM-MS). Plasma concentrations reached a maximum of ~250µM at 20min post-injection, remained elevated for at least 6h, ultimately returning to control levels within 24h (Figure 5a, Figure 6a-c). At 2h post injection, CMPF was detected in metabolic tissues including the kidney, liver, skeletal muscle, adipose and pancreas (Figure 6b). Based on these kinetic studies, CMPF was injected into mice IP for seven days, followed by an oral glucose tolerance test (OGTT). After three days of injections, there was no difference in either fasting blood glucose or plasma insulin levels in CMPF-treated mice compared to vehicle-injected controls (Figure 5b,c). However, after seven days CMPF-treated mice had both significantly higher fasting blood glucose and plasma insulin levels (Figure 5d,e). OGTTs revealed glucose intolerance in both three- and seven-day CMPF-treated mice (Figure 5b,d), and this corresponded to a significant inhibition of glucose-stimulated insulin secretion (GSIS) with significantly lower plasma insulin levels during glucose challenge at both timepoints (Figure 5c,e). Therefore, elevated plasma CMPF causes glucose intolerance and impaired insulin secretion.

Interestingly, despite defective GSIS, seven-day CMPF treatment was associated with fed-state hyperinsulinemia and hyperglucagonemia (Figure 5f,g, Figure 6d), prompting us to measure insulin sensitivity. Despite elevated insulin levels, no significant difference in insulin sensitivity was observed between CMPF and vehicle-treated mice by insulin-tolerance testing (ipITT) (Figure 5e). To more thoroughly examine integrated glucose homeostasis, hyperinsulinemic euglycemic clamps were performed. CMPF-treated mice required a significantly lower glucose infusion rate compared to vehicle controls (Figure 5h), which was attributed to a lower glucose appearance (Ra) rate in the basal period, and a lower glucose disappearance rate (Rd) in the post-insulin clamp period (Figure 5i,j). This corresponded to a lower glycolytic rate during both the pre- and post-insulin clamp (Figure 5k). However, the whole-body insulin response was not significantly different between CMPF- and vehicle-treated mice, as there was no difference in
the incremental glucose turnover (post-insulin infusion/basal) (Figure 5l, Figure 6f). Overall, this demonstrates that seven-day CMPF treatment does not induce insulin resistance, but does reduce glucose utilization, consistent with impaired glucose tolerance.

Figure 5. CMPF impairs glucose tolerance, GSIS and glucose utilization in mice.

a) CMPF concentration in mouse plasma following IP injection as measured by ELISA or selected reaction monitoring mass spectrometry (SRM-MS) using a stable isotope labeled standard (N=2-5). b) Blood glucose and (c) corresponding plasma insulin levels during an OGTT after three-day treatment with CMPF (N=8). d) Blood glucose and (e) corresponding plasma insulin levels during an OGTT after seven-day treatment (N=8). Fasting values inset for both (d) and (e). f) Random-fed plasma insulin on each day of injection, and (g) corresponding blood glucose (N=8). h) Glucose infusion rate, (i) glucose appearance and (j) disappearance rates, (k) glycolytic rate, and (l) glucose turn around rate (post/basal) during hyperinsulinemic euglycemic clamps on seven-day treated mice (N=4-5).

Values are mean +/- SEM. *P<0.05, **P<0.001.

*Clamp studies performed by Ying Liu
Figure 5

(a) CYP2E1 (nM)
(b) Blood Glucose (mM)
(c) Insulin (ng/mL)
(d) Blood Glucose (mM)
(e) Insulin (ng/mL)
(f) Insulin (ng/mL)
(g) Blood Glucose (mM)
(h) Glucose Infusion Rate (mg/kg/min)
(i) Glucose Appearance Rate (mg/kg/min)
(j) Glucose Disappearance Rate (mg/kg/min)
(k) Glycolytic Rate (mg/kg/min)
(l) Glucose Turnaround Rate (Post/Pre)
Figure 6. Quantification of CMPF in plasma and tissues and the effect on glucose homeostasis

a) Sample chromatogram showing the spectral peak of CMPF in the plasma of CMPF-injected mice (solid line) and stable isotope labeled control (dotted line).  
b) Concentrations of CMPF in the plasma and tissues of CMPF injected mice 2hrs post injection as determined by SRM-MS.  
c) Mouse plasma CMPF before and after i.p. injection over seven consecutive days.  
d) Fasting and re-fed plasma glucagon levels after seven days of injection (N=8/group) (*P<0.05, student’s t-test).  
e) Blood glucose during ipITT (N=8/group) (n.s., two-way ANOVA).  
f) Glycolytic rate (post/basal) during hyperinsulinemic euglycemic clamps on seven day injected mice (N=4-5/group) (n.s., two-way ANOVA). Values mean +/- SEM. *SRM-MS Performed by AFBM
2.3.4 CMPF impairs β cell function *in vivo*

The dysregulated insulin secretion observed in CMPF-treated mice compelled us to examine β cell function. No difference in insulin- or glucagon-positive area was observed by immunohistochemical staining, suggesting that seven-day CMPF treatment did not induce significant changes in β- or α-cell mass (Figure 7a-d). There was also no significant difference in islet size between CMPF- and vehicle-treated mice (Figure 8a). However, total insulin content from both isolated islets and whole pancreas was significantly reduced in CMPF treated mice (Figure 8b,c). Consistent with *in vivo* observations, islets isolated from the seven-day CMPF-treated mice had significantly elevated insulin secretion under sub-stimulatory concentrations of glucose (2.8mM), and significantly lower insulin secretion under high glucose (16.7mM) conditions (Figure 8d), corresponding to a significantly decreased fold change in GSIS (Figure 8e). Concurrently, high glucose failed to inhibit glucagon secretion in isolated islets (Figure 8f).
Figure 7. Pancreatic alpha and beta-cell morphology in CMPF treated mice.

a) Representative pancreatic cross sections stained for insulin. b) Beta-cell area calculated by positive pixel count of stained cross sections. c) Glucagon staining and d) alpha cell area (n.s., student’s t-test) N = 4 mice per group and 3 sections from each mouse were analyzed. Control (black bars), CMPF treated (white bars). Values are mean +/- SEM
Figure 8. CMPF impairs GSIS and decreases insulin content in vivo.

Islets isolated from seven-day treated mice were assessed for (a) islet size (N=5-7 islets/mouse, 4 mice) and (b) total insulin content (N=4). c) Total pancreatic insulin content from seven day treated mice (N=3). d) Glucose-stimulated insulin secretion (N=8), (e) fold change in insulin secretion in response to high glucose over low glucose, and (f) glucagon secretion (N=4) in isolated islets from seven-day treated mice. Values mean +/- SEM. *P<0.05, **P<0.01, ***P<0.001.
2.3.5 CMPF directly impairs beta cell function

To determine whether the alteration in glucose homeostasis was due to a direct effect of CMPF on the β cells, a series of experiments were performed on isolated human and mouse islets, as well as a β cell line (MIN6). Initially, mouse islets were treated with varying doses of CMPF for 24h to determine if there was any effect on GSIS. CMPF inhibited GSIS in a dose-dependent manner, with a significant reduction in high glucose-stimulated secretion after treatment with 150, 200 or 250µM CMPF (comparable to GDM and T2D circulating levels) for 24h as compared to vehicle and 50µM CMPF (comparable to NGT circulating levels) (Figure 9a). Conjugation to varying concentrations of BSA had no effect on 200µM CMPF-mediated inhibition of GSIS from mouse islets (Figure 9b). Islets and MIN6 cells were then treated for 4 or 24h with vehicle control, 20µM or 200µM CMPF to correspond to NGT and diabetic values respectively. In both human and mouse islets 24h treatment with 200µM CMPF significantly decreased GSIS compared to both control and 20µM CMPF treatment conditions (Figure 9c,d). Mouse islets also exhibited significantly increased insulin secretion under sub-stimulatory glucose concentrations (Figure 9c), consistent with *ex vivo* findings (Figure 8d). Impaired GSIS was observed in MIN6 cells treated for 4h (Figure 9e).

To determine the specificity of the effect of CMPF, mouse islets were treated with varying doses of the fatty acid eicosapentaenoate (EPA), or the structurally related compound estrone sulfate (ES). CMPF is produced through the metabolism of furan fatty acids, which are produced from PUFAs (Batna et al., 1993). The PUFA EPA was also shown to be significantly elevated in both GDM and T2D plasma compared to NGT controls (Figure 3b, 4a). However, treatment of islets with NGT control (10µM), diabetic (20µM) or supraphysiological (50µM) concentrations of EPA for 24h had no effect on GSIS (Figure 10a). Similarly, treatment of islets with either 10µM or 50µM ES, both supraphysiological doses, had no effect on GSIS (Figure 10b), suggesting that the effect of CMPF is specific.

Decreased insulin secretion under high glucose conditions could be explained by fatty acid toxicity (Kharroubi et al., 2004), however mouse islets treated for 24h with vehicle control, 20µM or 200µM CMPF did not show any signs of inducing apoptosis or necrosis, either alone or in the presence of cytokines TNFα, IF-γ and IL-1, or Tunicamycin (2ng/ml) (Figure 9f, Figure
In addition, there was no indication of dedifferentiation, as there was no difference in expression of Ngn3, Nanog or Oct4, which have previously been associated with a loss of β cell character (Figure 10d) (Talchai et al., 2012). In the absence of cell death or dedifferentiation, CMPF could be impairing insulin secretion by affecting insulin biosynthesis or its release. Consistent with in vivo findings, total insulin content was significantly reduced by treatment with 200µM CMPF (Figure 9g). Total glucagon content was not significantly changed (Figure 9h). Transmission electron microscopy (TEM) was used to determine whether CMPF was impairing insulin processing/packaging into granules. Mouse islets treated for 24h with 200µM CMPF showed normal granule morphology but had a dramatically decreased number of insulin granules compared to controls (Figure 9i,j Figure 10e).

Figure 9. CMPF impairs GSIS and decreases insulin biosynthesis in vitro.

a) Dose response curve showing the effect of CMPF on GSIS (N=3) and b) the effect of 200µM CMPF with varying concentrations of BSA (N=3). GSIS from (c) mouse islets (N=16), (d) human islets (N=10) and (e) MIN6 cells (N=8) incubated for 24h with vehicle control, 20µM or 200µM CMPF. f) Percentage of annexin V positive mouse islet cells following 24h treatment (N=3). g) Total insulin (N=8) and (h) glucagon (N=5) content in 24h treated islets. (i) Quantification of dense core granule numbers in (j) TEM images of 24h treated mouse islets (N=4). White arrows indicate insulin granules. Values are mean +/- SEM. *P<0.05, **P<0.01, ***P<0.001.
Figure 9
Figure 10. Chemical controls confirm specificity of the effect of CMPF, with no induction of apoptosis or dedifferentiation.

a) Dose response showing effect of EPA on GSIS (N=4). b) GSIS from islets treated with estrone sulphate and CMPF (N=5). c) Cleaved caspase 3/7 activity in islets treated for 24h with 200µM CMPF with or without cytokines (TNFα, IF-γ and IL-1) or 2ng/ml tunicamycin (N=4). d) Quantitative PCR looking at dedifferentiation markers Ngn3, Nanog and Oct4 in mouse islets treated in vitro for 24h (N=3/group). Control (black bars), CMPF treated (white bars). e) Transmission electron microscopy images from 24h vehicle-control and CMPF treated islets. 5,000 times magnification. N=6-8 islets from 3-4 mice/group. Values mean +/- SEM. *P<0.05, **P<0.01.
2.3.6 CMPF is metabolized to increase ROS

Lower total insulin content may be due to β cell exhaustion caused by over-stimulation and/or defective insulin production. To determine the mechanism through which CMPF diminishes insulin content, we measured insulin in the islet media following 24h treatment. There was no difference in accumulation of total insulin (data not shown), however there was substantially more proinsulin in the media of CMPF-treated islets compared to controls, consistent with an altered proinsulin:insulin ratio observed in T2D patients (Kamoda et al., 2006) (Figure 11a). Impaired insulin processing has previously been associated with elevations in ER stress and/or reactive oxygen species (ROS) production (Kashemsant and Chan, 2006). CMPF treatment did not induce changes in markers of ER stress including *Chop*, *Bip* and *XBP-1* as examined by microarray and qPCR (Figure 12a,b). We then examined whether CMPF was metabolized by β cells, as excess substrate for the electron transport chain leads to the formation of ROS (Joseph et al., 2004; Robson-Doucette et al., 2011; Saadeh et al., 2012). CMPF was acutely added to mouse islets and changes in mitochondrial membrane potential (MMP) were measured. CMPF caused a transient membrane hyperpolarization consistent with an increase in proton motive force generated through β-oxidation (Figure 11b). This corresponded to a 2-fold increase in ROS in CMPF-treated islets over controls after 4 and 24h of treatment (Figure 11c). Increased antioxidant gene expression, including uncoupling protein 2 (*Ucp2*) and catalase (*Cat*) after 24h of CMPF treatment suggests that the cells are compensating for the oxidative stress (Figure 11d,e) (Robson-Doucette et al., 2011). The increase in ROS production caused by CMPF-treatment was inhibited by co-treatment with the antioxidant N-Acetyl-Cysteine (NAC) (Figure 11f). Importantly, co-treatment of islets with NAC and CMPF rescued insulin secretion and insulin content to near control levels (Figure 11g,h). Therefore, oxidative stress caused by CMPF metabolism impairs GSIS and insulin processing, and is reversible through reducing ROS generation.
2.3.7 CMPF Impairs Insulin Biosynthesis

Elevated ROS has been shown to modulate insulin transcription (Poitout and Robertson, 2008; Robertson, 2004) through alterations in AKT and GSK3β activity (Boucher et al., 2006; Kawamori et al., 2003; Kawamori et al., 2006; Ryu et al., 2011). To determine the mechanism through which CMPF decreases insulin biosynthesis we examined its effect on these key regulators. Under normal conditions, AKT and GSK3β have reciprocal activities. Active pAKT phosphorylates and inactivates GSK3β, preventing phosphorylation of downstream targets (Figure 12d) (Humphrey et al., 2010). However, under conditions of oxidative stress AKT is inactive (Kawamori et al., 2006). Both AKT and GSK3β phosphorylation were significantly impaired after 24h CMPF treatment compared to controls (Figure 11i,j), indicating decreased AKT and increased GSK3β activity. We observed no significant difference in either total ERK1/2 or pERK1/2 (Figure 12c) confirming specificity for the AKT pathway. To determine if these changes had an effect on insulin transcription, we examined the localization of two key insulin transcription factors, PDX1 and FOXO1 using immunofluorescent staining. FOXO1 is normally sequestered in the cytosol due to phosphorylation by AKT (Kitamura et al., 2005). In CMPF-treated islets, FOXO1 is translocated to the nucleus (Figure 11k,m), reflecting decreased AKT activity (Kawamori et al., 2006). Conversely, PDX1, which is directly phosphorylated by GSK3β (Boucher et al., 2006), is sequestered outside of the nucleus, consistent with increased GSK3β activity (Figure 11l,n). Absolute levels of these transcription factors were not significantly different, indicating that altered localization is not due to changed protein abundance (Figure 11o). Translocation of both FOXO1 and PDX1 was prevented by treatment with that antioxidant NAC, indicating that oxidative stress contributes to defective insulin biosynthesis (Figure 11k-n). Altered activity of these transcription factors was further confirmed by decreased mRNA levels of key target genes including Ins1, transcription factors Pdx1 and Mafa, proinsulin processing enzymes CpE, Pc1 and Pc2, and the glucose transporter Glut2 (Boucher et al., 2006; Kaneto et al., 2008) (Figure 13a).
Figure 11. CMPF is metabolized by β cells which increases ROS production and reduces insulin biosynthesis.

a) Proinsulin in the media of 24h treated islets (N=4). b) Mitochondrial membrane potential (MMP) following acute addition of vehicle control or 200µM of CMPF (N=3). c) ROS levels in 4- and 24h treated mouse islets with representative images (N=10-15 islets/mouse from 4 mice). d) Expression of antioxidant genes Cat and (e) Ucp2 (N=4). f) ROS accumulation in 24h-treated islets treated with 500µM NAC (N=10 islets/mouse from 4 mice). g) GSIS and (h) total insulin content from 24h-treated islets co-treated with NAC (N=4). Western blots showing (i) Ser9 phosphorylation of GSK3β, and (j) Ser473 phosphorylation of AKT (N=3). k) Immunofluorescent staining showing nuclear translocation of FOXO1 and (l) PDX1 after CMPF and NAC treatment (N=3-6). Quantification of nuclear percentage of (m) FOXO1 and (n) PDX1 based on immunofluorescent staining with CMPF and NAC treatment. o) Western blots showing protein abundance of PDX1 and FOXO1. Values are mean +/- SEM. *P<0.05, **P<0.01, ***P<0.001.
Figure 11

(a) Relative levels of Pfk (mg/mg DNA) in control versus 200mM CMPF.

(b) Graph showing MMP (Rho123 RFU) over time with 2.8mM Glucose and 2.8mM Glucose + Treatment.

(c) ROS (DCF RFU) over time with various conditions.

(d) Gene expression of Cat.

(e) Gene expression of Ucp2.

(f) Graph showing ROS levels with CMPF and NAC.

(g) Graph showing Insulin levels with CMPF and NAC.

(h) Graph showing Insulin (mg/mg DNA) with glucose concentrations.

(i) Western Blot showing protein expression with CMPF and p38/GSK3.

(j) Western Blot showing protein expression with CMPF and pAkt.

(k) Immunofluorescence images showing FOXO1/INS/DAPI with different treatments.

(l) Immunofluorescence images showing PDX1/INS/DAPI with different treatments.

(m) Bar graph showing nuclear percentage of FOXO1 with different treatments.

(n) Bar graph showing nuclear percentage of PDX1 with different treatments.

(o) Western Blot showing protein expression with FOXO1 and PDX1.
Figure 12. Proposed model for how CMPF impairs insulin biosynthesis.

Quantification of markers of ER stress from (a) microarray (N=3/group) and (b) qPCR (N=3) in islets treated for 24hrs with vehicle control, 200μM CMPF or 2ng/ml tunicamycin as a positive control. c) Western blots showing ERK1/2 and pERK1/2 in islets treated for 24hrs with vehicle control or 200μM CMPF (N=3). d) Under normal conditions AKT is phosphorylated and activated by autocrine insulin signaling. Activated AKT phosphorylates and inactivates GSK3β, and phosphorylated FOXO1 to cause it to be sequestered in the cytosol. In the presence of diabetic-levels of CMPF, increased ROS production impairs AKT phosphorylation, causing it to be inactive. This prevents the phosphorylation of GSK3β, promoting its activity. FOXO1 is not phosphorylated and translocated to the nucleus, while PDX1 is phosphorylated by active GSK3β and sequestered in the cytosol to be degraded. This results in diminished insulin biosynthesis and lower total insulin content. Values mean +/- SEM. *P<0.05.
2.3.8 CMPF Alters Glucose Metabolism

Given that CMPF causes hyperinsulinemia during the fed-state in vivo, and exaggerated secretion of insulin from islets under non-stimulatory conditions in vitro, we rationalized that there must be a defect in glucose-sensing and/or secretion of insulin that could not be solely explained by effects on insulin production. Dispersed mouse islets treated for 24h with 200µM CMPF showed significantly greater hyperpolarization of the MMP under sub-stimulatory glucose concentrations, and significantly reduced MMP hyperpolarization under high glucose conditions when compared to vehicle treated controls (Figure 13b). These data suggest that CMPF metabolism leads to excessive proton motive force resulting in increased insulin secretion under low glucose conditions combined with impaired glucose metabolism and thus reduced GSIS. Consistent with these findings, CMPF increases basal and impairs high glucose-stimulated ATP production in 24h CMPF treated islets (Figure 13c). To confirm changes in mitochondrial function, we analyzed 24h 200µM CMPF-treated islets by microarray. Overall, 6.2% of transcripts were significantly differentially expressed following CMPF treatment. When organized based on biological process, the largest differentially expressed cluster encompassed genes involved in metabolism (38%) (Figure 13d). Specifically, we observed a significant upregulation of genes related to fat oxidation (Figure 14), which suggests a ‘switch’ from glucose oxidation-driven metabolism to fat oxidation (Elks, 1993). Such a switch can decrease the β cell’s ability to sense and metabolize glucose (Hue and Taegtmeyer, 2009), limiting their capacity for GSIS and thus contributes to CMPF’s effect on β cells.
Figure 13. CMPF causes decreased expression of insulin biosynthesis genes, and alters metabolic pathway genes.

a) Expression of insulin, insulin processing enzymes, insulin transcription factors and glucose-sensing genes in 24h treated mouse islets (N=5-6).

b) Mitochondrial Membrane Potential (MMP) following 24h incubation, and change in fluorescence (N=4).

c) Intracellular ATP content is islets treated for 24h with vehicle control, 200µM CMPF or 400µM palmitate.

d) Classification of significantly changed genes by microarray in 24h treated mouse islets based on biological function (N=3, P<0.05). Values are mean +/- SEM. *P<0.05, **P<0.01
Figure 14. CMPF-treated islets have significantly increased expression of mitochondrial-related genes and genes involved in FFA metabolism.

a) Fold change of significantly changed genes classified based on biological function in 24-hour treated mouse islets (N=3/group). Values are means, P<0.05.
2.3.9 CMPF Enters the β Cell Through OAT3

Dibasic urofuran acids, including CMPF, are normally secreted in the urine (Deguchi et al., 2005), and CMPF is known to be elevated in the plasma of uremic patients due to reduced expression of the organic anion transporters (OATs) which are responsible for their clearance (Sassa et al., 2000). OAT3 (Slc22a8) and OAT1 (Slc22a6) transport CMPF into the kidney proximal tubule cells and require the co-transporter NaDC3 to function (Figure 15a) (Deguchi et al., 2004). OAT4 (Slc22a11) is an efflux transporter that removes CMPF from the proximal tubule into the kidney lumen in humans (Deguchi et al., 2005). We therefore investigated whether CMPF also enters the β cell through these transporters. Microarray analysis of human islets showed expression of all four transcripts at levels comparable to that of the β cell KATP channel Kcnj11 (Figure 16a). This was further validated by quantitative PCR (Figure 16b) and immunoblotting (Figure 16c) in human islets. To determine protein localization, we performed immunofluorescent staining in dispersed human islet cells (Figure 16d). OAT3 and NaDC3 show strong staining in insulin-positive β cells. OAT1 and OAT4 are predominantly expressed in insulin-negative cells. Co-staining with glucagon revealed that OAT4 was also not expressed in glucagon-positive cells (Figure 15b). Therefore, the OAT transporters are expressed in the islet, with the influx transporters OAT3 and NaDC3 being strongly expressed in insulin-positive cells.

To determine if OATs are responsible for CMPF transport into β cells, we utilized inhibitors of OAT function. Probenecid is a non-specific OAT-blocker (Miyamoto et al., 2012). Treatment of islets with 1mM probenecid for 24h rescued insulin secretion from CMPF-treated islets to control levels (Figure 16e). Importantly, probenecid was unable to rescue palmitate-induced impairment of GSIS, supporting the importance of CMPF transport in mediating the effect on GSIS, as well as suggesting that OAT inhibition alone is not sufficient to prevent FFA-induced defects in GSIS. To determine which OAT is primarily responsible for CMPF transport into β cells, we treated islets with 300µM of benzylpenicillin (PCG), an OAT3-specific inhibitor, or 50µM of p-aminohippurate (PAH), an OAT1-specific inhibitor (Deguchi et al., 2005). Consistent with previous reports that OAT3 is the dominant CMPF transporter (Deguchi et al., 2005), PCG was able to rescue insulin secretion and insulin content from CMPF-treated islets to control levels (Figure 16f,h). However, treatment with PAH had no effect on secretion (Figure 16g). To
further support this observation, islets were isolated from whole body OAT3 knockout mice (OAT3KO). Deletion of OAT3 from the islets was confirmed by qPCR and western blot as compared to wildtype controls (Figure 16i,j). Importantly, treatment of OAT3KO islets with 200µM CMPF for 24h had no effect on GSIS or insulin content (Figure 16k,l). Therefore, CMPF is transported into the β cell through OAT3, and blockage of this transporter prevents CMPF from impairing GSIS and insulin biosynthesis.
Figure 15. CMPF is transported through OAT transporters in the proximal tubule cells of the kidney.

a) Schematic of CMPF transport in the proximal tubule cells of the human kidney. b) Immunofluorescent staining for glucagon (red) and OAT4 (green) in dispersed human islet cells. N=4 islet donors. c) GSIS from islets treated for 48hrs with 400µM palmitate, 1mM probenecid or a combination of palmitate and probenecid (N=4). Values mean +/- SEM. *P<0.05.
Figure 16. CMPF enters the β cell via Organic Anion Transporter 3

a) Microarray analysis of human islets showing expression of OAT transporters and β cell genes (N=3). Validation of OAT expression in human islets by (b) qPCR, (c) western blot, and (d) immunofluorescent staining with insulin (N=3-5). GSIS from murine islets co-treated with OAT inhibitors (e) Probenecid, (f) benzylpenicillin (PCG) and (g) p-aminohippurate (PAH) (N=4). h) Total insulin content from murine islets co-treated with CMPF and PCG (N=4). Validation of knockout of OAT3 in OAT3KO mice compared to wildtype C57Bl/6 controls by (i) qPCR (N=4) and (j) western blot (N=3). k) GSIS and (l) total insulin content from control and OAT3KO islets treated for 24h with vehicle control or 200µM CMPF (N=5). Values mean +/- SEM. *P<0.05, **P<0.01, ***P<0.001.
2.4 DISCUSSION

The underlying cause of both GDM and T2D is a failure of the β cell to respond to changing metabolic demands; namely increased insulin resistance (Buchanan, 2001; Kahn, 2003; Prentki and Nolan, 2006). Here we demonstrate that the furan fatty acid metabolite CMPF impairs pancreatic β cell function at concentrations observed in patients with GDM, T2D, and IGT, correlating with a progressive decline in β cell function during this period (Retnakaran et al., 2010). Treatment with CMPF recapitulates many key characteristics of diabetes, including basal hyperinsulinemia (Wijendran et al., 1999) with impaired GSIS and reduced whole-body glucose utilization (Bowes et al., 1996; Kuhl, 1991). Interestingly, uremia in both humans and rodent models is associated with elevated plasma CMPF (200-400µM (Niwa et al., 1988)), as well as basal hyperglycemia and hyperinsulinemia combined with impaired first phase insulin secretion, consistent with our in vivo observations (Allegra et al., 1994; De Marchi et al., 1987; DeFronzo, 1978; Nakamura et al., 1985). In vitro, we demonstrate that CMPF metabolism causes β cell dysfunction through impairment of mitochondrial function and ATP synthesis, as well as inducing oxidative stress. Elevated ROS levels altered the activity of key kinases AKT and GSK3β (Kawamori et al., 2006), changing transcriptional activity, and ultimately reduced insulin transcription and post-translational processing. This mechanism is outlined in Figure 17. Therefore, elevated plasma CMPF may play an important causal role in β cell dysfunction associated with GDM, T2D and the progression from GDM to T2D.

The effect of CMPF can be rescued by two distinct approaches: blocking CMPF entry into the β cell, and reducing ROS accumulation. Here we show for the first time that CMPF enters the β cell through the OAT3 transporter, which has previously only been functionally characterized on the basolateral membrane of kidney proximal tubules cells (Deguchi et al., 2005), with low levels of expression reported in the liver and brain (Deguchi et al., 2006; Sweet et al., 2002). We show that CMPF transport can be blocked through genetic deletion of OAT3, or by using the commonly prescribed drugs probenecid, a ubiquitous OAT inhibitor, and benzylpenicillin, a specific inhibitor of OAT3 transport. Interestingly, we show that while human β cells express influx transporter OAT3, the efflux transporter OAT4 is absent. Thus during diabetes when plasma CMPF is elevated it can presumably enter the β cell but not exit, promoting its...
metabolism and associated effects. Therefore, preventing CMPF influx, or increasing CMPF efflux from the β cell are attractive avenues for future research into the prevention of β cell failure.

Once inside the β cell, CMPF is metabolized, causing impaired glucose utilization and increased ROS production. Low-level ROS potentiates GSIS, aiding the β cell in responding to acute increases in nutrients including FFAs (Poitout and Robertson, 2008; Robson-Doucette et al., 2011; Saadeh et al., 2012). However, longer-term, the β cell is particularly vulnerable to oxidative stress due to relatively low expression of antioxidant enzymes relative to other tissue types (Robertson, 2004). Treatment with anti-oxidants has been proposed as a promising approach for the treatment of T2D, and has been shown to attenuate islet fibrosis and apoptosis and improve glucose tolerance and insulin sensitivity in rodent models (Lee et al., 2011). Our finding that pre-treatment with the antioxidant NAC prevents CMPF from inducing β cell failure suggests that antioxidant treatment may also be used in the prevention and/or treatment of GDM.
Figure 17. Proposed Mechanism of Acute Inhibition of Insulin Biosynthesis by CMPF
2.5 MATERIALS AND METHODS

2.5.1 Identification and quantification of CMPF

Fasting plasma samples were collected from women at 24-28 weeks gestation at the time of diagnosis to minimize differences in diet and medications that may be associated with diabetes management. Glucose tolerance was tested first using a glucose challenge test, followed by a 3h 100g-glucose OGTT. Samples were tested using selected reaction monitoring mass spectroscopy (SRM-MS) in combination with either gas chromatography (GC) or liquid chromatography (LC) for separation of the analytes comparing GDM and NGT patients (performed by Metabolon Inc.) as previously described (Allister et al., 2013). Two independent cohorts with 12 samples/group/cohort were investigated, and 342 biochemicals were quantified in each sample. A low q-value (<0.1) was used to determine confidence in results. Patients were matched for age, race, BMI, and family history of diabetes. Plasma and tissue CMPF levels were quantitatively determined by competitive enzyme-linked immunosorbent assay (ELISA) or SRM-MS. The ELISA was performed following manufacturer’s instructions (NovaTein Biologicals, USA) and detects total CMPF. SRM-MS was performed by the Analytical Facility for Bioactive Molecules of The Centre for the Study of Complex Childhood Diseases, The Hospital for Sick Children, Toronto, Canada. Plasma samples from a subset of the same women used for the GDM/NGT studies were taken one year post-partum for analysis (5/group). Women with IGT had GDM during pregnancy, NGT patients remained NGT at both timepoints.

2.5.2 SRM-MS for the quantification of CMPF

Briefly, mice were injected with 6mg/kg CMPF IP. After 2h mice were sacrificed and blood and tissues collected for analysis. Tissues were rinsed with PBS and flash frozen in liquid nitrogen. Plasma and tissues were stored at -80C until use. CMPF standards (1 – 500 ng) and samples were spiked with 25 ng of CMPF-d₅ (Cayman Chemical, USA) internal standard. A surrogate matrix of 4% BSA in PBS was used for standards. Plasma (20µL) and standards (20µL matrix surrogate) were diluted with 480 µL of ultrapure water. 20µL of 80% phosphoric acid was added, mixed by vortexing then 1.5 mL of ethyl acetate (EtOAc) was
added. Samples were chilled on ice, and then centrifuged. The upper EtOAc layer was collected and re-extracted with another 1.5 mL of EtOAc. Samples were vortexed, chilled on ice, and centrifuged. The upper EtOAc layers were removed and combined with the previous extract. The combined EtOAc layers were dried and residues were reconstituted in 500 μL of acetonitrile and analyzed by LC-MS/MS using an Agilent 1200 HPLC with an API 4000 mass spectrometer (AB Sciex). For the liver samples, the extraction protocol is the same except that the liver was homogenized in 1:1 water:ethanol at a concentration of 100 mg/mL and the equivalent of 20 mg was extracted (200 μL). 300 μL water was added instead of 480 μL. The curve range for the liver was 0.5–100 ng and the curve was extracted from control liver.

2.5.3 CMPF Preparation

CMPF was purchased from Cayman Chemical (product number 10007133) and dissolved in 100% ethanol to stock concentrations of 200mM and 20mM. CMPF was stored at -20°C.

2.5.4 Intraperitoneal injection of CMPF and Tolerance Tests

Mice were injected intraperitoneally (IP) with 6mg/kg CMPF or vehicle control at 24h intervals for seven days and assessed for body weight, blood glucose and plasma insulin immediately prior to each injection. Mice were fasted for 14h following the final injection before an oral glucose tolerance test (OGTT) or measurement of fasted plasma glucagon. Re-fed plasma glucagon was measured 2h after gavage with 1g/kg glucose. OGTTs, and measurement of plasma insulin and glucagon were performed as previously described (Allister et al., 2013). IP insulin tolerance tests were performed following a 4h fast. 1.5IU/kg insulin was injected and blood glucose was measured at 0, 10, 20, 30, 45, 60 and 120 minutes. All experiments were approved by the Animal Care Committee (University of Toronto or York University, where applicable) and animals handled according to the Canadian Council of Animal Care guidelines.
2.5.5 Hyperinsulinemic Euglycemic Clamps

Hyperinsulinemic euglycemic clamps were performed as previously described (Liu et al., 2012) following 7 days of IP injections with CMPF or vehicle. Internal and external cannulation was performed on day 3 of the injection protocol.

2.5.6 Immunohistochemistry and islet morphology

Pancreata were weighed and fixed in 10% neutral buffered formalin. Tissue processing and immunostaining for insulin and glucagon have been described previously (Luu et al., 2013). Images of each section were acquired using Aperio Imagescope at 40x magnification. The β- or α-cell area was calculated by using positive pixel count analysis (Aperio Image scope).

2.5.7 Human Islets

Human islets from review board approved healthy donors were provided by the IsletCore and Clinical Islet Laboratory (University of Alberta, Canada). Islets were picked into low glucose DMEM media (Gibco, ref#11885-084) with 10% FBS 1% penicillin/streptomyosin, 1% L-glutamine and cultured overnight before use.

2.5.8 Glucose-Stimulated Insulin Secretion

Glucose-stimulated insulin secretion (GSIS) was assessed as previously described (Luu et al., 2013; Robson-Doucette et al., 2011) using 2.8mM and 16.7mM glucose. Mouse islets were isolated as previously described (Hardy et al., 2009) and incubated in standard media (RPMI-1640 with 10% FBS, 1% penicillin/streptomyosin, 1% L-glutamine) overnight. Islets were incubated for 24h in standard media with 0.1% BSA plus either ethanol control (1:1000 ethanol), 20µM (1:1000 20mM CMPF in ethanol), or 200µM (1:1000 200mM CMPF in ethanol) CMPF, as previously described (Everts et al., 1995). CMPF was combined with fatty acid free BSA in standard media for 6h at 37°C before the addition of islets. Islets isolated from vehicle or CMPF-treated mice immediately underwent GSIS following isolation. To induce ER stress, islets were treated for 24h with 2ng/ml Tunicamycin (T7765 Sigma Aldrich) dissolved in DMSO. The final concentration of DMSO was 0.1%. Cytokines
were dissolved in PBS supplemented with 0.1% endotoxin free bovine serum albumin (A8806, Sigma Aldrich), according to manufacturer instructions. 10ng/ml TNFα (H8916, Sigma Aldrich), 150pg/ml IL-1β (H6291, Sigma Aldrich), and 5ng/ml IFN-γ (285-IF, R&D Systems) were added to islets for 24h.

Insulin and glucagon concentrations were measured in secretion samples and total content samples using an HTRF kit (Insulin, Cisbio) on the Pherastar plate reader (Thermo Fisher) or glucagon RIA kit (Cedarlane Labs, Burlington, Canada) and normalized to DNA. Total islet insulin content was determined by incubating islets overnight in an acid ethanol solution. Samples were dried down and resuspended in ultrapure water and insulin content assayed by insulin HTRF as above and normalized to DNA. For calculation of pancreatic insulin content, pancreata were isolated, rinsed in PBS, and flash frozen in liquid nitrogen. Pancreata were crushed using a mortar and pestle and 1ml acid ethanol added. Samples were refrigerated at 4°C overnight and dried down. Pancreata were resuspended in 300ul ultrapure water and spun a 10,000rpm for 5 minutes. Supernatant was collected and insulin content determined by insulin ELISA as previously described (Allister et al., 2013) and normalized to DNA content.

2.5.9 Apoptosis and necrosis

Dispersed isolated islets were treated for 24 or 48h with vehicle control or CMPF. Treatment was washed away before staining with Annexin V (0.5µg/ml), Propidium Iodide (2µg/ml) and Hoechst (2µM) for 20mins at 37°C. Images were acquired and analyzed on a Thermo Fisher Cellomics ArrayScan VTI HCS Reader using iDEV software. The filter settings for each dye were BGRFR 485-20 for Annexin V, BGRFR 549-15 for PI and BGRFR 386-23 for Hoechst. Cleaved caspase 3/7 assay was performed according to manufacturers instructions using 10 islets treated for 24h (G8091, Promega, USA).

2.5.10 Gene Expression

Total RNA was extracted from 24h vehicle or CMPF-treated islets using the Qiagen RNeasy Plus mini kit (Hilden, Germany). Microarray analysis was performed as previously described (Basford et al., 2012) using the Affymetrix Mouse 430 2.0 Gene Chip at the
University Health Network microarray center (Toronto, Canada). Significant changes were defined as $P<0.05$. Data is deposited as GEO record GSE55028. Reverse transcription from total RNA and quantitative real time PCR (qPCR) analysis was performed as previously described (Basford et al., 2012). Primers were designed using Primer3 software (NCBI) and are listed in Table 7. Data were normalized to β actin mRNA. Human islet microarray data was previously described in Basford et al., 2012 and deposited as GEO record GSE40709.

2.5.11 Transmission Electron Microscopy

Isolated islets were treated with either vehicle or 200µM CMPF for 24h, fixed and images acquired as previously described (Basford et al., 2012). Granule number was manually quantified using ImageJ software (Lu et al., 2010).

2.5.12 ROS accumulation and islet size in isolated islets

The level of H$_2$O$_2$ was determined using 2’,7’-dichlorodihydro fluorescein diacetate (CM-H$_2$-DCFDA) (Molecular Probes, Invitrogen, Canada) as previously described (Robson-Doucette et al., 2011) in isolated islets treated with either vehicle control or 200µM CMPF for 4 or 24h. Bright field images were used to determine islet size.

2.5.13 Mitochondrial Membrane Potential (MMP)

Dispersed isolated islets were treated with either vehicle control or 200µM CMPF for 24h prior to loading with rhodamine 123 (25 µg/ml, 10 min) in 2.8mM glucose imaging buffer. 5mM NaN$_3$ was added to fully depolarize the MMP (Diao et al., 2008).

2.5.14 Western Blotting

CMPF-treated and control mouse islets were lysed in RIPA buffer (Cell Signaling, Danvers, MA, USA) containing protease inhibitor cocktail (Roche, Mississauga, ON, Canada). Lysates were spun at 12,000rpm and supernatant was loaded onto a 4-15% SDS-PAGE gradient gel (BioRad, Mississauga, ON, Canada) and transferred onto PVDF membrane using a Turbo Blotter (BioRad). The membrane was probed with antibodies as listed in Table 8, and imaged using Kodak Imager 4000pro (Carestream, Rochester, NY, USA).
2.5.15 Immunoﬂuorescent staining

The cellular localization of FOXO1 and PDX1 were determined in dispersed CD1 mouse islet cells using immunofluorescence. The presence and localization of the organic anion transporters (OATs) was determined in dispersed human islet cells. Antibodies used are detailed in Table 8. Staining was performed as previously described (Diao et al., 2008). Images were acquired using a confocal microscope (Quorum Wave FX Spinning Disc; Perkin Elmer, Waltham ML, USA) and Volocity software (Perkin Elmer). Colocalization with Hoescht nuclear stain was determined with Volocity software.

2.5.16 Pharmacological Inhibition of OATs

Islets were treated with OAT inhibitors for 24h with or without 200µM CMPF. Probenecid (P36400, Invitrogen), PCG (13752, Sigma Aldrich), and PAH (A3759, Sigma Aldrich) were dissolved in ultrapure water. ES (E0251, Sigma Aldrich) was dissolved in 100% methanol. Palmitate was conjugated to BSA prior to treatment of islets at a final concentration of 0.4mM. In all cases treatments were compared to vehicle control.

2.5.17 ATP Measurements

Isolated islets were equilibrated in 2mM glucose KRB for 1 h prior to stimulation with 1mM or 20mM glucose for 5 min. Islets were then treated with 100ul of 1x ATP extraction buffer from the StayBrite™ Highly Stable ATP Bioluminescence Assay Kit (Biovision, Milpitas, CA, USA) and homogenized. Homogenate was spun at 10,000rpm for 2 min and supernatant was collected for ATP measurements following manufacturer’s protocol.

2.5.18 Type 2 Diabetic (T2D) Plasma

T2D and NGT control samples were collected from mixed-gender, mixed-race patients. Samples used for global metabolomic profiling were obtained at 6th People’s Hospital in Shanghai, China. For ELISA quantification, a subset of the samples were purchased from Bioreclamation Inc. Remaining samples were obtained from the MRI Biorepository (IRB# 1011004282 (04-093)), by Eli Lilly (Indianapolis, IN, USA).
2.5.19 Statistics

Statistical significance was assessed using either the Student’s t-test, Welsh t-test, or a two-way ANOVA for repeated measures followed by a Bonferroni post-test comparison where required. $P<0.05$ was considered significant. All data is mean ± SEM unless otherwise specified.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
</tr>
</thead>
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<tr>
<td>mB-Actin</td>
<td>CTGAATGGCCCAAGTCTGA</td>
<td>CCCTGGGCTGCTCATACAC</td>
</tr>
<tr>
<td>mIns</td>
<td>GGTGGCCCATCCAGTACACCCCA</td>
<td>GAAGCCACGCTC CCCCAACAC</td>
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<td>mPdx1</td>
<td>ACAAATACATCTCCCCGCCC</td>
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</tr>
<tr>
<td>mMafA</td>
<td>CTGGTATCCATGTCCGTGC</td>
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<td>mHnf4a</td>
<td>CTTCAACCCTGAAGGACAC</td>
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<tr>
<td>mPc1</td>
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<td>mPc2</td>
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<td>mCpE</td>
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<td>mNanog</td>
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Table 7. Primers used for quantitative PCR.
### Table 8. Antibodies used for immunoblotting and immunofluorescent staining

<table>
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<tr>
<th>Antibody</th>
<th>IF Dilution</th>
<th>Western Dilution</th>
<th>Supplier</th>
<th>Cat. Number</th>
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<tr>
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<td>Dako</td>
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<td>Mouse α Insulin</td>
<td>1:200</td>
<td></td>
<td>Sigma</td>
<td>I1208</td>
</tr>
<tr>
<td>Mouse α Glucagon</td>
<td>1:150</td>
<td></td>
<td>Sigma</td>
<td>G2654</td>
</tr>
<tr>
<td>Rabbit α FOXO1</td>
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<td>1:1000</td>
<td>Cell Signaling</td>
<td>2880S</td>
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<tr>
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<td>1:1000</td>
<td>Abcam</td>
<td>Ab47308</td>
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<tr>
<td>Rabbit α SLC22A6</td>
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<td>1:1000</td>
<td>Novus Biologicals</td>
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<td>Rabbit α SLC13A3</td>
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<td>1:1000</td>
<td>Abcam</td>
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<td>Rabbit α α-actinin</td>
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<td></td>
<td>Santa Cruz</td>
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<td>Cell Signaling</td>
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<tr>
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2.6 CONCLUDING REMARKS

The studies described in this chapter are the first to identify a panel of metabolites that correlate with gestational diabetes as compared to normal glucose tolerant pregnant controls. The comparison between the GDM population and the T2DM population examined reveal a significant number of similarities, as well as a surprising number of differences. While the similarities likely reflect the common underlying pathology of these two forms of diabetes, the differences are likely due to the duration of diabetes, medications, and population differences. The metabolomics approach is inherently limited for its ability to compare different populations due to the strong influence of environmental factors such as diet on the metabolome.

While a strength of this study is that it examined a number of different populations, these populations are small and limited in scope. Patients all had a BMI of <30, excluding an extremely large proportion of the diabetic population that is obese. Furthermore, the study of prediabetic patients was limited to only 5 individuals. Further studies are necessary to determine when CMPF is elevated in the development of diabetes, and to determine whether this metabolite can be used as a predictive biomarker of diabetes development.

The effect of CMPF on beta cell function is profound, inducing a near complete loss of 1st phase insulin secretion in vivo and more than 50% reduction in glucose-stimulated insulin secretion in vitro. The role of altered transcription factor localization in this mechanism should be explored further. The observed effects could be caused by either a loss of nuclear PDX1, as well as a loss of total protein caused by both increased degradation and decreased synthesis. Furthermore, the translocation of FOXO1 and the role of FOXO1 in glucose metabolism with CMPF remains to be elucidated, as while there is an increase in nuclear FOXO1, total protein and cytosolic levels remain relatively consistent with CMPF treatment. Future work should characterize the phosphorylation status of both PDX1 and FOXO1 transcription factors with CMPF.

Finally, these studies were performed in lean, chow-fed animals that have no component of insulin resistance. Due to the critical role that insulin resistance plays in the development of GDM and T2DM, it will be important to characterize the role of CMPF under these conditions. Investigating how CMPF impacts beta cell compensation before and after the induction of insulin resistance is explored in Chapters 3 and 4. Furthermore, the effect of CMPF on other metabolic tissues is examined in Chapter 5.
Chapter 3- Rapid Elevation in Plasma CMPF is a Risk Factor for Diabetes in Humans and Accelerates Diabetes Development in Rodent Models

The following chapter has been submitted:


*Co-first authorship on the presented manuscript

Contributions by co-authors to the figures presented are stated in the figure legends
3.1 ABSTRACT

Type 2 diabetes (T2D) is a progressive disease characterized by a significant decline in beta cell function. CMPF, a furan fatty acid metabolite, is elevated in the plasma of T2D patients and associated with beta-cell dysfunction. However, the timing of CMPF elevation, and its role in the deterioration of beta-cell function during diabetes progression is unknown. Here, plasma CMPF was evaluated in a prospective cohort of individuals who were diagnosed as prediabetic or diabetic during a 5-year follow-up period. Individuals who had the largest increase in CMPF during follow-up had the highest risk of developing diabetes. To determine whether rapid elevation in plasma CMPF levels participate in the pathological progression of diabetes, animal models predisposed to diabetes due to either high fat diet feeding or genetics were treated with CMPF and islet function was studied. CMPF accelerated diabetes progression, including causing worsened glucose intolerance, and diminishing glucose-stimulated insulin secretion (GSIS) \textit{in vivo} and \textit{ex-vivo}. This was associated with impaired glucose metabolism, reduced beta-cell size, increased glycoprotein formation, and defective insulin granule formation characteristic of human diabetes. Thus, rapid elevation of CMPF is a strong risk factor that accelerates beta cell dysfunction during the pathological progression of diabetes.
3.2 INTRODUCTION

Type 2 diabetes (T2D) is a progressive disease characterized by a significant decline in beta cell function, ultimately resulting in insufficient insulin secretion to meet metabolic demands (Weir and Bonner-Weir, 2004). In the early stages, increased demand due to insulin resistance induces a state of beta cell compensation mediated by increases in beta cell mass and insulin secretion to maintain euglycemia (Butler et al., 2003; Kahn, 2003; Weyer et al., 1999). Chronically high insulin demand in combination with environmental and genetic factors are thought to contribute to the initial stages of beta cell dysfunction, manifesting as impaired fasting glucose (IFG) and impaired glucose tolerant (IGT) phenotypes, collectively termed prediabetes (Kahn, 2003; Prentki and Nolan, 2006). These individuals have abnormalities in fasting blood glucose and/or a mild impairment during a glucose tolerance test (Bansal, 2015). Importantly, individuals with prediabetes are at a significantly elevated risk of developing T2D if their beta cell function continues to decline, with approximately 70% of prediabetic individuals eventually developing T2D (Pan et al., 1997).

Interestingly, individuals with prediabetes can maintain a state of glucose intolerance for years before developing overt diabetes (Tuomilehto et al., 2001). The deterioration to T2D occurs at vastly different rates for different individuals, though the conversion is directly correlated with a sudden and drastic impairment in glucose-stimulated insulin secretion, independent of variables including BMI and insulin resistance (Ferrannini et al., 2004; Weir and Bonner-Weir, 2004). The mechanism underlying this “tipping point” of beta cell function, mediating the conversion from prediabetes to T2D, remains largely unknown. It has been suggested that chronic factors including glucolipotoxicity, inflammation, ER stress, and/or oxidative stress may collectively exhaust the beta cell compensatory capacity, reducing beta cell mass and insulin secretion over time until compensation is no longer feasible (Jonas et al., 2009; Poitout and Robertson, 2008; Prentki and Nolan, 2006). However, this chronic picture does not account for acute instances of beta cell failure in response to insulin resistant conditions. Gestational diabetes, a closely related condition to T2D that is also characterized by beta cell failure in response to insulin resistance, occurs acutely within weeks of insulin resistance onset and is rapidly resolved following
parturition (Buchanan and Xiang, 2005b). Therefore, we believe there is an underlying factor that acts to accelerate beta cell dysfunction in the conversion from prediabetes to overt diabetes.

Recently, we identified 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) as being significantly elevated in the plasma of patients with gestational and T2D (Prentice et al., 2014). Further studies in animal models revealed that this furan fatty acid metabolite functions to directly impair beta cell function through reduced glucose-stimulated insulin secretion and insulin biosynthesis, suggesting a potential causal role in diabetes development. However, the timing of the elevation in CMPF, and the role of CMPF in the deterioration of beta cell function during the progression to diabetes, before diabetes onset, has not yet been investigated. Here we performed a prospective study examining 196 participants, wherein 156 individuals maintained or developed either prediabetes or diabetes during a 5 year follow up period. CMPF was significantly elevated in patients with either overt type 2 diabetes or prediabetes. Interestingly, individuals who had the largest increase in circulating CMPF levels during the follow up period had the highest risk of developing diabetes, indicating that rapid elevation in CMPF levels is a strong risk factor for future diabetes development.

In order to determine whether rapid elevation in plasma CMPF levels participate in the induction of beta cell failure during the pathological progression of diabetes, we used high fat diet and genetically predisposed animal models with and without elevation of serum CMPF to study islet function. Pathologically elevated circulating CMPF in these predisposed animal models accelerated the progression of diabetes, shown as worsened glucose intolerance, diminished insulin secretion during an intraperitoneal glucose tolerance test (ipGTT) \textit{in vivo}, and impaired glucose stimulated insulin secretion \textit{ex-vivo}. This was also associated with reduced islet size, specifically beta cell mass and a dramatically increased number of proinsulin containing premature insulin granules. Through studying the isolated islets, we discovered that CMPF causes a significant reduction in glucose utilization, thus deteriorating glucose-stimulated insulin secretion to produce the worsened glucose excursions. Thus, CMPF is a strong risk factor that causes beta-cell dysfunction during the pathological progression of diabetes.
3.3 RESULTS

3.3.1 Demographic and Metabolic Features of Participants in the Shanghai Diabetes Study II Population

We examined a large prospective population of 4886 participants who presented at clinics in six regions of Shanghai, China in 2007-2008. The population was assessed for clinical parameters, including participating in a standard oral glucose tolerance test (OGTT) to confirm non-diabetic status and monitor insulin secretion (Table 9) (Bao et al., 2010). Five years following the initial evaluation, 1923 participants were re-evaluated for clinical characteristics and underwent a second OGTT. In this examination 507 individuals were determined to be either diabetic or prediabetic (Table 9) based on ADA criteria (American Diabetes, 2014). From this cohort, we selected controlled subpopulations of newly diagnosed diabetics (n=71), pre-diabetics (n=75), and normal glucose tolerant (n=50) individuals matched for age, gender, weight, and BMI at the follow-up visit (Table 10,11). Exclusion criteria included evidence of cardiovascular, liver, and/or kidney disease. Significant alterations in glucose tolerance, insulin secretion, HbA1c, and free fatty acid levels were observed between the populations at the follow up visit, as anticipated based on glucose tolerance status (Table 11).
Table 9. Shanghai Diabetes Study II

<table>
<thead>
<tr>
<th>Baseline (2007-2008)</th>
<th>Normal (n=1416)</th>
<th>Prediabetes (n=256)</th>
<th>Diabetes (n=251)</th>
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<tr>
<td>Follow Up (2011-2012)</td>
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<td>Diabetes (n=63)</td>
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<td>Prediabetes (n=75)</td>
<td>Diabetes (n=71)</td>
<td>Matched Prospective Cohort (n=196)</td>
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Table 10. Baseline Clinical Parameters of Shanghai Diabetes Study II-Matched Prospective Cohort in 2007-2008

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<th>Clinical Parameters (2007-2008)</th>
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<tr>
<td>Age (yr)</td>
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<td>Female (%)</td>
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<td>2hr Insulin (mU/L)</td>
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<td>TG (mg/dl)</td>
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<td>1.95 ± 0.81</td>
<td><strong>2.31 ± 1.57</strong>*</td>
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<tr>
<td>LDL (mmol/L)</td>
<td>3.02 ± 0.64</td>
<td>3.13 ± 0.66</td>
<td>3.10 ± 0.70</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.33 ± 0.32</td>
<td>1.22 ± 10.26</td>
<td>1.25 ± 0.28</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>21.74 ± 8.05</td>
<td>21.64 ± 7.36</td>
<td>23.92 ± 9.25</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>20.43 ± 11.08</td>
<td>22.25 ± 11.51</td>
<td>27.01 ± 17.09</td>
</tr>
<tr>
<td>Uric Acid (umol/L)</td>
<td>312.30 ± 86.18</td>
<td>325.10 ± 70.88</td>
<td>332.40 ± 79.72</td>
</tr>
</tbody>
</table>

Values are mean ± SD, bold indicate values are pathologically elevated and statistically significant compare to normal control population. *P<0.05, **P<0.01, ***P<0.001
Table 11. Clinical Parameters of Shanghai Diabetes Study II - Matched Prospective Cohort in 2011-2012

<table>
<thead>
<tr>
<th>Clinical Parameters (2011-2012)</th>
<th>Normal (n=50)</th>
<th>Prediabetes (n=75)</th>
<th>Diabetes (n=71)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>63.26±10.62</td>
<td>64.11±9.22</td>
<td>62.67±10.33</td>
</tr>
<tr>
<td>Female (%)</td>
<td>64.3</td>
<td>69.1</td>
<td>58.3</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>65.50±10.40</td>
<td>66.66±8.676</td>
<td>64.55±10.36</td>
</tr>
<tr>
<td>BMI</td>
<td>25.32±3.06</td>
<td>26.05±2.821</td>
<td>25.26±2.965</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mmHg)</td>
<td>127.7±14.19</td>
<td>131.6±15.54</td>
<td>132.7±14.66</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (mmHg)</td>
<td>81.21±8.162</td>
<td>80.90±8.458</td>
<td>80.85±8.517</td>
</tr>
<tr>
<td>Fasting Glucose (mM)</td>
<td>5.024±0.401</td>
<td>5.846±0.538***</td>
<td>7.520±2.383***</td>
</tr>
<tr>
<td>2hr Glucose (mM)</td>
<td>5.726±1.202</td>
<td>7.792±1.632***</td>
<td>12.27±4.148***</td>
</tr>
<tr>
<td>Fasting Insulin (mU/L)</td>
<td>5.504±2.293</td>
<td>7.676±4.724*</td>
<td>10.69±11.54*</td>
</tr>
<tr>
<td>2hr Insulin (mU/L)</td>
<td>27.92±22.09</td>
<td>73.82±70.94***</td>
<td>67±65.49***</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.234±0.546</td>
<td>1.992±1.229***</td>
<td>4.136±6.610***</td>
</tr>
<tr>
<td>HOMA-β</td>
<td>77.32±37.25</td>
<td>69.88±48.48</td>
<td>56.58±39.33***</td>
</tr>
<tr>
<td>FFA (mmol/L)</td>
<td>385.0±161.50</td>
<td>436.5±138.8</td>
<td>528.4±199.0***</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>1.448±0.642</td>
<td>1.952±1.156</td>
<td>2.460±2.231**</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>3.214±0.721</td>
<td>3.154±0.894</td>
<td>3.169±0.815</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.380±0.350</td>
<td>1.237±0.238</td>
<td>1.289±0.359</td>
</tr>
<tr>
<td>C-Reactive Protein (mg/L)</td>
<td>1.444±1.554</td>
<td>2.340±3.179</td>
<td>2.165±2.755</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>23.53±10.56</td>
<td>23.74±7.874</td>
<td>25.56±10.63</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>21.36±22.63</td>
<td>24.37±15.48</td>
<td>28.28±21.45</td>
</tr>
<tr>
<td>Uric Acid (umol/L)</td>
<td>323.4±84.15</td>
<td>336.5±78.49</td>
<td>335.4±76.56</td>
</tr>
</tbody>
</table>

Values are mean ± SD, bold indicate values are pathologically elevated and statistically significant compare to normal control population. *P<0.05, **P<0.01, ***P<0.001
3.3.2 Evaluation of Plasma CMPF In a Prospective Population

Plasma samples from the selected cohort of individuals at baseline and follow-up were evaluated for CMPF concentration. As anticipated, at the follow up visit the newly diagnosed diabetic population had significantly greater plasma CMPF concentration compared to normal glucose tolerant (NGT) controls (103.9+/−11.38uM in diabetics vs. 37.14+/−6.350uM in NGTs, P<0.001 ANOVA; Figure 18). Interestingly, the prediabetic individuals also had a significantly greater CMPF concentration than normal controls at this time point (84.14+/−10.90uM vs. 37.14+/−6.350uM, P<0.01 ANOVA; Figure 18), suggesting that CMPF is associated with both diabetes and prediabetes.

Analysis of CMPF plasma samples from the same population at baseline, when all subjects were non-diabetic, revealed a non-significant correlation between plasma CMPF and future diabetes/prediabetes status. Individuals who were prediabetic or diabetic at the follow up visit 5 years later both tended to have increased levels of CMPF compared to controls at baseline (P=0.025 future prediabetics vs. future NGT, P=0.0718 future diabetics vs. future NGT, ANOVA; Table 12), though this may associated with a subset of patients having some degree of glucose intolerance at the baseline visit. Interestingly, however, when we evaluated the change in plasma CMPF concentration between baseline and follow up we determined that individuals with the greatest increase in plasma CMPF concentrations between these time points were at the highest risk of developing overt diabetes overall (P=0.0004; Table 13). Those in the 3rd and 4th quartiles of highest change in CMPF levels, when adjusted for age, sex, and BMI, had a highly significantly greater risk of developing diabetes compared to those in the lower quartiles. The normal glucose tolerant population had no significant change in their CMPF concentrations through the follow-up period (Table 12). Therefore, a large elevation in circulating CMPF concentrations is associated with increased risk of diabetes development.
Figure 18. Plasma CMPF is Elevated in Prediabetic and Diabetic Populations

Plasma CMPF level evaluated in human fasting plasma samples collected in 2011-2012 and shown in A). bar graph and B). dot plot. n=50, 75, 71 for normal, prediabetes and diabetes groups respectively. Values are mean ± SEM, *p<0.05, **p<0.01, ***p<0.001. *ELISA performed by Ying Liu
Table 12. Fasting Blood Glucose And CMPF Levels In Shanghai Diabetes Study II- Matched Prospective Cohort At Baseline (2007-2008) And Follow-Up Visit (2011-2012)

<table>
<thead>
<tr>
<th></th>
<th>Normal (n=48-50)</th>
<th>Prediabetes (n=68-75)</th>
<th>Diabetes (n=59-71)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong> (2007-2008)</td>
<td>Fasting Glucose (mM)</td>
<td>4.98 ± 0.53</td>
<td>5.28 ± 0.58</td>
</tr>
<tr>
<td></td>
<td>CMPF (uM)</td>
<td>43.99 ± 2.855</td>
<td>83.31 ± 12.72</td>
</tr>
<tr>
<td><strong>Follow up</strong> (2011-2012)</td>
<td>Fasting Glucose (mM)</td>
<td>5.024 ± 0.401</td>
<td>5.846 ± 0.538 ***</td>
</tr>
<tr>
<td></td>
<td>CMPF (uM)</td>
<td>37.14 ± 6.350</td>
<td>84.14 ± 10.90 **</td>
</tr>
</tbody>
</table>

Values are mean ± SEM for CMPF and SD for fasting glucose, bold indicate values are pathologically elevated and statistically significant compare to normal control population. *P<0.05, **P<0.01, ***P<0.001
Table 13. Relationship Between Change In CMPF Levels And Risk Of Future Diabetes Development Adjusted For Age, Sex, And BMI

<table>
<thead>
<tr>
<th>Change in CMPF levels during 4-5 years follow up period</th>
<th>Diabetes</th>
<th>Likelihood Ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st quartile</td>
<td>Base line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd quartile</td>
<td></td>
<td>1.83</td>
<td>0.1760</td>
</tr>
<tr>
<td>3rd quartile</td>
<td></td>
<td>4.5</td>
<td>0.0340</td>
</tr>
<tr>
<td>4th quartile</td>
<td></td>
<td>13.9</td>
<td>0.0002</td>
</tr>
<tr>
<td>Continuous</td>
<td></td>
<td>12.6</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

Values are odds ratios (95% CIs) for diabetes, from conditional logistic regressions. All models are adjusted for age, sex and BMI.

*Statistics Performed by Katherine Leavey
3.3.3 Elevated CMPF Potentiates the Development of Diabetes in Rodent Models

To examine the role of rapid elevation in plasma CMPF in the context of pathological progression of diabetes, we utilized two models of diabetes development; high fat diet (HFD) feeding to produce diet-induced obesity (DIO) (Wang and Liao, 2012), and the genetic ob/ob mouse model (Lindstrom, 2010). Both models mimic the dyslipidemia, and beta cell compensation for peripheral insulin resistance that is characteristic of diabetes development in the human population. These compensatory responses include increases in beta cell mass and insulin secretion, and occur prior to the de-compensation and beta cell failure of overt diabetes (Lindstrom, 2010; Wang and Liao, 2012).

To establish our DIO diabetic model we fed male CD1 mice with a HFD comprised of 60% kcal from fat, or a sucrose-matched control chow diet for 6 weeks. To examine the role of CMPF in potentiating diabetes development, we then treated DIO mice with 6mg/kg CMPF (Prentice et al., 2014) once daily for an additional two weeks while maintaining the mice on the HFD. DIO and chow mice injected with vehicle were used as controls (Figure 19a). Eight week old male ob/ob mice were used as a genetic model of diabetes development. These mice were injected with 6mg/kg CMPF once daily for two weeks, as in the DIO model. Controls were treated with vehicle (Figure 19b). There was no difference in body weight (Figure 20) or food consumption (data not shown) at the end of the treatment period in either model.

At the end of the treatment period DIO mice treated with CMPF (DIO-CMPF) exhibited even greater fasting blood glucose concentrations than DIO control mice, with no significant difference in fasting plasma insulin levels (Figure 19c,d). Interestingly, unlike the DIO model, CMPF had no additive effect on fasting hyperglycemia and hyperinsulinemia observed in the ob/ob-Control mice, perhaps due to the existing severe hyperglycemia in ob/ob-Controls (Figure 19e,f). As expected, DIO control mice exhibited impaired glucose tolerance during an intraperitoneal glucose tolerance test (ipGTT) as compared to chow controls (Figure 19g). This corresponded to a reduced glucose-stimulated insulin response during the GTT (Figure 19i). Interestingly, DIO mice treated with CMPF (DIO-CMPF) had an even greater impairment in glucose tolerance than DIO controls, with a complete lack of glucose-stimulated insulin secretion (GSIS) during GTT (Figure 19g,i). These results were mirrored in the ob/ob mice treated with CMPF (ob/ob-CMPF). Despite significant glucose intolerance in ob/ob-Control mice, treatment
with CMPF acted to further impair glucose tolerance (Figure 19h), which again correlated to a significant reduction in GSIS during the GTT (Figure 19j). Together, this suggests that CMPF acts to impair beta cell function to potentiate diabetes development.
Figure 19. Elevated CMPF Potentiates the Development of Diabetes in Rodent Models

CMPF was delivered to animals via intraperitoneal injection at a dosage of 6mg/kg/day for the period of 2 weeks in A). Diet Induced Obesity (DIO) and B). Ob/Ob mouse models; Fasting plasma glucose and insulin levels were checked in C,D). DIO and E,F). Ob/Ob mouse models respectively; Glucose tolerance test (GTT) was performed and correspondent insulin secretion was checked during ipGTT on G,I). DIO and H,J). Ob/Ob mouse models respectively. n= 5-8 for GTT and n= 20-32 for others within each group. Values are mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.
Figure 20. CMPF Treatment Does not alter body weight in either DIO or ob/ob mice

Body weight in A). Diet Induced Obesity (DIO) and B). Ob/Ob mouse models before and after the two week injection period with either CMPF or vehicle control. n= 12-14 within each group. Values are mean ± SEM.
3.3.4 Treatment With CMPF Impairs Glucose-Stimulated Insulin Secretion

To verify that the observed worsening in glucose tolerance is due to impaired beta cell function, we isolated islets from both models and performed glucose-stimulated insulin secretion (GSIS) assays \textit{ex vivo}. Consistent with the observed impairment in insulin secretion during GTT, when we examined the glucose sensitivity of islets from individual mice, we found that islets from DIO-CMPF and ob/ob-CMPF mice had significantly reduced amplification in insulin secretion when stimulated with high glucose concentrations as compared to controls (Figure 21a,b). Interestingly, the observed defect in GSIS did not correspond to a reduction in total insulin content in either model (Figure 21c,d), suggesting an alternate source of beta cell dysfunction such as defective glucose utilization and/or loss of beta cell mass.
Figure 21. Treatment with CMPF Impairs Glucose-Stimulated Insulin Secretion

Glucose stimulated insulin secretion and total insulin content was examined in islets isolated from A,C). DIO and B,E). Ob/Ob mouse models respectively. Quantitative analysis of positive insulin staining on pancreata sections from D). DIO and F). Ob/Ob mouse models respectively. n=6-11, Values are mean ± SEM, *p<0.05, **p<0.01, ***p<0.001. GSIS: Glucose Stimulated Insulin Secretion.
3.3.5 CMPF Treatment Impairs Glucose Metabolism

To test this, we first assessed glucose metabolism through measuring changes in mitochondrial membrane potential (MMP) in response to the acute addition of glucose. Islets from both chow and DIO control mice exhibited a robust hyperpolarization of the MMP upon addition of 20mM glucose (Figure 22a). Islets from DIO-CMPF mice, however, had a significantly blunted response (Figure 22a), corresponding to the observed impairment in GSIS. Remarkably, when the same assay was performed with the acute addition of 400uM of palmitic acid, the DIO-CMPF islets exhibited a greater hyperpolarization of the MMP than either control (Figure 22b). This same trend was observed in islets from the ob/ob-CMPF mice compared to ob/ob-controls (Figure 22c,d). To confirm this effect of enhanced fatty acid utilization was not specific to murine islets, we treated human islets with vehicle, 400uM palmitate, or 200uM CMPF for 24 hours and evaluated their capacity for glucose oxidation. Pre-treatment with palmitate had no effect on glucose response compared to vehicle controls, however, human islets pre-treated with CMPF had a significantly blunted response to the addition of glucose, consistent with our previous findings (Figure 22e). However, when the effect of palmitate treatment was evaluated, we again observed a significantly enhanced response from CMPF pre-treated islets compared to both controls (Figure 22f). In all assays there was no effect on mitochondrial oxidative capacity with CMPF treatment (Figure 22e-f) or markers of mitochondrial biogenesis (data not shown). Overall, we have determined that CMPF induces a state of enhanced fatty acid utilization in islets, which reduces glucose metabolism, resulting in impaired GSIS.
Figure 22. CMPF Treatment Impairs Glucose Metabolism

Glucose-induced hyperpolarization of the mitochondrial membrane potential (MMP) was compared among islets isolated from the A). DIO mouse model and B). Ob/Ob mouse model. Palmitic acid-induced hyperpolarization of the mitochondrial membrane potential (MMP) was compared among islets isolated from C). DIO and D). Ob/Ob mouse models. Representative trace from a seahorse experiment performed on human islets with E). 20mM glucose and F). 150uM palmitic acid. n=4-12 for animal work and n= 3-4 for human islet work. Values are mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.
3.3.6 CMPF Treatment Increases AGEs and Oxidative Stress

This result prompted us to further examine glucose utilization in islets from CMPF-treated mice. We first investigated whether CMPF induced changes in glucose uptake or metabolism by determining expression of the glucose transporter Glut2, and the rate-limiting enzyme in glycolysis, glucokinase (Gck). Interestingly, we did not observe any differences at either the mRNA (Figure 23a) or protein levels (Figure 23b), suggesting that CMPF likely does not alter the ability of cells to uptake glucose or generate glucose-6-phosphate. Previous studies have demonstrated that under hyperglycemic conditions glucose can be shunted toward alternate pathways including glycogen synthesis (Malaisse et al., 1993), the pentose phosphate pathway (PPP) (Goehring et al., 2011; Spegel et al., 2013), and/or the hexosamine biosynthesis pathway (HBP) (Kaneto et al., 2001; Shankar et al., 1998; Tang et al., 2000), which are all associated with beta cell dysfunction. Interestingly, we did not observe alterations in transcript abundance of any of the key rate-limiting enzymes regulating these processes, including glucose-6 phosphate (G6P), glucose-6 phosphate dehydrogenase (G6PD) (Zhang et al., 2010), gluconolactonase (RGN), and glutamine fructose-6-phosphate amidotransferase (GFAT) (Kaneto et al., 2001), indicating that these pathways are not utilizing the excess glucose (Figure 23a). We did, however, observe an increase in total glycoprotein content in the islets of CMPF-treated mice compared to controls (Figure 23c). To determine if this is due to enhanced protein glycosylation through the HBP, we quantified total protein O-linked glycosylation in the islets of ob/ob-CMPF mice compared to ob/ob-controls. We observed a significant reduction in total O-linked glycosylation with CMPF treatment (Figure 23d). Therefore, the increase in glycoprotein content is likely due to aberrant protein glycation. Advanced glycation end-products (AGEs) are characteristic of diabetes progression and form through the addition of glucose to proteins without enzyme involvement (Singh et al., 2001). Consistent with an increase in AGE formation (Coughlan et al., 2011), as well as increased rates of beta-oxidation, islets isolated from DIO-CMPF and ob/ob-CMPF mice both exhibited significantly increased levels of ROS compared to controls (Figure 23e). Importantly, this was not associated with the induction of islet apoptosis as quantified by cleaved caspase 3/7 activity in islets ex vivo (Figure 23f). Furthermore, TUNEL staining of pancreatic sections following two weeks of CMPF injection showed a reduction in positive staining in ob/ob-CMPF mice compared to Controls, suggesting a protection against obesity-induced apoptosis. Thus, CMPF increases beta-oxidation and decreases glycolysis, increasing the formation of AGEs to increase oxidative stress and induce beta cell dysfunction.
Figure 23. CMPF Treatment Increases Advanced Glycation End-Products and Oxidative Stress

A). Quantitative PCR analysis and comparing the gene expression levels of genes involved in glucose utilization in islets isolated from the DIO mouse model; B). Representative image and quantitative analysis of western blot comparing GLUT2 protein expression in islets isolated from the Ob/Ob mouse model. Representative image and quantitative analysis of western blot analyzing C). total glycoprotein and D). O-linked glycosylation level in islets isolated from the Ob/Ob mouse model. E). Representative images and quantitative analysis of ROS measurements taken by PTI Fluorescence Microscopy in islets isolated from DIO and Ob/Ob mouse models. F). Caspase 3/7 activity was measured in islets isolated from DIO mouse model. n=4-15. Values are mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.
Figure 23

A. Relative Expression to B-actin (%)

B. Relative Expression (GLUT2/B-actin) (%)

C. Band Intensity (x1000)

D. Relative Expression (O-Glycosylation/B-actin) (%)

E. Relative Oxygen Species (DCF, FLU x1000)

F. Apoptosis Caspase 3/7 Activity (Luminescence x1000)
3.3.7 CMPF Alters Beta Cell Morphology

Increases AGES and correspondent reactive oxygen species (ROS) generation in the beta cell are known to impair multiple aspects of beta cell function (Robertson, 2004; Shu et al., 2011). In the absence of apoptosis at the conclusion of the study, we monitored the effect of CMPF on islet size, beta cell morphology, and insulin biosynthesis. Pancreata from chow and DIO controls, as well as DIO-CMPF mice were isolated at the end of the treatment period and stained for insulin (Figure 24a). As expected, DIO controls had significantly larger islets than chow controls, characteristic of compensation for increased insulin resistance (Figure 24c). Interestingly, however, we observed a significant reduction in islet size in DIO-CMPF pancreata compared to both DIO and chow controls (Figure 24c). This same trend was observed in the islets of ob/ob-CMPF mice compared to ob/ob-controls (Figure 24d,f). The observed smaller islet size corresponded to a slight reduction in insulin positive staining in the DIO model, with no change in ob/ob mice (Figure 24b,e). When stratified based on islet size, we found that DIO control mice had a greater percentage of large islets compared to chow controls, particularly extremely large islets (>50,000μm²). With CMPF treatment we observed a shift in islet size distribution, with a significant increase in the percentage of extremely small islets (<1,000μm²) and a reduction of large islets compared to HFD controls (Figure 24g). This trend was also observed in the islet size from ob/ob-CMPF mice compared to ob/ob-controls, despite ob/ob mice having overall significantly larger islets than those observed in the DIO model (Figure 24h).
Figure 24. CMPF Alters Beta Cell Morphology

Representative image and quantitative analysis of insulin positive staining on pancreatic sections from A,B). DIO and D,E). Ob/Ob mouse models respectively. Individual islet size was measured and islet size distribution was calculated from insulin stained mouse pancreatic sections from C,D). DIO and F,H). Ob/Ob mouse models respectively. n=3-4, Values are mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.


3.3.8 CMPF Reduces Beta Cell Hypertrophy to Reduce Islet Size

To further determine the cause of the increased number of extremely small islets, we investigated whether CMPF stimulates islet neogenesis. We began by simultaneously treating the mice in the DIO model with BrdU for one or two weeks of the injection period to monitor cell proliferation. As predicted, the DIO control mice had significantly more BrdU positive cells per islet than chow controls, indicating greater islet proliferation as a mechanism of beta cell compensation. Interestingly, the DIO-CMPF mice had no significant difference in the number of BrdU positive cells within islets compared to chow controls, and had significantly less than DIO-controls at both time points, indicative of beta cell decompensation or reduced compensation during the treatment period (two week treatment in DIO mice as representative, Figure 25a). There was also no difference in total pancreatic BrdU staining between chow controls and DIO-CMPF mice, indicating that there is also no difference in proliferation of the ductal cells (data not shown), which would be characteristic of islet neogenesis (Bonner-Weir et al., 2012). Finally, we explored whether CMPF treatment may be inducing beta cell dedifferentiation by staining the pancreata for neurogenin 3 (Ngn3), an early beta cell transcription factor known to be expressed during islet neogenesis, and beta cell dedifferentiation (Talchai et al., 2012). We did not observe any differences in Ngn3 staining between groups (Figure 25b), nor any difference in Ngn3 transcript levels from isolated islets (data not shown). Therefore, CMPF does not alter islet size through induction of apoptosis or through promoting islet neogenesis. Further, the observed defect in GSIS is likely not due to islet dedifferentiation.

In the absence of alterations in apoptosis, proliferation, neogenesis, or dedifferentiation, we wanted to determine if the reduction in islet size was due to reduced individual islet cell size. Under DIO or ob/ob conditions, beta cells are known to become hypertrophic as they increase insulin production to compensate for hyperglycemia (Bock et al., 2003; Hull et al., 2005). We therefore assessed individual beta cell size through transmission electron microscopy (TEM) images, as well as through quantification of number of nuclei per insulin positive area in histological sections. We observed using both methods that there was a significant increase in beta cell size in the DIO controls as compared to chow controls, as expected (Figure 25c). Remarkably, DIO-CMPF islets contained beta cells that were significantly reduced in size compared to DIO controls (Figure 25c). Consistent with reduced TUNEL staining with CMPF treatment, we also observed more beta cells per islet with CMPF treatment (Figure 25d). Thus,
CMPF reduces islet size through inducing beta cell hypotrophy, while maintaining a larger number of cells per islet as compared to DIO and ob/ob controls.
Figure 25. CMPF Reduces Beta Cell Hypertrophy to Reduce Islet Size

A). Representative images and quantitative analysis of islet specific BrdU positive staining in pancreatic sections from the DIO mouse model. B). Representative images and quantitative analysis of islet-specific Ngn3 positive staining in pancreatic sections from the Ob/Ob mouse model. C). Individual cell size and D). total cell number were measured and counted from insulin stained pancreatic sections. Black arrows point to the positive staining. n=3-4. Values are mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.
3.3.9 CMPF Treatment Impairs Insulin Granule Processing

Curiously, despite impaired beta cell glucose sensitivity, reduced GSIS, and beta cell hypertrophy in CMPF-treated mice we observed no reduction in total insulin content compared to controls (Figure 21c,d). To more closely examine the dynamics of insulin biosynthesis and storage with CMPF treatment we performed TEM experiments. Islets from DIO-control mice exhibited characteristics of early beta cell dysfunction including dilated ER (indicative of ER stress), hypertrophic mitochondria, and diminished insulin crystallization within granules (Figure 26a). This morphology was also observed in the islets of ob/ob-Control mice (Figure 26b). Unexpectedly, the islets from CMPF-treated mice contained fewer typical insulin granules, characterized by a white halo surrounding a dense black core, than either control (Figure 26a,c). Of the observed granules, there were a large number of membrane-bound structures with homogenous content, not typical of mature secretory granules. The number of these structures was significantly increased with CMPF treatment relative to the number of typical insulin granules, particularly in the ob/ob model (Figure 26b,d). Given the size and orientation of the atypical granules, particularly their close relationship to the golgi and plasma membrane, we predict that they may be misprocessed or premature insulin granules (Figure 26b). The increased level of protein glycation, which is known to alter protein aggregation, further supports this hypothesis (Hull et al., 2004). To determine if this is the case, we performed immunogold electron microscopy (immunogold-EM) with antibody staining against insulin in islets from the DIO-Control and CMPF mice. We found that all membrane-bound granule-like structures in islets from both mice contained insulin (Figure 26e). Given that the antibodies used for immunogold-EM and quantification of total insulin content are unable to distinguish between proinsulin and mature insulin, we finally quantified the amount of total proinsulin within the islets of CMPF treated mice compared to controls. Consistent with the atypical granules being premature or containing misprocessed insulin, islets from CMPF-treated mice contained nearly twice as much proinsulin as controls (Figure 26e). Thus, an increase in proinsulin due to granule misprocessing is likely responsible for the increased insulin content within islets.
Figure 26. CMPF Treatment Impairs Insulin Granule Processing

Representative transmission electron microscopy (TEM) images of islets isolated from A). DIO and B). Ob/Ob mouse models. Quantitative analysis of the relative distribution of dense core versus premature insulin granules within beta cells from C). DIO and D). Ob/Ob mouse models. E). Fasting plasma proinsulin content was measured in islets isolated from the DIO mouse model. F). Representative images of immunogold-EM insulin staining. White arrows point to the premature insulin granules. n=3 for TEM and n=2 for immunogold-EM. n= 10-23 for proinsulin content. Values are mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.
3.3.10 Premature Insulin Granules are Due to Protein Misfolding and are Capable of Being Secreted

Misprocessed or immature proinsulin-containing granules are characteristic of diabetes progression in humans based on numerous examinations of islets isolated from individuals with T2D postmortem (Masini et al., 2012; Sempoux et al., 2001). Furthermore, patients with T2D typically exhibit an increased proinsulin to insulin ratio, which has been suggested to be involved in impaired insulin signaling in diabetes (Grill et al., 2002). To examine the underlying cause of the increased number of immature granules, we began by investigating markers of impaired protein sorting in the granule maturation process. In spite of significantly increased proinsulin within the islets of CMPF-treated mice, circulating proinsulin was not significantly changed between DIO-CMPF and DIO-controls in the fasting state, suggesting the immature granules are not part of the constitutive pathway of secretion (Figure 27a). Levels of circulating proinsulin were, however, significantly increased in the fed state, indicating that these immature granules are capable of being secreted (Figure 27b). Together this indicates that the defect does not lie in granule sorting or exocytosis. Immature granules may accumulate due to differences in granule degradation (Uchizono et al., 2007). However, markers of lysosomal activity, the degradation machinery for misprocessed granules, including COX2 and LC3 were not significantly altered in the islets from DIO-CMPF mice compared to controls (Figure 27c&d). Further, there is no indication of elevated ER stress or activation of the unfolded protein response that would indicate aggregation of misfolded insulin within the ER, a process that would also signal for increased protein degradation (Figure 27c). Finally, we did not observe any differences in the transcript levels of the proteolytic genes responsible for insulin cleavage including PC1/2 or CpE (Figure 27e). In conclusion, the insulin biosynthetic pathway appears to be intact in islets from CMPF-treated mice with DIO or on an ob/ob background. Increased generation of immature insulin granules is likely attributed to increased levels of protein glycation, which impacts protein sorting and folding within granules, without impacting the exocytotic capacity.
Figure 27. CMPF Treatment Increases Immature Insulin Granule via Impaired Protein Sorting and Folding in Granules

A). Fasting and B). Fed circulating levels of proinsulin were analyzed in the DIO mouse model. Quantitative PCR analysis comparing gene expression levels of C). genes involved in oxidative and ER stress, and E). genes regulating insulin biosynthesis in islets isolated from the DIO mouse model. D). Representative image and quantitative analysis of western blot comparing LC3BI&II protein expression level in islets isolated from the DIO mouse model. n=4-10. Values are mean ± SEM, *p<0.05.
3.4 DISCUSSION

The ultimate cause of type 2 diabetes (T2D) is a failure of the pancreatic beta cells to compensate for increased demand associated with insulin resistance. The pathological progression to diabetes is characterized by increases in beta cell mass, insulin biosynthesis, and insulin secretion in the compensatory period, followed by a decline in function and mass, ultimately resulting in overt diabetes (Weir and Bonner-Weir, 2004). This decline in beta cell function has been largely attributed to glucolipotoxicity, a combination of hyperglycemia and hyperlipidemia acting on the beta cell to increase ROS production, ER stress, and ultimately induce apoptosis (Poitout et al., 2010). However, glucolipotoxic conditions are not prevalent in the early stages of diabetes progression, and even in the prediabetic period, when there are only mild changes to glucose tolerance and circulating free fatty acid levels (Bansal, 2015). Additionally, in acute onset forms of T2D, such as gestational diabetes, a model of chronic deterioration in beta cell function does not match the clinical evolution of the disease.

In the present study, we examined the Shanghai Diabetes Study II (SHDS II) prospective cohort (Bao et al., 2010) for changes in plasma CMPF levels as patients were diagnosed with diabetes or prediabetes. We found that subjects who had rapid, large increases in plasma CMPF levels during the 5 year follow up were at a significantly greater risk of developing overt diabetes in that short period. Based on our previous study into the effect of CMPF on impairing beta cell function (Prentice et al., 2014), we examined whether this dramatic increase in CMPF contributes to diabetes progression through promoting beta cell dysfunction in islets that are in a compensatory state. We utilized two models of diabetes development, high fat diet-induced obesity (DIO) and the ob/ob mouse, and treated them with CMPF for two weeks to determine the effect of rapid CMPF elevation on diabetes progression. In both DIO and ob/ob control mice we observed beta cell compensation including increased islet size, dilated mitochondria, and markers of ER and oxidative stress. Treatment with CMPF produced a rapid decline in beta cell function including worsened glucose tolerance, impaired GSIS, increased beta-oxidation with impaired glucose metabolism, and reduced islet mass. Interestingly, these changes were not associated with induction of apoptosis or beta cell dedifferentiation. Impaired glucose metabolism was, however, associated with increased protein glycation, and subsequent insulin granule misprocessing resulting in aberrant proinsulin accumulation. Overall, we conclude that rapid
elevations in circulating CMPF induce beta cell dysfunction through increasing beta-oxidation, resulting in altered glucose utilization, impaired insulin biosynthesis, and reduced GSIS to potentiate diabetes development (Figure 28).

Normal pancreatic beta cell function is dependent on appropriate insulin cycling, including adequate insulin biosynthesis, appropriate insulin sorting and crystallization, as well as insulin degradation to maintain a readily releasable pool (Uchizono et al., 2007). As newly formed secretory granules are formed from the trans-golgi network (TGN) the budding structures contain various secretory proteins including, but not limited to proinsulin and lysosomal proteins (Hou et al., 2009). As granules mature, sorting occurs and granules divide to remove non-proinsulin proteins into the lysosomes or the constitutive secretory pathway, leaving proinsulin to be cleaved into mature insulin and stored in releasable insulin pools. Previous work has demonstrated that this maturation process is perturbed in IGT and diabetes states in human patients, resulting in enhanced proinsulin accumulation and release (Grill et al., 2002; Sempoux et al., 2001). In the islets of CMPF-treated DIO and ob/ob mice we observe a profound accumulation of premature granules, and an abundance of proinsulin content. These granules are capable of exocytosis and mice exhibit a significant elevation in circulating proinsulin in the fed state compared to controls, similar to the human condition. We hypothesize that CMPF is altering the process of granule processing in part through impairment of carbohydrate utilization. A combination of impaired glycolysis, associated loss of signals to potentiate insulin biosynthesis, increased AGEs, and elevated ROS likely act to impair insulin processing. Studies utilizing pharmacological or genetic inhibition of glucokinase have demonstrated that glycolysis is required for potentiation of proinsulin biosynthesis, and that addition of alternate fuel sources, such as fatty acids, are not capable of stimulating this process (Jonas et al., 2009; Uchizono et al., 2007). In the absence of glucose metabolism with CMPF treatment, glucose accumulates within the cell, resulting in abnormal formation of advanced glycation end-products (AGEs). AGEs are characteristic of diabetes progression, as well as numerous other disorders including chronic renal failure and Alzheimer’s disease (Singh et al., 2001; Vitek et al., 1994). Interestingly, uremia is the only other condition where CMPF is known to be elevated (Niwa et al., 1988). In Alzheimer’s, AGEs are associated with increased protein cross-linking, potentiating plaque formation and neuronal dysfunction (Vitek et al., 1994). In the beta cell, increased AGEs may be associated with aberrant insulin crosslinking, preventing appropriate folding and cleavage, to
result in increased accumulation of premature insulin granules. Further, induction of oxidative stress associated with AGE accumulation can also act to impair insulin biosynthesis. ROS reduces Akt activation, resulting in diminished activity of key beta cell transcription factors PDX1 and MAFA, and enhanced nuclear translocation of FOXO1, decreasing insulin transcription and processing (Kawamori et al., 2003). This is consistent with what was previously observed with CMPF treatment in chow-fed mice (Prentice et al, 2014). Altogether, altered insulin granule processing/maturation in islets from CMPF-treated mice is likely caused by alterations to glucose utilization.

CMPF-mediated impairment in glucose utilization is likely also responsible for the observed shift in islet mass, with a disproportionate loss of extremely large islets and an increase in very small ones in both DIO and ob/ob mice. Interestingly, this same shift in islet size distribution is also observed in pancreata from diabetic humans (Kilimnik et al., 2011). In a study by Kilimnik and colleagues, the distribution of endocrine cell types was examined in whole pancreas sections from diabetic and normal glucose tolerant donors. The investigators determined that diabetic patients had a significant loss of large islets as compared to normal glucose tolerant individuals, and that beta cell mass within existing large islets was also significantly reduced. This loss in beta cell mass is associated with glucotoxicity, caused by enhanced glucose flux into the beta cell acting to both potentiate cell growth and insulin synthesis through increased metabolism, as well as increasing AGE formation and enhance ROS. Conversely, reduced glucose availability and metabolism are both sufficient to induce opposing alterations in beta cell size. Studies in isolated islets have shown that altering the glucose concentrations in the media of isolated islets from 28mM to 3.3mM is sufficient to stimulate crinophagy and reduce total insulin content and cell mass (Sandberg and Borg, 2007). Similarly, in a genetic model of impaired glucose metabolism where a beta cell specific knockout of pyruvate dehydrogensase alpha (PDHa) was used to block the conversion of pyruvate to acetyl-CoA for use as a mitochondrial substrate, islets were hypotrophic with reduced insulin content and reduced insulin secretion despite hyperglycemia in the mice (Patel et al., 2014). Overall, this indicates that impairment of glucose metabolism by CMPF is sufficient to induce alterations in beta cell size, resulting in the observed shift in islet mass, independent of glycemic status.

The observation that rapid elevations in CMPF are more strongly associated with diabetes development than concentrations that are constantly moderately high suggest that the body is
able to compensate for the effects of CMPF to some extent. The ability of the beta cell to compensate for stress associated with insulin resistance and metabolic disturbances for years in the prediabetic state speaks to the resilience of this cell type. Interestingly, the rapid elevation of CMPF in models of diabetes progression accelerates diabetes development and produces a beta cell phenotype that closely resembles the human condition including reduced insulin sensitivity (Mari et al., 2010), loss of first phase insulin secretion (Cheng et al., 2013), elevation in proinsulin content and secretion (Grill et al., 2002; Sempoux et al., 2001), AGE formation (Singh et al., 2001), and disproportionate loss of large islets (Kilimnik et al., 2011). Thus, elevated circulating CMPF is a major risk factor for the development of beta cell failure in the progression of diabetes.
Figure 28. Schematic Diagram Illustrating the Proposed Mechanism of CMPF Potentiated Beta-cell Dysfunction during the Pathological Progression of Diabetes
### 3.5 MATERIALS AND METHODS

#### 3.5.1 Study Population and CMPF Quantification

Fasting plasma samples were obtained from the Shanghai Diabetes Study II (SHDS II) Cohort of patients representing a cross sectional population from six communities in Shanghai, China (Bao et al., 2010). Patient samples were collected at baseline in 2007-2008 prior to completing a standard 75g oral glucose tolerance test (OGTT) to characterize glucose tolerance in subjects without known diabetes. Follow-up samples were collected from the same patients in 2011-2012 prior to another OGTT. At each visit a complete physical examination was conducted and clinical parameters evaluated. Each participant gave written informed consent for the study. Plasma samples were stored at -80°C following collection and prior to analysis. CMPF was quantified by enzyme-linked immunosorbant assay (ELISA) according to manufacturer’s instructions (NovaTein Biologicals, USA) (Prentice et al., 2014).

#### 3.5.2 Logistic Regression Analysis

Samples with either a missing baseline, outcome or both values for CMPF were removed and the data was normalized by log2 transformation. To assess the predictive power of the change in CMPF between the baseline and outcome measurements on the progression to an overt diabetic state, the data was subjected to exact conditional logistic regression analysis using the `clogit` function (`survival` package from CRAN) in R 3.1.3. Patients who developed diabetes by the second time point (starting from a NGT or prediabetic state) were compared to subjects who maintained their NGT or prediabetes diagnosis over the course of the study. Values were treated as both continuous and categorical (split by quartile of CMPF change), and were stratified by baseline age (binned: ≤35, 35-49, 50-64, or ≥65 years), sex (male or female), and BMI (binned: <18.5, 18.5-25, 25-30, or 30-40 kg/m²).
3.5.3 Animal Models

All animals were housed in an environmentally controlled facility on a standard 12-hour light/dark cycle with free access to food and water. Five-week-old male mice were purchased from Charles River (CD1 mice) or Jackson Labs (ob/ob mice) and allowed to acclimate for one week prior to beginning the study. CD1 mice were placed on a 60% kcal from fat high fat diet (HFD; OpenSource diets D12492, Research Diets Inc, USA) or a matched sucrose chow diet with 10% kcal from fat (OpenSource diets D12450J, Research Diets Inc, USA) for 6 weeks. Mice were maintained on their respective diets while injected intraperitoneally (i.p.) once daily for two weeks with 6mg/kg CMPF or vehicle (ethanol) (Prentice et al., 2014). CMPF (Cayman Chemical, USA) was prepared by dissolving in 100% ethanol to a stock concentration of 100mM and diluted in sterile saline for injection. Ob/ob mice were maintained on a standard chow diet (Teklad diet 2018, Harlan Laboratories, USA) throughout the two weeks of i.p. injection with 6mg/kg CMPF or vehicle. For quantification of islet proliferation mice were injected with BrdU (50ug/g body weight) once daily in addition to CMPF or vehicle. Mice were monitored for body weight weekly. All experiments were approved by the Animal Care Committee at the University of Toronto, and animals handled according to the Canadian Council of Animal Care guidelines.

3.5.4 Glucose Tolerance Tests

At the end of the injection period intraperitoneal glucose tolerance tests (IpGTTs) were performed on mice fasted for 14 hours overnight (Allister et al., 2013). Mice were injected i.p. with 1g sucrose per kilogram body weight. Blood samples (<25ul) were drawn at time 0 (fasting), 10, 20, and 30 minutes post injection for insulin and glucose measurements and additionally at times 60 and 120 for glucose measurements. Plasma insulin was quantified by ELISA according to manufacturer’s instructions (AIS, HongKong, Cat#32270).

3.5.5 Islet Isolation, Insulin Secretion, and Apoptosis

Mice were anesthetized using isoflurane. Total blood volume was collected from the chest cavity following removal of the heart. Tissues were collected and flash frozen in liquid nitrogen for future analysis or fixed in a 4% formaldehyde solution for histology. Islets were isolated through pancreatic perfusion and collagenase digestion as previously described(Luu et al., 2013). Islets were hand picked three times and allowed to recover overnight in RPMI (1640; Sigma)
supplemented with 10% FBS prior to analysis. Glucose stimulated insulin secretion (GSIS) assays were performed as previously described (Prentice et al., 2014). Briefly, 30 islets from each mouse were picked into Kreb’s Ringer Buffer (KRB) containing 2mM glucose and for one hour. The media was then disposed of and fresh 2mM glucose KRB added for 30 minutes at 37°C. The supernatant was collected and stored at -20°C, and 20mM glucose KRB was added to the islets for 30 minutes at 37°C for high glucose stimulation. For palmitate-stimulated insulin secretion (PSIS), KRB containing 2mM glucose and 400uM palmitate conjugated to BSA was added instead. Supernatant was again removed and stored. Islets were then lysed with acid ethanol at 4°C for 24 hours, samples dried down using a speedvac and resuspended in 60ul ultrapure water for quantification of total DNA (Basford et al., 2012). Insulin content in secretion samples was evaluated using an insulin HTRF assay, according to manufacturers instructions (Cisbio, USA). Insulin HTRF was quantified on a BMG Pherastar plate reader, as previously described (Prentice et al., 2014). Cleaved caspase 3/7 assay was performed according to manufacturers instructions using 30 islets (G8091, Promega, USA).

3.5.6 Histology

Pancreata were fixed in 40% formaldehyde immediately following dissection. Tissue processing and immunostaining were performed as described previously (Luu et al., 2013). Images of each section were acquired using Aperio Imagescope at 40x magnification. Quantification of staining was calculated by using positive pixel count analysis (Aperio Image scope).

3.5.7 Transmission and Immunogold Electron Microscopy

Isolated islets were fixed, processed, and images acquired as previously described (Basford et al., 2012). Images were acquired at 2000x and 10000x magnification to examine whole-cell and subcellular morphology, respectively. Beta cell area and insulin granule number was manually quantified using ImageJ software (Lu et al., 2010).

3.5.8 Gene Expression

Total RNA was extracted from isolated islets using the Qiagen RNeasy Plus mini kit (Hilden, Germany). Reverse transcription from total RNA and quantitative real time PCR was performed
as previously described (Basford et al., 2012). Primers were designed using Primer3 software (NCBI) and are listed in Table 14. Data were normalized to β actin mRNA.

3.5.9 Western Blotting

Mouse islets were washed in sterile PBS, and then lysed in RIPA buffer (Cell Signaling, USA) containing protease inhibitor cocktail (Roche, Canada) and stored at -20C prior to use. Lysates were spun at 12,000rpm and supernatant was evaluated for protein content by Bradford assay (BioRad, Canada). Equal amount of protein were then combined with sample buffer containing DTT and loaded onto a 4-15% SDS-PAGE gradient gel (BioRad, Canada) and run at increasing voltages of 50V for 30 minutes, 75V for 30 minutes, and then 100V for 30 minutes. Proteins were then transferred onto PVDF membrane using a Turbo Blotter (BioRad, Canada). The membrane was probed with antibodies as listed in Table 15, and imaged using Kodak Imager 4000pro (Carestream, USA).

3.5.10 Mitochondrial Membrane Potential (MMP)

Islets isolated from mice were loaded with rhodamine 123 (25 ug/ml) in imaging buffer without glucose for 10 minutes. Islets were washed and placed in imaging chambers containing imaging buffer with no glucose. Images were taken at 10s intervals at ex:511nm by an Olympus IX70 inverted epi-fluorescence microscope in combination with an Ultrapix camera and a computer with PTI imaging software, as previously described. Glucose was added to a final concentration of 20mM to observe the correspondent change of MMP in cells. Alternately, palmitate conjugated to fatty acid free BSA was added to a final concentration of 400uM. 5mM NaN_3 was added to fully depolarize the MMP (Diao et al., 2008).

3.5.11 Reactive Oxygen Species (ROS)

The level of H_2O_2 was determined using 2',7'-dichlorodihydro fluorescein diacetate (CM-H_2-DCFDA) (Invitrogen, Canada) as previously described (Robson-Doucette et al., 2011). Briefly, isolated islets were loaded with DCFDA for 25 minutes in 2mM glucose imaging buffer at 37C. Islets were washed and imaged at ex:475nm for 20ms once.
3.5.12 Human Islets and Oxygen Consumption Measurements

Human islets from review board approved healthy donors were provided by the IsletCore and Clinical Islet Laboratory (University of Alberta, Canada). Islets were picked into low glucose DMEM media (Gibco, ref#11885-084) with 10% FBS 1% penicillin/streptomyosin, 1% L-glutamine and treated with either vehicle control, 200uM CMPF, or 400uM palmitate for 24 hours (Prentice et al., 2014). 70 Islets were then picked into each well of XF24 islet plates (Seahorse Bioscience, USA) and secured with a mesh cover, as previously described (Wikstrom et al., 2012). Cells were incubated in 2mM glucose KRB without bicarbonate for one hour prior to being loaded into the XF24 machine. Oxygen consumption rates (OCR) were measured at 2mM glucose, followed by either 20mM glucose or 400uM palmitate to measure substrate utilization. All islets were then treated with 5uM oligomycin, 5uM FCCP, and 5uM rotenone and 5uM antimycin A. Raw traces were normalized to basal respiration at 2mM glucose.

3.5.13 Statistics

Statistical significance was assessed using either the Student’s t-test or a one way/two-way ANOVA for repeated measures followed by a Bonferroni/Dunnett/Tukey post-test comparison where required. \( P<0.05 \) was considered significant. All data is mean ± SEM unless otherwise specified.
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<th>Gene Name</th>
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<th>Reverse (5’-3’)</th>
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Table 15. Antibodies used for western blotting

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3.6 CONCLUDING REMARKS

This chapter establishes rapid elevation in plasma CMPF as a risk factor for the development of diabetes in a human population. Furthermore, we demonstrate that elevating CMPF to diabetic concentrations in rodent models of prediabetes accelerates diabetes development through causing rapid a decline in beta cell function, resulting in a beta cell phenotype that closely resembles human diabetes. The use of murine models of obesity and insulin resistance are more physiologically relevant to the diabetic conditions where CMPF is elevated in humans as compared to the model used in Chapter 2. Interestingly, while many of the effects of CMPF on the beta cell are similar to those observed in lean animals, there are distinct differences, indicating that the starting health of the beta cell is a factor in determining the effect of CMPF. For example, while CMPF impairs glucose metabolism, glucose-stimulated insulin secretion, and increases proinsulin content in both conditions, obese animals do not exhibit significant reductions in insulin transcription. This is potentially due to the enhanced function of the beta cell in the compensating state seen in obesity.

The population examined in this chapter was significantly larger than the cohorts where we originally identified CMPF, as well as had the benefit of being taken from a longitudinal study where we were able to monitor CMPF levels over a 5 year follow up period. Interestingly, CMPF was significantly elevated in prediabetic patients compared to normal glucose tolerant controls, suggesting that it may be a biomarker for prediabetes. Larger and more diverse populations need to be examined to determine if this is widely applicable. Interestingly, levels of CMPF in patients who were prediabetic at both time points examined were consistently elevated compared to normal glucose tolerant controls, but did not change over time. This suggests that there may be a threshold concentration of CMPF that is required to induce beta cell function. Also, it indicates that the beta cell is able to compensate for minor elevations in CMPF as long as this level does not increase. Large changes in CMPF significantly increase risk of diabetes development, consistent with CMPF being a “tipping point” in the induction of beta cell failure in the transition between prediabetes and overt diabetes. Further examination of larger populations at more time points are necessary to characterize exactly when this elevation in CMPF occurs, and how this relates to beta cell function in humans.
The cause of the significant increase in CMPF is also an important question for further study. The short time period and lack of correlation with changes in BMI or diet would indicate that the increase is likely due to impaired clearance rather than increased consumption in the diet or endogenous production. However, this remains to be elucidated. While CMPF does not correlate with changes in markers of kidney function that were evaluated in this population including uric acid, creatine, and creatinine, it is possible that there is a specific alteration in OAT transporter function that is responsible for CMPF accumulation, rather than kidney dysfunction in general. Examination of other metabolites that are cleared through these transporters would indicate if this were a possible explanation.

Finally, a consistent finding in both the diet induced obese (DIO) and leptin-deficient ob/ob mouse models used in this chapter was that treatment with CMPF ameliorated steatosis. Liver fat was almost completely absent in the animals treated with CMPF and this correlated to significantly improved insulin sensitivity by insulin tolerance test. Therefore, it is difficult to elucidate which aspects of the beta cell phenotype are due to CMPF directly, and which are occurring in response to the reduction in insulin resistance. Improved insulin sensitivity may be responsible for the shift in islet size for example, as islet hypertrophy is associated with compensation for insulin resistance. Utilizing genetic models with consistent hepatic insulin resistance may be required to elucidate this phenotype.
Chapter 4- CMPF Induces Persistent Impairment in Beta Cell Function due to Metabolic Inflexibility

The following chapter is prepared for submission:


Contributions by co-authors to the figures presented are stated in the figure legends.
4.1 ABSTRACT

Gestational diabetes (GDM) significantly increases future risk of developing type 2 diabetes (T2D). While this transition is attributed to impaired beta cell function, the underlying cause remains unknown. Here, we explore whether temporal elevation in the furan fatty acid metabolite CMPF has long-term effects contributing to future diabetes risk. Previous CMPF exposure impairs glucose tolerance and glucose-stimulated insulin secretion for at least 4 months following elimination of CMPF from circulation, accelerating diabetes development under both lean and insulin resistant conditions. Mechanistically, this is due to persistent metabolic inflexibility, with islets preferentially utilizing fatty acids as a substrate over glucose resulting in chronically reduced expression of glucose transporter and glucokinase. Interestingly, CMPF-treated islets are capable of rescuing glucose-stimulated insulin secretion upon inhibition of beta-oxidation. Thus, CMPF exposure during GDM may induce chronic beta cell metabolic inflexibility, reducing the ability to compensate for future metabolic challenge and accelerating development of T2D.
4.2 INTRODUCTION

Gestational diabetes (GDM) is a condition of transient glucose intolerance that occurs in women with no previous history of diabetes. It is caused by a failure of the pancreatic beta cells to compensate for the increased insulin demand of late pregnancy (Buchanan and Xiang, 2005b). In the early stages of gestation there is no change in insulin sensitivity, though an increase in circulating insulin levels promote fat deposition (Barbour et al., 2007). During the transition between the second and third trimester there is a massive induction of insulin resistance that occurs to reprogram maternal metabolism toward a reliance on the stored fatty acids (Catalano et al., 1999). This transition is required to prevent the antilipolytic activity of insulin, and promotes an increase in circulating free fatty acid (FFA) levels for maternal fuel supply in order to preserve glucose for the developing fetus. This 2-3 fold increase in insulin resistance is mediated by a combination of placental hormones placental lactogen (PL) and placental growth hormone (PGH), as well as increased adipose tissue mass, inflammation, and reduced physical activity (Barbour et al., 2007; Handwerger and Freemark, 2000). In uncomplicated pregnancy, this rapid and severe induction of insulin resistance is compensated by an increase in beta cell mass and improved beta cell function to increase insulin secretion as much as 200-250% (Catalano et al., 1999; Kuhl, 1991). Thus, glucose tolerance can be maintained at prepregnancy levels. When this compensation fails to occur, GDM develops and women become hyperglycemic in the final stages of pregnancy. This acute form of diabetes resolves rapidly following delivery as the placental hormones are lost from circulation.

Importantly, women with GDM have an extremely high risk of developing type 2 diabetes (T2D), with up to 50% of women with GDM developing T2D within 5 years postpartum, and up to 70% within 10 years (Bellamy et al., 2009). The development of T2D following a GDM pregnancy is directly correlated to a decline in beta cell function, as measured by an ISSI-2 score (Retnakaran et al., 2010). This is demonstrated in studies following women the first year postpartum, when insulin resistance has returned to prepregnancy levels. Women who maintain normal glucose tolerance throughout pregnancy and the postpartum period maintain beta cell function, while women with GDM who go on to develop T2D continue to experience a decline in
beta cell function, independent of insulin resistance. The cause of this persistent beta cell dysfunction is largely unknown.

Recently, we utilized a global metabolomics screening approach to identify circulating metabolites that are dysregulated in GDM compared to normal glucose tolerant pregnant women. We identified the furan fatty acid metabolite CMPF as being dramatically elevated in plasma of women with GDM (Prentice et al., 2014). Further investigation revealed that acute CMPF treatment induces glucose intolerance, reduces whole-body glucose utilization, and impairs insulin biosynthesis secretion. Thus, CMPF may play a causal role in the pathogenesis of GDM. Here we examine if elevated CMPF during GDM may play a causal role in the high rate of progression to T2D. We demonstrate that CMPF induces beta cell dysfunction that persists for months following exposure, despite the absence of CMPF from circulation and tissues. This beta cell dysfunction is caused by metabolic inflexibility, an impaired ability to switch from fatty acid to carbohydrate substrates, and thus an impaired response to glucose challenge. Further, CMPF impairs the ability of the beta cell to compensate for future metabolic challenge, accelerating the development of diabetes in response to metabolic stress.
4.3 RESULTS

4.3.1 CMPF Induces Persistent Glucose Intolerance

To determine the duration of effect of CMPF treatment we began by treating mice with CMPF once daily for seven consecutive days, the equivalent time of the final trimester of pregnancy when GDM is present (Figure 319a)(Prentice et al., 2014). Intraperitoneal injection of 6mg/kg CMPF elevated circulating levels of CMPF to concentrations observed in GDM patients within 20 minutes of injection (Figure 30a). Controls were treated with an equal volume of vehicle, or 6mg/kg oleic acid to ensure that any changes were not due to increases in circulating free fatty acids. CMPF was completely cleared from circulation and tissues within 24 hours of each injection. Following the final day of injection, CMPF was no longer detectable in the mice at any time point examined (Figure 30a). Mice were maintained on a standard chow diet with no further treatment for 16 weeks. Mice treated with CMPF appeared phenotypically normal during the follow up period. While CMPF-treated mice gained slightly but significantly less weight than both vehicle and oleate controls, they had no differences in random blood glucose or food consumption (Figure 30b-d).

To establish if CMPF has persistent metabolic effects we performed oral glucose tolerance tests (OGTTs) on the mice at 6 and 15 weeks following final injection to evaluate glucose tolerance and insulin secretion. Remarkably, mice treated with CMPF were severely glucose intolerant at both time points compared to vehicle controls (Figure 30c,f). Oleate treatment had no effect on glucose tolerance at the 6 week timepoint, suggesting this effect is not a consequence of fatty acids in general (Figure 30e). Interestingly, fasting blood glucose levels were not significantly different between groups at either time point, consistent with the phenotypically normal appearance of the mice.
4.3.2 Glucose Intolerance is Due to Loss of Glucose-Stimulated Insulin Secretion

Consistent with our previous findings, glucose intolerance during OGTT corresponded with a complete absence of glucose-stimulated insulin secretion (GSIS) at both timepoints (Figure 30g,h). This was particularly associated with a loss of first-phase insulin secretion, as observed in human diabetic patients. To further examine the source of glucose intolerance we evaluated insulin sensitivity by insulin tolerance test (ITT) at both 6 and 15-week time points. There was no difference in insulin sensitivity between any of the groups at either time point (Figure 30i,j). This is further supported by no difference in fasting or random plasma insulin or blood glucose levels at both 6 and 15 weeks post treatment (Figure 30g,h). Therefore, CMPF induces persistent glucose intolerance due to impaired GSIS, despite its absence from tissue and circulation, for at least 4 months post treatment.
Figure 29. Treatment protocol for Investigating the Long-Term Effect of CMPF

a) Mice were injected once daily for seven consecutive days. Mice were then maintained on a standard chow diet for up to 16 weeks post final injection.
Figure 30. CMPF Induces Long-Term Impairment in Glucose Tolerance.

a) CMPF concentration in mouse plasma following intraperitoneal injection as determined by selected reaction monitoring mass spectrometry (SRM-MS) and a stable isotope labeled standard. b) Body weight and (c) weekly food consumption during the follow up period. Blood glucose levels during ipGTT at (d) six weeks and (e) 15 weeks following the final injection. Corresponding plasma insulin levels during ipGTT at (f) six weeks and (g) 15 weeks post final injection. Blood glucose levels following bolus of insulin during insulin tolerance test (ITT) at (h) six and (j) 15 weeks following final injection. Random blood glucose values at (i) six and (j) 15 weeks following injection. N=8-12 for all. Values are mean +/- SEM. *P<0.05, **P<0.01, ***P<0.001.
Figure 30

A

B

C

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Islets isolated from CMPF treated mice six weeks following final injection assessed for (a) islet size, (b) glucose-stimulated insulin secretion, and (c) total insulin content. (d) Insulin granule number and distribution evaluated by transmission electron microscopy. N=8 mice per group for insulin secretion and content. N=5-8 islets/mouse with 8 mice/group for islet size. N=3/group for TEM. Values mean +/- SEM. *P<0.05, **P<0.01.
4.3.3 The Insulin Secretory Defect is Inherent to the Beta Cell

To confirm that the loss of GSIS is inherent to the beta cell and not caused by signaling from another tissue type, we isolated islets from mice 6 weeks post final injection. Interestingly, islets from CMPF-treated mice were significantly larger than those from either vehicle or oleate controls, suggesting a potential compensatory mechanism (Figure 31a). Consistent with in vivo findings during OGTT, islets from CMPF-treated mice had significantly impaired GSIS ex vivo compared to both control groups (Figure 31b). Despite larger islet size, this defect in GSIS corresponded to a significant reduction in total insulin content (Figure 31c). Ultrastuctural examination of the islets confirmed this finding, with a reduction in mature insulin granule number compared to both control groups as evaluated by transmission electron microscopy (Figure 31d,e). Together, this suggests that CMPF induces a persistent impairment in GSIS and insulin biosynthesis that acts to activate a compensatory increase in islet size to prevent the development of overt diabetes.
4.3.4 CMPF-Treatment Worsens Response to Metabolic Challenge

The persistence of beta cell dysfunction prompted us to examine whether prior CMPF exposure might accelerate the development of diabetes under the conditions of metabolic challenge. While the development of T2D following a GDM pregnancy is primarily associated with beta cell dysfunction, it occurs in the presence of insulin resistance and frequently obesity (Buchanan and Xiang, 2005b). Therefore, we challenged the 7-day CMPF-treated mice with a 60% kcal from fat high fat diet (HFD) for 4 weeks beginning on the day after the final injection (Figure 32a). Mice were then evaluated for glucose tolerance and beta cell function as compared to vehicle treated controls maintained on chow (Chow-controls) or HFD (HFD-controls) respectively.

As anticipated, mice fed a HFD for 4 weeks gained significantly more weight than chow-fed mice over the duration of the feeding period (Figure 33a). Consistent with results in long-term chow-fed CMPF-treated mice, however, CMPF-treated HFD-fed (CMPF-HFD) animals gained slightly but significantly less weight than HFD-controls, though this did not correspond to differences in food consumption (Figure 33a,b). Despite lower body weight, CMPF-HFD mice displayed significantly elevated fasting blood glucose compared to both Chow- and HFD-controls, suggesting metabolic impairment (Figure 33c). This was confirmed by glucose tolerance testing where CMPF-HFD mice had worsened glucose tolerance compared to both controls (Figure 33d). HFD-controls had significant glucose intolerance compared to chow, as expected. In line with our previous observations, this glucose intolerance again corresponded to a complete absence of GSIS during GTT (Figure 33e). In the fasting state, plasma insulin levels in the CMPF-HFD group were comparable to those of the HFD-control group, suggesting that the effect is beta cell specific.

To further validate the specificity of action of CMPF we utilized a second furan acid as a control under the same experimental protocol. 2,5-Furandicarboxylic acid (FA) is comprised of a furan ring with two carboxylic acid side chains, similar to CMPF, making it an ideal control (Figure 33f). Mice were injected with 6mg/kg FA for 7 days, as described for CMPF, and maintained on a HFD for 4 weeks. FA treatment had no effect on weight gain or food consumption over the treatment period compared to HFD-Controls (Figure 33g, data not shown). Interestingly, when challenged by GTT, FA-treated mice had a modest improvement in glucose tolerance compared to HFD-controls (Figure 33h). Overall, this suggests that the effect of CMPF on impairing beta cell function long-term is specific.
A

**CMPF Followed By High-Fat Diet**

- CD1 mouse (8wks age)
- Vehicle
- CMPF
- HFD
- 1wk
- 4wks Diet
- Chow
- HFD
- Control-Chow
- Control-HFD
- CMPF-HFD

**Figure 32. Protocol for Investigating the Long-Term Effect of CMPF with High Fat Diet Exposure**

a) Mice were injected once daily for seven consecutive days. Mice were then changed to either a 60% kcal from fat diet (HFD) or sucrose-matched control (chow) for four weeks.
Figure 33. Prior CMPF Treatment Worsens Glucose Tolerance on a High Fat Diet

a) Weekly body weight and (b) food consumption per cage of 4 mice per week while mice are maintained on respective diets. Blood glucose levels at (c) fasting and (d) during intraperitoneal glucose tolerance test. (e) Corresponding plasma insulin during fasting and ipGTT. N=12-16/group. Values mean +/- SEM. *P<0.05, **P<0.01, ***P<0.001.
4.3.5 CMPF Protects Against Apoptosis though Compromises Insulin Secretion

Given the modest increase in islet size following CMPF treatment in chow-fed animals, we wanted to examine how beta cell compensation for a HFD was affected by CMPF. Similar to the chow-fed condition, insulin positive area was increased in the CMPF-treated mice compared to both control groups (Figure 34a). Interestingly, this corresponded to a significant decrease in TUNEL positive area compared to HFD-controls, suggesting that CMPF may protect beta cells against apoptosis (Figure 34b). This prevention of apoptosis is likely responsible for the increase in islet mass, as there was no indication of increased islet proliferation, as assessed by Ki67 staining, or neogenesis as assessed by staining for neurogenin 3 (NGN3), a marker of immature beta cells (Figure 34c,d). Furthermore, a lack of change in NGN3 positive area indicates that the beta cells are not undergoing dedifferentiation as an explanation for the reduced insulin secretion during GTT (Talchai et al., 2012).

In spite of increased beta cell mass, islets isolated from CMPF-HFD mice secreted significantly less insulin with glucose challenge than HFD-controls, consistent with in vivo findings (Figure 34e). This again corresponded to a significant reduction in total insulin content compared to HFD-controls (Figure 34f). Interestingly, there was no significant difference in either GSIS or insulin content in the CMPF-HFD mice compared to Chow-controls. This indicates that mice treated with CMPF are able to compensate for increased metabolic stress to some degree, however the defect in GSIS remains profound and exacerbates glucose intolerance.
Figure 34. CMPF Treatment Increases Islet Size but Reduces Insulin Secretion and Content

a) Total insulin positive area in pancreatic sections. Percentage of total cells positive for (b) TUNEL, (c) Ki67, or (d) Ngn3 in pancreatic sections. (e) Glucose-stimulated insulin secretion (GSIS) and (f) total insulin content within isolated islets. N=4/group for histology. N=12/group for secretion and content. Values mean +/- SEM. *P<0.05.
4.3.6 Impairment of Insulin Biosynthesis and Secretion is Partially ROS Independent

In acute studies with CMPF we have demonstrated that increased ROS, potentially generated through CMPF metabolism, is largely responsible for the observed defects in insulin biosynthesis and secretion through alterations in Akt signaling and increasing mitochondrial uncoupling through uncoupling protein 2 (UCP2)(Prentice et al., 2014). Co-treatment of CMPF with the antioxidant N-Acetylcysteine (NAC) was able to largely reverse the observed defects. Interestingly, in both long-term models ROS is not significantly changed despite the persistence of the effect of CMPF (Figure 35a,b). Expression of the antioxidant genes catalase (CAT) and UCP2 remain significantly increased in the islets, however, suggesting activation of an antioxidant profile to combat CMPF exposure, which may chronically impact beta cell function (Figure 35c). To further elucidate the relationship between ROS, mitochondrial uncoupling, and CMPF, we isolated islets from beta cell specific UCP2 knockout mice (UCP2BKO) and control mice expressing Cre under the rat insulin promoter (RIP-CRE) and treated them with CMPF for 24 hours. UCP2BKO islets have increased insulin secretion, which is attributed to increased ROS signaling, and strong mitochondrial coupling resulting in more efficient generation of ATP(Robson-Doucette et al., 2011). Evaluation of ROS in UCP2BKO islets confirmed a nearly 2-fold increase in ROS compared to RIP-CRE controls (Figure 35d). Treatment of RIP-CRE islets with CMPF for 24hrs increased ROS to the same level as UCP2BKO islets; however, the same treatment had no effect on ROS in UCP2BKO islets, likely due to an increased antioxidant profile associated with UCP2 deletion(Robson-Doucette et al., 2011) (Figure 35d). Acute addition of CMPF to both RIP-CRE and UCP2BKO islets stimulated an identical hyperpolarization of the mitochondrial membrane potential (MMP), indicating that the lack of ROS generation in UCP2BKO islets is not caused by impairment in CMPF metabolism (Figure 35e). Consistent with previous findings, 24hr treatment with CMPF significantly impaired GSIS from both RIP-CRE islets and UCP2BKO islets compared to controls (Figure 35f). Intriguingly, however, this defect was greatly reduced in the islets from UCP2BKO animals, with comparable insulin secretion under high glucose stimulation between the
RIP-CRE controls and the UCP2BKO islets treated with CMPF. This suggests that increased ROS and induction of UCP2 is partially responsible for the defect in GSIS. However, the persistent defect in GSIS indicates that CMPF has further effects on alternate aspects of the GSIS pathway.
Figure 35. UCP2BKO Mice are Partially Protected Against CMPF Treatment

Reactive Oxygen Species (ROS) levels in islets from mice (a) six weeks following final injection on a chow diet and (b) four weeks after treatment while on a HFD. c) mRNA expression of UCP2 and Catalayse (CAT) in islets from the HFD model. d) ROS levels in UCP2BKO mice and RIP-CRE controls treated with 200uM CMPF or vehicle control. e) Mitochondrial membrane potential (MMP) hyperpolarization with the acute addition of CMPF in UCP2BKO mice and RIP-CRE controls. Glucose-stimulated insulin secretion (GSIS) from UCP2BKO and RIP-CRE islets treated with vehicle or CMPF. g) Glucose-induced hyperpolarization of the MMP in CMPF or vehicle control treated UCP2BKO and RIP-CRE islets. N=8-12/group. Values mean +/- SEM. *P<0.05, **P<0.01.
4.3.7 CMPF Impairs Glucose Metabolism

To determine where the defect in GSIS might be, we began by stimulating islets from CMPF-treated mice six weeks post final injection with the secretagogue KCl. KCl closes the $K_{ATP}$ channels, resulting in membrane depolarization, calcium influx and insulin exocytosis in a glucose-independent manner (Figure 36). There was no significant difference in insulin secretion with KCl treatment in islets from CMPF-treated mice in either chow- or HFD-fed groups (Figure 37a,b). This indicates that the defect in insulin secretion is not due to defects in any mechanism downstream of the $K_{ATP}$ channels including the reduced intracellular insulin content.

These findings prompted us to examine the effect of CMPF on the most proximal aspect of the GSIS pathway, glucose metabolism. We began by testing the capacity of islets from CMPF-treated mice to metabolize glucose. First, we measured hyperpolarization of the MMP in response to an acute bolus of glucose. As anticipated islets from both chow and HFD control groups exhibited a robust hyperpolarization under 20mM glucose conditions (Figure 37c,d). Interestingly, this glucose response was significantly blunted in islets from CMPF-treated mice in chow- and HFD-fed groups (Figure 37c,d). Interestingly, and consistent with defective glucose metabolism as a mechanism of impaired GSIS, CMPF treatment of both RIP-CRE and UCP2BKO islets also elicited a blunted glucose-induced MMP response compared to respective controls (Figure 35g). The defect in glucose metabolism was further validated through measurements of glucose oxidation in isolated human islets. Treatment with CMPF for 24hrs caused a 50% reduction in glucose oxidation compared to both vehicle and palmitate-treated controls (Figure 37e). In all experiments the maximal respiratory capacity of the islets was not changed with CMPF treatment, suggesting that mitochondrial function in general is not compromised by CMPF treatment. Thus, CMPF induces and acute impairment in glucose metabolism that persists long-term.
Figure 36. Model of First Phase Glucose-Stimulated Insulin Secretion.
Figure 37. CMPF Impairs Glucose Metabolism but Potentiates Palmitate Metabolism

KCl-stimulated insulin secretion from islets isolated from (a) chow-fed mice 6 weeks post final injection and (b) HFD-fed model four weeks post final injection. Glucose-stimulated hyperpolarization of the mitochondrial membrane potential (MMP) in (c) chow and (d) HFD-fed models of long-term CMPF effect. Oxygen consumption rate (OCR) evaluated by Seahorse in human islets treated with vehicle or 200uM CMPF for 24 hours with the addition of (e) 20mM glucose or (f) 200uM palmitic acid. Hyperpolarization of the MMP in response to acute addition of palmitic acid in islets from the (g) chow and (h) HFD-fed models. i) Palmitate-stimulated insulin secretion from islets isolated from the HFD model. N=8-12 per group for mouse studies. N=3 for human islets. Values mean +/- SEM. **P<0.01, ***P<0.001.
4.3.8 Fatty Acid Metabolism is Enhanced by CMPF

Importantly, insulin secretion is preserved under fasting and low glucose stimulatory conditions, indicating that response to other nutrient stimuli such as free fatty acids may be preserved in the CMPF-treated mice. The concept of metabolic inflexibility, an impaired ability to switch between fatty acids and carbohydrate substrates, has been proposed to explain the loss of both insulin sensitivity and 1\textsuperscript{st} phase insulin secretion in diabetic subjects (Kim-Muller et al., 2014). To determine if CMPF may be inducing a preferential state of fatty acid utilization, we performed the same series of experiments utilizing palmitic acid as a substrate in place of glucose. Beginning with acute studies, human islets treated with CMPF had a robust enhancement of fatty acid oxidation compared to vehicle and palmitate treated controls (Figure 37f). Remarkably, this same finding was observed in islets from CMPF-treated mice under both long term chow-fed and HFD conditions, which exhibited a significantly enhanced hyperpolarization of the MMP in response to palmitate compared to all control groups (Figure 37g,h). Interestingly, this enhanced response to palmitate treatment translates in enhanced insulin secretion. Islets isolated from CMPF-treated mice six weeks post final injection or CMPF-HFD mice both exhibited significantly enhanced insulin secretion in response to palmitate challenge than controls (Figure 37i). Thus, CMPF treatment appears to induce a preferential state of fatty acid utilization, which is capable of stimulating insulin secretion.
4.3.9 CMPF Alters Metabolic Gene Expression

To further elucidate the cause of this metabolic switch, we examined key genes required for glucose uptake and metabolism including the glucose transporter GLUT2, and the rate-limiting enzyme for glycolysis, glucokinase (Gck). Expression of both GLUT2 and Gck were significantly reduced in both CMPF-chow and CMPF-HFD mice compared to respective controls, while Cpt1b, the primary mitochondrial fatty acid transporter, was significantly increased (Figure 38a). Alterations in expression of these genes are attributable to altered activity of key beta cell transcription factors HNF4 and PDX1, which also regulate insulin biosynthesis. PDX1 was previously shown to be downregulated with CMPF treatment, and may partially explain the observed phenotype. Consistent with these findings, as well as the reduced insulin content, insulin transcripts were also significantly reduced following CMPF treatment on both chow and HFD (Figure 38b).
4.3.10 Preferential Fatty Acid Utilization is Reversible

Enhanced fatty acid utilization may be due to either an inability to metabolize glucose, caused by impaired uptake or metabolism, or due to a preference for fatty acids over glucose. To test this, we established an in vitro model where we treated isolated islets with CMPF for 24 hours followed by a 24 hour washout period to mimic the long term in vivo treatment conditions (Figure 38c). After the washout period, islets treated with CMPF maintained a significant impairment in GSIS, similar to our ex vivo findings (Figure 38d). To determine if CMPF-treated islets were able to utilize glucose, we treated islets with 1uM etomoxir, an inhibitor of the mitochondrial fatty acid transporter Cpt1b, for either the full 24 hour washout period, or one hour prior to the GSIS assay. Interestingly, etomoxir treatment for either one or 24 hours rescued GSIS from CMPF-treated islets to control levels (Figure 38d). Etomoxir treatment had no significant effect on insulin content of the isolated islets, suggesting that alteration in insulin content is independent of metabolic inflexibility (Figure 38e). Therefore, CMPF treatment induces a state of preferential fatty acid utilization that is reversible upon the inhibition of beta oxidation.
Figure 38. Preferential Fatty Acid Oxidation Induced by CMPF is Reversible

Quantitative PCR expression of glucose metabolic genes (a) GLUT2, GCK, and (b) Insulin in the HFD-fed model. c) Experimental design for etomoxir treatment of isolated islets in *in vitro* long-term protocol. d) Glucose-stimulated insulin secretion from long-term vehicle or CMPF-treated islets treated with etomoxir for (A) 24 hours or (B) 1 hour prior to GSIS, and (e) corresponding total insulin content. N=8/group for PCR. N=3 for GSIS. Values mean +/- SEM. *P<0.05, **P<0.01, ***P<0.001.
4.4 DISCUSSION

The acute nature of gestational diabetes (GDM) means that it is not associated with the same severe complications as type 2 diabetes such as cardiovascular disease, neuropathy, or retinopathy. It is, however, associated with a significantly elevated risk of future development of T2D, with up to 70% of women with GDM going on to develop T2D within 10 years postpartum (Bellamy et al., 2009; Mitanchez, 2010; Reece, 2010). Immediately following delivery, levels of insulin resistance drop significantly with elimination of the placenta and insulin desensitizing placental hormones. With the improvement in insulin sensitivity, glucose tolerance dramatically improves, even with the decline in beta cell function to prepregnancy levels (Buchanan, 2001; Retnakaran et al., 2010). While in most cases beta cell function is not further characterized until the patient presents with prediabetes or overt T2D, studies monitoring women with GDM in the postpartum period report significant, persistent impairment in beta cell function during glucose challenge, even when women are maintaining relatively normal glucose tolerance (Homko et al., 2001; Kautzky-Willer et al., 1997). The cause of this persistent beta cell dysfunction is largely unknown.

Recently we identified the furan fatty acid metabolite CMPF as being significantly elevated in the plasma of women with GDM (Prentice et al., 2014). In vivo and in vitro studies demonstrated that CMPF impaired beta cell function through diminishing insulin biosynthesis, as well as impairing glucose-stimulated insulin secretion, and thus may contribute to the pathogenesis of GDM. Here, we investigated whether CMPF has long-term effects on beta cell function and thus may contribute to future risk of T2D development. Mice were treated with CMPF for seven days and followed for up to 4 months after the end of the injection period. The seven day treatment period is the equivalent duration of the final trimester of pregnancy, thus mimicking the exposure to CMPF during a GDM pregnancy. In the follow up period, the mice appear to be completely phenotypically normal, with no difference in food consumption and a minor decrease in body weight gain, which is normally associated with improved metabolic health. We observed no significant difference in random or fasting blood glucose or plasma insulin, and no effect on insulin sensitivity. Overall, CMPF treatment does not greatly impact overall health. It is only under the conditions of glucose challenge during the ipGTT that a defect in glucose tolerance and
insulin secretion is observed, similar to what is observed in human patients following GDM pregnancy.

Development of T2D is largely associated with the development of insulin resistance. Given the lack of impact of CMPF on insulin resistance in lean animals, we investigated the effect of prior CMPF exposure on the ability to compensate for metabolic stress and insulin resistance. Therefore, we treated mice for seven days with 6mg/kg/day of CMPF and then placed the mice on a 60% kcal from fat HFD. Mice fed the HFD developed elevated fasting blood glucose and plasma insulin, and had glucose intolerance and elevated insulin secretion during ipGTT, consistent with prediabetes (Wang and Liao, 2012). Prior treatment with CMPF exacerbated glucose intolerance, which was associated with a lack of glucose-stimulated insulin secretion, suggesting that previous exposure to CMPF decreases the compensatory capacity of the beta cell, resulting in accelerated development of diabetes. This may explain the high rate of progression to T2D in women with GDM if they are exposed to further metabolic challenge following pregnancy.

CMPF appears to induce beta cell dysfunction through at least two mechanisms. In the acute setting, CMPF increases reactive oxygen species (ROS) production, resulting in altered transcription factor localization and activity and reduced insulin biosynthesis (Prentice et al., 2014). Interestingly, the effect of CMPF is potentiated for months following treatment, even when CMPF is no longer present and ROS levels have returned to normal. However, the consequence of elevated ROS persists, including increased expression of antioxidant genes, as well as the mitochondrial protein uncoupling protein 2 (UCP2). Physiologically increased UCP2 acts to uncouple mitochondrial respiration, decreasing both ROS generation and ATP production (Kashemsant and Chan, 2006; Robson-Doucette et al., 2011; Zhang et al., 2001). Furthermore, chronic genetic overexpression of UCP2 results in significantly decreased GSIS due to reduced ATP generation (Chan et al., 1999). Thus, increased UCP2 may partially explain the effect of CMPF. To test this, we treated islets from beta cell specific UCP2 knockout mice (UCP2BKO) with CMPF and evaluated GSIS. Deletion of UCP2 was associated with a partial rescue of GSIS, suggesting that UCP2 and antioxidant gene expression partially mediate the
effect of CMPF, and that an alternate pathway is likely also contributing to the observed phenotype.

The increase in UCP2 may also account for the protective effect of CMPF against obesity-induced beta cell apoptosis. Increased beta-oxidation under conditions of elevated free fatty acids is typically associated with increased ROS production, ultimately leading to oxidative stress activation of the JNK and NF-κB pathways and induction of beta cell apoptosis (Poitout et al., 2010; Poitout and Robertson, 2008). The altered antioxidant profile of CMPF-treated islets, including increased UCP2, allows compensation for this ROS, protecting against the proapoptotic signals. This may explain why islets from CMPF-treated mice are significantly larger and exhibit reduced TUNEL positive staining.

The alternate pathway we propose for the impairment of glucose-stimulated insulin secretion involves the induction of metabolic inflexibility, an impaired ability to switch between fatty acid and carbohydrate substrates (Kim-Muller et al., 2014). Reduced ability to metabolize glucose is apparent in islets from CMPF-treated mice both acutely and months following exposure, as well as mouse and human islets exposed to CMPF in vitro. While this is associated with reduced expression of key genes involved in glucose metabolism including the glucose transporter GLUT2 and the rate-limiting enzyme in glycolysis, glucokinase, the effect is reversible, meaning sufficient glucose is able to enter the cell and be metabolized when required. Thus, instead of being unable to metabolize glucose, CMPF induces a state of preferential fatty acid utilization, where cells will utilize fatty acids as their primary fuel source, even in the presence of hyperglycemia as during a GTT. Furthermore, the ability to utilize fatty acids is enhanced, with greater fatty acid oxidation when given the same amount of substrate as compared to controls. The mechanism underlying how CMPF mediates this effect, especially when it is no longer present as in the long-term follow-up conditions, remains to be elucidated.

The Randle Cycle describes the competition between glucose and fatty acids for substrates in metabolism, and has been proposed to play an important role in both type 2 diabetes (T2D) and insulin resistance (Hue and Taegtmeyer, 2009). Under conditions of increased beta-oxidation there is increased production of fatty acid-derived acetyl-CoA. This acetyl-CoA feeds back to down-regulate glucose metabolism through inhibition of the pyruvate dehydrogenase complex (PDH), providing a signal that the cell has sufficient substrate supply (Bowker-Kinley et al.,
This reduces the ability of pyruvate to enter into the TCA cycle, thus inhibiting glucose metabolism. Chronic beta-oxidation further inhibits glycolysis upstream of pyruvate formation as flux through the TCA cycle increases production of citrate, which acts to inhibit both phosphofructo-1-kinase (PFK), decreasing production of fructose-1,6-bisphosphate, as well as glucose transporter itself. Furthermore, long-chain acyl-CoA derivatives directly inhibit glucokinase, further downregulating glucose uptake and metabolism (Dawson and Hales, 1969; Garland et al., 1963). This is consistent with the observed reductions in GLUT2 and glucokinase observed in the CMPF treated mice, as chronic inhibition may lead to decreased expression over time. Under fasting conditions, the beta cell is capable of utilizing fatty acids through this pathway to ensure survival without induction of insulin secretion, which could be lethal to the organism. However, chronically decreased glucose metabolism, such as under extended starvation, results in many of the same alterations observed in the islets of CMPF-treated animals followed long-term. These include diminished insulin biosynthesis, and an altered transcriptional profile to one that enhances beta-oxidation and reduces glucose metabolism (Sugden et al., 2001; Vara and Tamarit-Rodriguez, 1986; Zhou and Grill, 1994). Thus, CMPF may induce a persistent fasting-like state by reducing glucose uptake and altering the transcriptional profile to a preferential fatty acid oxidation profile.
4.5 MATERIALS AND METHODS

4.5.1 CMPF Preparation

CMPF was purchased from Cayman Chemical (product number 10007133) and dissolved in 100% ethanol to a stock concentration of 100mM. CMPF was stored at 4C. For injections, CMPF was dissolved in 100ul sterile saline within an insulin syringe for injection.

4.5.2 Intraperitoneal injection of CMPF and Tolerance Tests

Seven week old male CD1 mice were purchased from Charles River and allowed to acclimate for one week prior to the beginning of experiments. Mice were injected intraperitoneally (IP) with 6mg/kg CMPF or vehicle control at 24h intervals for seven days, as previously described (Prentice et al., 2014). For the chow-fed model, mice were then maintained on a standard chow diet (Alpco) for up to 16 weeks following final injection. Mice were weighed individually once weekly, and food consumption was measured by measuring the weight of food remaining in the cage per week. Mice were housed with 4 mice per cage. For the high-fat diet (HFD) model, mice were maintained on standard chow diet for the injection period. 24 hours following the final injection, mice were switched to either a 60%kcal from fat HFD (HFD; OpenSource diets D12492, Research Diets Inc, USA) or a sucrose-matched control (OpenSource diets D12450J, Research Diets Inc, USA) for 4 weeks. Mice were also monitored weekly for individual body weights and whole-cage food consumption.

At the end of the follow-up period, mice were fasted overnight for 14h before an intraperitoneal glucose tolerance test (ipGTT). Mice were injected ip with 1g/kg sucrose. IpGTTs, and measurement of plasma insulin were performed as previously described (Allister et al., 2013). Blood (<25ul) was collected from the tail vein at 0 (fasting), 10, 20 and 30 minutes from quantification of plasma insulin and blood glucose. Blood glucose was also measured at 60 and 120 minutes post injection. IP insulin tolerance tests (ipITT) were performed following a 4h fast. 1.5IU/kg insulin was injected and blood glucose was measured at 0, 10, 20, 30, 45, 60 and 120 minutes. All experiments were approved by the Animal Care Committee at the University of Toronto and animals handled according to the Canadian Council of Animal Care guidelines.
4.5.3 Selected Reaction Monitoring Mass Spectrometry (SRM-MS)

Briefly, mice were injected with 6mg/kg CMPF intraperitoneally, as described. Blood was collected 20 minutes, 2 hours, 24 hours, or 4 weeks following final injection. 20 minute and 2 hour blood samples were taken from separate mice due to the large volume (~60ul) required. CMPF standards (1 – 500 ng) and samples were spiked with 25 ng of CMPF-d₅ (Cayman Chemical, USA) internal standard. A surrogate matrix of 4% BSA in PBS was used for standards. Plasma (20µL) and standards (20µL matrix surrogate) were diluted with 480 µL of ultrapure water. 20µL of 80% phosphoric acid was added, mixed by vortexing then 1.5 mL of ethyl acetate (EtOAc) was added. Samples were chilled on ice, and then centrifuged. The upper EtOAc layer was collected and re-extracted with another 1.5 mL of EtOAc. Samples were vortexed, chilled on ice, and centrifuged. The upper EtOAc layers were removed and combined with the previous extract. The combined EtOAc layers were dried and residues were reconstituted in 500 µL of acetonitrile and analyzed by LC-MS/MS using an Agilent 1200 HPLC with an API 4000 mass spectrometer (AB Sciex).

4.5.4 Islet Size and ROS Quantification

At the end of the protocol, mice were fasting overnight before sacrifice. Mice were anesthetized using isofluorane and total blood volume was collected from the chest cavity following removal of the heart. Tissues were collected and flash frozen in liquid nitrogen for future analysis or fixed in a 10% neutral buffered formalin solution for histology. Islets were isolated through pancreatic perfusion and collagenase digestion as previously described (Luu et al., 2013). Islets were hand picked three times and allowed to recover overnight in RPMI (1640; Sigma) supplemented with 10% FBS and 1% Pen/Strep prior to analysis. The level of H₂O₂ was determined using 2',7'-dichlorodihydro fluorescein diacetate (CM-H₂-DCFDA) (Molecular Probes, Invitrogen, Canada) as previously described (Robson-Doucette et al., 2011). Briefly, ilstes were loaded with dye for 25 minutes prior to imaging on an Olympus IX70 inverted epi-fluorescence microscope in combination with an Ultrapix camera and a computer with PTI imaging software. Islets were exposed for 25ms with one exposure per islet. Bright field images were used to determine islet size.
4.5.5 Beta Cell Specific Uncoupling Protein 2 Knockout Mice

Beta cell specific uncoupling protein 2 knockout mice (UCP2BKO) were generated by crossing UCP2 floxed mice to mice expressing Cre under the rat insulin promoter (RIP-CRE), as described previously (Robson-Doucette et al., 2011). Deletion of UCP2 is established embryonically and was confirmed by PCR. Islets were isolated from 8-10 week old male mice, picked into standard RPMI media, and allowed to recover overnight. Islets were then treated with either vehicle, or 200uM CMPF for 24 hours. CMPF was conjugated to fatty acid free BSA for 4 hours prior to addition to islets.

4.5.6 Glucose-Stimulated Insulin Secretion

Glucose-stimulated insulin secretion (GSIS) was assessed as previously described (Luu et al., 2013; Robson-Doucette et al., 2011) using 2.8mM and 16.7mM glucose in sequential secretion assays. 16.7mM Glucose with 30mM KCl was used to stimulate maximal secretion (Basford et al., 2012). Mouse islets were isolated as previously described (Hardy et al., 2009) and incubated in standard media (RPMI-1640 with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine) overnight prior to GSIS. Insulin concentrations were measured in secretion samples and total content samples using an HTRF kit (Insulin, Cisbio) on the Pherastar plate reader (Thermo Fisher) and normalized to DNA. Total islet insulin content was determined by incubating islets overnight in an acid ethanol solution. Samples were dried down and resuspended in ultrapure water and insulin content assayed by insulin HTRF as above and normalized to DNA.

4.5.7 Transmission Electron Microscopy

Isolated islets were fixed and images acquired as previously described (Basford et al., 2012). Images were taken at 5,000x and 10,000x for each region examined. Granule number was manually quantified using ImageJ software in images taken at 10,000x magnification (Lu et al., 2010).
4.5.8 Immunohistochemistry and islet morphology

Pancreata were weighed and fixed in 10% neutral buffered formalin at the time of sacrifice. Tissue processing and immunostaining for insulin have been described previously (Luu et al., 2013). Briefly, pancreata were dehydrated, cleared and embedded in paraffin. Paraffin sections were cut at 4μm from the middle of the pancreas. Sections were then dried and deparaffinized for staining. TUNEL, Ki67 and Ngn3 staining was performed according to the same protocols. Images of each section were acquired using Aperio Imagescope at 40x magnification. The β-cell area and other positive staining was calculated by using positive pixel count analysis (Aperio Imagescope).

4.5.9 Gene Expression

Total RNA was extracted from isolated islets using the Qiagen RNeasy Plus mini kit (Hilden, Germany). Reverse transcription from total RNA and quantitative real time PCR (qPCR) analysis was performed as previously described (Basford et al., 2012). Reverse transcription was performed using a M-MLV kit according to manufacturers instructions (Sigma Aldrich, Canada). Primers were designed using Primer3 software (NCBI) and are listed in Table. Data were normalized to β actin mRNA.

4.5.10 Mitochondrial Membrane Potential (MMP)

Islets isolated from mice were loaded with rhodamine 123 (25 μg/ml) in imaging buffer without glucose for 10 minutes. Islets were washed and placed in imaging chambers containing imaging buffer with no glucose. Images were taken at 10s intervals at ex:511nm by an Olympus IX70 inverted epi-fluorescence microscope in combination with an Ultrapix camera and a computer with PTI imaging software, as previously described. Glucose was added to a final concentration of 20mM to observe the correspondent change of MMP in cells. Alternately, palmitate conjugated to fatty acid free BSA was added to a final concentration of 400uM, or CMPF was added to a final concentration of 200uM. 5mM NaN₃ was added to fully depolarize the MMP (Diao et al., 2008).
4.5.11 Human Islets and Oxygen Consumption Measurements

Human islets from review board approved healthy donors were provided by the IsletCore and Clinical Islet Laboratory (University of Alberta, Canada). Islets were picked into low glucose DMEM media (Gibco, ref#11885-084) with 10% FBS 1% penicillin/streptomycin, 1% L-glutamine and allowed to recover overnight (Prentice et al., 2014). 70 Islets were then picked into each well of XF24 islet plates (Seahorse Bioscience, USA) and secured with a mesh cover, as previously described (Wikstrom et al., 2012). Cells were incubated in 2mM glucose KRB without bicarbonate for one hour prior to being loaded into the XF24 machine. Oxygen consumption rates (OCR) were measured at 2mM glucose, followed by either 20mM glucose or 400uM palmitate to measure substrate utilization. All islets were then treated with 5uM oligomycin, 5uM FCCP, and 5uM rotenone and 5uM antimycin A. Raw traces were normalized to basal respiration at 2mM glucose.

4.5.12 Pharmacological Inhibition of CPT1b

Islets were treated with or without 200µM CMPF or vehicle control for 24 hours Islets were then picked into fresh media and cultured for an additional 24 hours with or without 1uM etomoxir (Sigma Aldrich, Canada). On the third day, islets were treated with vehicle or 1uM etomoxir for 1 hour prior to GSIS.

4.5.13 Statistics

Statistical significance was assessed using either the Student’s t-test, or a two-way ANOVA for repeated measures followed by a Bonferroni post-test comparison where required. \( P<0.05 \) was considered significant. All data is mean ± SEM unless otherwise specified.
Table 16. Primers Used for Quantitative PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-Actin</td>
<td>CTGAATGGCCAGGTTCTGA</td>
<td>CCCTGGCTGCCTCAACAC</td>
</tr>
<tr>
<td>Ins</td>
<td>GGTGGGCATCCAGTAAACCCCA</td>
<td>GAAGCCACGCTCCCAACACA</td>
</tr>
<tr>
<td>Glut2</td>
<td>CCTTGGGCCTTACGTGTCTTCT</td>
<td>TTGTACAGCAGCTTTGCGTG</td>
</tr>
<tr>
<td>GCK</td>
<td>GAGATGGATGTGGTGCAAT</td>
<td>ACCAGCTCCACATTCTGCAT</td>
</tr>
<tr>
<td>Cat</td>
<td>TGAGAAGCCAAGAAGCAATTC</td>
<td>CCCTTCGAGCCATGTG</td>
</tr>
<tr>
<td>Ucp2</td>
<td>CAGCCAGCGCCAGTACC</td>
<td>CAATGGGAGCGGAGGCAAAGC</td>
</tr>
</tbody>
</table>
Table 17. Antibodies used for Immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Western Dilution</th>
<th>Supplier</th>
<th>Cat. Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit α Neurogenin 3</td>
<td>1:250</td>
<td>Abcam</td>
<td>Ab38548</td>
</tr>
</tbody>
</table>
4.6 CONCLUDING REMARKS

In this chapter, we explore the long-term impact of CMPF treatment on beta cell function under both lean and obese conditions. Additionally, we explored the mechanism of action of CMPF and determined that there are multiple contributing factors, including acute elevation of ROS and the associated transcriptional changes, as well as induction of a state of preferential fatty acid utilization and an inability to switch between fatty acid and carbohydrate substrates under physiologically relevant conditions. The multiple mechanisms of CMPF action make the elucidation of the contribution of each of these multiple pathways difficult. Here we began by using beta cell specific UCP2 knockout mice (UCP2BKO) to determine the contribution of ROS and mitochondrial uncoupling to CMPF-induced dysfunction. While these mice provide important insight into the role of increased UCP2 in the reduced GSIS, they are also complicated by the confounding factors of endogenously increased ROS and increased expression of antioxidant enzymes. Thus, the ability of UCP2 knockout to only partially rescue CMPF treatment may also be attributable to the altered oxidant state of the islet. Furthermore, the increased mitochondrial coupling in UCP2BKO islets alters the baseline mitochondrial membrane potential, making study of differences in glucose and fatty acid metabolism complicated as compared to wild type controls (Robson-Doucette et al., 2011). Utilization of UCP2 inhibitors, such as Genipin, or conditional deletion of UCP2 may represent better models for studying the role of UCP2 in the activity of CMPF as there is reduced compensation from the antioxidant profile under these conditions.

The long-term effect of CMPF may also be mediated by an as yet undetermined metabolite of CMPF that is retained in the islet to mediate the function. Our quantification of circulating and tissue CMPF was performed using a targeted mass spectrometry approach, meaning that it was only investigating the parent ion of CMPF and not any possible derivatives. It is possible that CMPF is metabolized or converted into another form within the cell. It has been proposed that CMPF may form an ester to the plasma membrane, and exist in this state long-term. We are currently investigating this possibility using untargeted mass spectrometry screens, as well as working with Eli Lilly to generate CMPF analogs that can be tested for functionality.

In an independent study we have now evaluated the concentration of CMPF in a cohort of women who had GDM during pregnancy at 6-8 weeks postpartum when glucose tolerance had
returned to normal (the SWIFT cohort, outlined in (Gunderson et al., 2011). Interestingly, CMPF levels had reduced dramatically during this short timespan though was still elevated compared to normal glucose tolerant individuals in all of the other cohorts we have examined (60-70uM in SWIFT compared to 20-40uM in other populations). This validates the use of our model with short-term exposure to CMPF followed by complete elimination of CMPF from circulation to evaluate its role in future T2D risk. This also suggests that CMPF may not represent a useful tool as a companion diagnostic for evaluation of drug efficacy, as previous exposure to CMPF can have long term consequences, even when it is no longer in circulation.

Finally, we know that CMPF is able to enter into other metabolically active tissues, primarily the liver. A major limitation of the current study is the fact that the mice treated with CMPF never develop insulin resistance on the high fat diet despite significant weight gain. Thus the comparison between HFD-CMPF mice and HFD-controls is complicated. The effect of CMPF on the liver is explored in Chapter 5. In order to properly determine the long-term effect of CMPF under conditions of insulin resistance we should investigate models of permanent insulin resistance such as the liver-specific insulin receptor knockout mouse (LIRKO). The fact that the islet phenotype is nearly identical between the long-term chow- and HFD-fed models suggests that islet function is not significantly impacted by liver insulin sensitivity, however this remains to be tested.
Chapter 5- CMPF Enhances Lipid Metabolism and Induces FGF21 to Protect Against Steatosis

The following chapter is prepared for submission:

Prentice KJ, Liu Y, Eversley JA, Wang XS, and Wheeler MB.. CMPF Enhances Lipid Metabolism and Induces FGF21 to Protect Against Steatosis

Contributions by co-authors to the figures presented are stated in the figure legends
5.1 ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a condition of excess fat accumulation in the liver that is strongly correlated with insulin resistance, the development of diabetes, and cardiovascular disease. Options for the prevention or treatment of NAFLD are currently non-existent. Here we identify a novel role for the endogenous furan fatty acid metabolite CMPF in protecting insulin sensitivity and preventing the development of steatosis during exposure to a high fat diet. Acute treatment with CMPF increases future energy expenditure and lipid metabolism, resulting in decreased fat accumulation with no difference in food consumption, despite its absence from circulation and tissues. This corresponds to dramatically improved insulin sensitivity, and complete protection against the hepatic lipid accumulation. Mechanistically, we find that CMPF acts to drive lipolysis and potentiate lipid uptake into hepatocytes. Increased lipolysis induces expression and secretion of FGF21, which acts in an autocrine fashion to protect against the development of steatosis. Thus, CMPF is a novel, potent driver of lipid metabolism that may represent a novel target for the prevention of NAFLD.
5.2 INTRODUCTION

Excessive caloric intake combined with increasingly sedentary lifestyles is producing an epidemic of overweight and obesity, affecting more than 35% of the world’s adults (WHO data). The cluster of metabolic disturbances associated with increased adiposity, called the metabolic syndrome (MetS), includes diabetes, fatty liver disease, and atherosclerosis and is directly attributed to 1 in 5 deaths in the United States annually (Eckel et al., 2005; Masters et al., 2013a; Masters et al., 2013b). The underlying pathophysiology of MetS is based in dysregulated lipid metabolism, resulting in aberrant lipid storage in the liver and muscle, increased insulin resistance, and altered circulating lipoprotein levels (Avramoglu et al., 2006; Bergman and Ader, 2000; Cao et al., 2008; Ginsberg, 2006). While this dyslipidemia is largely attributed to a ubiquitous increase in release of free fatty acids (FFAs) from the adipose tissue, systemic lipid profiling has revealed that the development of MetS is strongly correlated with a specific fatty acid signature of serum lipids (Warensjo et al., 2005). Thus, while adipose dysfunction is certainly attributable to development of MetS, contribution of diet, microbiome, genetics, and environment to the plasma lipomic signature may play a more significant role in the regulation of systemic metabolic homeostasis than previously thought.

The furan fatty acid metabolite 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) is a relatively obscure small molecule originally identified as elevated in patients with uremia (Niwa et al., 1988). While the source of CMPF remains controversial (Nolan, 2014), its clearance is regulated by a family of organic anion transporters (OATs) in the proximal tubule cells of the kidney (Deguchi et al., 2005; Sassa et al., 2000; Spiteller, 2005). Recently, we identified CMPF as being significantly elevated in the plasma of patients with gestational (GDM) and type 2 diabetes (T2D) (Prentice et al., 2014). Treatment for 7 days with diabetic concentrations of CMPF resulted in impaired glucose tolerance, attributed to a significant impairment in glucose-stimulated insulin secretion. Interestingly, the effect of CMPF is was independent of changes in insulin sensitivity, as determined by intraperitoneal insulin tolerance test (IpITT) studies and hyperinsulinemic euglycemic clamps, despite fasting hyperinsulinemia. Clamp studies also revealed an overall impairment of glucose utilization, as indicated by a significantly lower glucose infusion rate with no difference in glycolytic rate in response to insulin. Thus, CMPF-
injected mice developed an increased reliance on non-glucose energy sources, suggesting a switch in metabolism from primarily carbohydrates to fats or amino acids. Importantly, this correlates with the switch in metabolism observed in gestational diabetes, which is established in order to preserve glucose for the developing fetus. This prompted us to continue our investigations to determine the effect of CMPF under HFD feeding conditions to examine its relationship to increased lipid metabolism and insulin sensitivity.
5.3 RESULTS

5.3.1 CMPF is Rapidly Cleared from Circulation

Acute treatment with the furan fatty acid metabolite CMPF has been shown to alter whole-body glucose utilization, including a reduction in both glucose appearance and disappearance rates in a hyperinsulinemic, euglycemic clamp condition (Prentice et al., 2014). Reduced glucose utilization suggests increased reliance on alternate energy sources, including fatty acids. To determine the role of CMPF in altered substrate utilization, we treated mice with CMPF acutely, as described previously (Prentice et al., 2014), and then placed the mice on a high fat diet (HFD) comprised of 60% kcal from fat for four weeks to induce insulin resistance (Figure 39a). Following intraperitoneal injection of CMPF, plasma concentrations peaked within one hour of injection to concentrations observed in diabetic populations (Prentice et al., 2014), and declined thereafter, returning to baseline within 24 hours of injection (Figure 39b). CMPF was not elevated in the plasma for the duration of dietary intervention.
5.3.2 CMPF Alters Fat Deposition and Utilization

Interestingly, the CMPF-HFD group gained slightly, but significantly less weight than the Control-HFD group, though both groups gained more than Control-Chow mice (Figure 39c). This did not correlate to any difference in food or water consumption at any time (Figure 39d,e). To determine if the difference in body weight was due to altered body composition, mice were placed in DEXA scanners to examine body fat distribution (Figure 39f). CMPF-HFD mice had significantly lower total fat area than Control-HFD mice, which was specifically due to a significant reduction in subcutaneous fat area (Figure 39g). There was no difference in visceral fat area. Mice were placed in metabolic CLAMs for 48hrs at the end of the diet period to assess changes in whole body metabolism. CMPF-HFD mice were significantly more active than Control-HFD mice, particularly during the light phase (Figure 39h). Consistent with a decrease in whole-body glucose utilization, CMPF-HFD had a significantly reduced respiratory exchange ratio (RER) compared to Control-HFD and Control-Chow mice, suggesting preferential fatty acid utilization (Figure 39i,j). In spite of reduced subcutaneous fat area, there were no differences in circulating leptin, adiponectin, free fatty acid, or triglyceride levels (Figure 39k-n).

Figure 39. Acute treatment with CMPF induces persistent changes in whole body metabolism.

a) Schematic of the treatment protocol of CMPF treatment followed by high fat diet (HFD). b) Plasma CMPF concentration following intraperitoneal injection (n=4-6). c) Weekly weight gain through injection period and following placement on 60% high fat diet (n=20/group). d) Food and (e) water intake over 24 hours four weeks following final injection (n=4/group). f) DEXA scan images and (g) quantification of fat distribution (n=4/group). h) Activity over 24 hour period in x, y, and z planes (n=4/group). i,j) Respiratory exchange ratio (RER) calculated as VCO₂/VO₂ over 24 hour period (n=4/group). k) Fasting plasma leptin, (l) adiponectin, (m) free fatty acid (FFA), and (n) triglyceride (TG) levels 4 weeks following final injection (n=8/group). *P<0.05, **P<0.01, ***P<0.001. All error bars SEM. *Adipose quantification performed by Serena Wang
5.3.3 CMPF Further Impairs Glucose Tolerance

Due to the persistent alterations in whole-body metabolism observed in the CMPF-HFD mice, we examined its effect on glycemic control. Consistent with previous observations, CMPF-HFD mice had impaired glucose tolerance during intraperitoneal glucose tolerance test (IpGTT) compared to both control groups (Figure 40a). As expected, Control-HFD mice had significantly impaired glucose tolerance compared to Control-Chow mice. Elevated blood glucose levels during IpGTT corresponded to a significant impairment in glucose-stimulated insulin secretion in CMPF-HFD mice compared to Control-HFD mice (Figure 40b). Therefore, the effect of CMPF on impairing beta cell function persists after CMPF is cleared from circulation.
5.3.4 Insulin Sensitivity is Protected with Previous CMPF Treatment

CMPF-HFD mice exhibited significantly elevated fasting blood glucose and plasma insulin levels (Figure 40a,b inset), prompting us to examine insulin sensitivity. Interestingly, despite glucose intolerance, CMPF-HFD mice exhibited dramatically improved insulin sensitivity compared to Control-HFD mice during intraperitoneal insulin tolerance test (IpITT) (Figure 40c). The rate of glucose clearance in response to insulin was equal in the Control-Chow and CMPF-HFD groups. To determine the source of increased insulin sensitivity, tissues were isolated from mice following an intravenous bolus of insulin. Enhanced Akt activation, a marker of insulin sensitivity, was observed in both skeletal muscle and liver in the CMPF-HFD mice compared to Control-HFD mice (Figure 40d). TUNEL staining was not increased in any tissue examined including liver, muscle, adipose, kidney, spleen, or pancreas, suggesting that CMPF is not inducing apoptosis (data not shown).
5.3.5 High-Fat Diet-Induced Steatosis is Prevented by CMPF

To determine the source of the increased insulin sensitivity, we compared the tissues from the CMPF-HFD and control mice following 4 weeks of diet intervention. Consistent with the previous observation that CMPF accumulates in the liver more than most other tissue types (Prentice et al., 2014), the most striking difference between CMPF-HFD and Control-HFD mice was in the appearance of the liver (Figure 40e). Remarkably, livers from the CMPF-HFD mice appear nearly identical to Control-Chow livers, with no visual evidence of fat deposits or inflammation. This was confirmed by histological analysis with both H&E and Oil red O staining for triglyceride revealing that the HFD induced massive steatosis in the Control-HFD livers, with little intracellular lipid accumulation in the CMPF-HFD (Figure 40f). In fact, livers from CMPF-HFD mice had reduced triglyceride accumulation compared to Control-Chow mice. There was no difference in any of the other tissues examined with the exception of significantly reduced adipocyte size within the subcutaneous fat pads, consistent with the reduction in area (data not shown).
Figure 40. CMPF treatment induces persistent glucose intolerance but protects insulin sensitivity and prevents steatosis on a high fat diet.

a) Blood glucose during intraperitoneal glucose tolerance test (IpGTT) (n=8/group). b) Corresponding plasma insulin levels during IpGTT. c) Blood glucose levels during intraperitoneal insulin tolerance test (IpITT) (n=8/group). d) Western blot and quantification of skeletal muscle and liver Akt activation following in vivo insulin stimulation (n=4/group). e) Representative liver morphology of freshly isolated livers from mice 4 weeks following final injection. f) H&E and oil red O staining of liver sections (n=4/group).*P<0.05, **P<0.01, ***P<0.001. All error bars SEM
5.3.6 CMPF Stimulates Fatty Acid Utilization in Hepatocytes

To examine the mechanism underlying the increase in fatty acid utilization and resulting reduction in hepatic lipid accumulation with CMPF treatment we utilized primary hepatocytes isolated from chow-fed mice. Treatment for 24 hours with CMPF stimulated fatty acid uptake compared to vehicle treatment (Figure 41a). This corresponded to a significant increase in beta-oxidation (Figure 41b), and a significant impairment in glucose oxidation (Figure 41c). TOFA, an allosteric inhibitor of acetyl-CoA carboxylase (ACC), the master regulatory proteins of lipolysis and lipogenesis, produced a similar increase in beta-oxidation with no effect on glucose oxidation compared to control, suggesting CMPF may act through a similar mechanism (Figure 41b,c). Specificity of the assay for measuring beta-oxidation was confirmed through co-treatment with the CPT1a inhibitor etoxomir, which reduced control and blocked any stimulation in beta-oxidation (Figure 41d). Acute 24 hour treatment with either CMPF or TOFA stimulated beta oxidation in the absence of an increase in ACC phosphorylation (Figure 41e), suggesting that inhibition of activity may be direct. Intriguingly, despite no change in phosphorylated ACC, we observed a significant increase in phosphorylated AMPK in hepatocytes treated with CMPF or TOFA compared to control (Figure 41f). Modulation of AMPK activity with the inhibitor Compound C, or the activator AICAR revealed that the acute effect of CMPF is independent of AMPK activity, as the fold increase in beta oxidation remained constant with AMPK manipulation (Figure 41g,h). The effect of TOFA to increase beta-oxidation, however, is dependent on AMPK activation, as co-treatment with Compound C eliminated the ability of TOFA to stimulate beta-oxidation (Figure 41g,h). Together, this suggests that CMPF may act through ACC inhibition to increase beta-oxidation.
Figure 41. Acute effect of CMPF on hepatocyte metabolism.

a) Fatty acid uptake and (b) oxidation per hour in isolated hepatocytes following 24 hour treatment (n=4/group). c) Glucose oxidation per hour (n=4/group). d) Hepatocyte fatty acid oxidation blockage with etomoxir co-treatment (n=4/group). Western blot and quantification of (e) ACC and (f) AMPK phosphorylation in isolated hepatocytes treated for 24 hours with CMPF or TOFA (n=4/group). g) Fatty acid oxidation rate and (h) fold change in fatty acid oxidation rate in isolated hepatocytes treated for 24 hours. *P<0.05, **P<0.01, ***P<0.001. All error bars SEM.
5.3.7 CMPF Induces FGF21 Expression to Prevent Steatosis

While acute ACC inhibition by CMPF during the 7 day injection period may be responsible for early increases in beta-oxidation, CMPF is eliminated both from circulation (Figure 39b) and the liver while the mice are maintained on a high fat diet (Figure 42a). To determine how CMPF protects against the development of steatosis \textit{in vivo} even when it is no longer present, we began by investigating the AMPK-ACC pathway in liver isolated from CMPF-HFD mice and controls at the end of the 4 week diet period. Activation of AMPK leads to downstream inhibition of ACC transcription, which may potentiate a feedback loop to promote beta-oxidation. Indeed CMPF-HFD mice had both increased levels of phosphorylated AMPK (Figure 42b), as well as lower total ACC protein, with a higher ratio of phosphorylated ACC to total ACC (Figure 42c). This, combined with the \textit{in vitro} data, suggests that the AMPK-ACC pathway is likely critical for the prevention of steatosis development by CMPF.

To further elucidate the mechanism underlying the loss of ACC abundance, we performed microarray analysis of livers isolated from Control-Chow, Control-HFD, and CMPF-HFD groups. Overall, significant differences in gene expression were observed between the Control-HFD and CMPF-HFD groups, particularly in terms of genes involved in lipid processing, as anticipated based on the loss of ACC. Remarkably, expression of FGF21 was found to be dramatically induced in the CMPF-HFD condition compared to both control groups (Figure 42d,e). This was further validated in the plasma of the CMPF-HFD group, where circulating levels of FGF21 were also found to be significantly elevated (~7-fold) (Figure 42f). FGF21 is known to attenuate hepatic steatosis through regulation of lipolysis. Closer examination of FGF21 target genes indicated that FGF21 acts locally on the liver in CMPF-HFD mice to regulate lipolysis. We observed pronounced alterations in expression of a number of genes including increased expression of leptin receptor (Lepr), PCK1, and CPT1a, as well as reduced expression of both ACC1 and ACC2, Gck, and Scd1 (Figure 42d,e). Therefore, increased lipolysis and diminished lipogenesis observed in CMPF-HFD mice is mediated through FGF21.
The induction of FGF21 expression with CMPF treatment may be acute or the result of chronically enhanced lipolysis rates. To determine when FGF21 expression is increased with CMPF treatment, we examined lysates from the livers of mice following 7 days of CMPF treatment while maintained on a chow diet (Figure 42g), as well as isolated hepatocytes from chow-fed mice treated with CMPF for 24 hours (Figure 42h). FGF21 was significantly increased with CMPF treatment under both conditions. In addition, expression of key genes regulating lipolysis and glucose metabolism were significantly altered in 24 hour treated hepatocytes, including ACC1 and ACC2, and Gck (Figure 42h). Together this suggests that CMPF induces the expression of FGF21 acutely following the start of treatment, and therefore, FGF21 is likely essential for the long-term prevention of development of steatosis when CMPF is cleared from circulation.

Figure 42. Persistent effect of CMPF is associated with increased FGF21 and decreased ACC abundance.

a) Concentration of CMPF in the liver over time as determined by SRM-MS (n=4-6). Western blot and quantification of (b) AMPK and (c) ACC activity and abundance in livers isolated from mice 4 weeks following final injection (n=3-4/group). d) Heat map showing significantly altered genes associated with lipogenesis and lipolysis in livers from mice isolated 4 weeks following treatment as determined by microarray (n=3/group). e) Quantitative PCR validation of differential gene expression in liver 4 weeks following final injection (n=8/group). f) FGF21 levels in media from 24 hour treated hepatocytes, and fasting plasma concentrations from mice immediately following 7 days of injection, and following 4 weeks of high fat diet. qPCR showing expression of FGF21 and target genes in (g) livers isolated from mice immediately following 7 day injection period (n=8/group) and (h) isolated hepatocytes treated for 24 hours with CMPF (n=4/group). *P<0.05, **P<0.01, ***P<0.001. All error bars SEM.
5.3.8 FGF21KO Mice Are Resistant to CMPF Treatment

To determine if FGF21 is required for the long-term prevention of the development of steatosis we treated mice globally lacking FGF21 (FGF21KO) and age-matched c57 controls with CMPF according to the same treatment protocol (Figure 43a). Mice were maintained on a HFD for 6 weeks prior to the start of experiments. At the end of the protocol insulin sensitivity was evaluated by ITT. C57 controls treated with CMPF had significantly improved insulin sensitivity compared to HFD controls (Figure 43b). CMPF treatment had no effect on insulin sensitivity in FGF21KO mice. The lack of improvement in insulin sensitivity corresponded to no difference in hepatic triglyceride content in FGF21KO mice (Figure 43c,d). CMPF treatment significantly reduced triglyceride content in HFD-fed c57 control mice. Consistent with induction of FGF21 regulating ACC expression and activity, livers from CMPF-treated FGF21KO mice had no significant difference in total ACC content, and no difference in expression of genes regulating glucose metabolism (Figure 43e,f). Therefore, CMPF prevents the development of steatosis through induction of FGF21.
Figure 43. CMPF Prevents Steatosis through FGF21

a) Schematic of the treatment protocol used for the treatment of FGF21KO and c57 control mice. b) Blood glucose during ipITT (n=6/group). c) Oil red O staining of liver sections (n=6/group) and (d) quantification of liver triglyceride content (n=6/group). e) Western blot and (f) quantitative PCR analysis of protein and gene expression, respectively, in isolated liver tissue (n=4-6/group). *P<0.05, **P<0.01, ***P<0.001. All error bars SEM. *Preliminary data obtained from Eli Lilly*
FGF21 expression is normally elevated under fasting or nutrient restrictive conditions (Galman et al., 2008). Treatment with CMPF may mimic this status in the liver of chow-fed mice through increases in beta-oxidation, the primary fuel source during fasting, as well as inhibition of glucose utilization. This “fasting-like state” is induced rapidly, as primary hepatocytes have elevated FGF21 expression and secretion with only 24hr CMPF treatment. The increased FGF21, combined with a persistent drive toward beta-oxidation induced by CMPF over the chronic 7-day treatment period likely activates a feedback loop that continues to perpetuate after CMPF is eliminated. FGF21 activates AMPK, resulting in inhibition of SREBP1c, and reduced expression of acetyl-CoA carboxylase (ACC) enzymes 1 and 2 (outlined in Figure 44) (Potthoff et al., 2009). These enzymes are rate-limiting for the production of malonyl-CoA, which is required for both triglyceride synthesis, as well as inhibition of beta-oxidation (Abu-Elheiga et al., 2001). Livers isolated from mice treated with CMPF have a significant reduction in total ACC levels, supporting this hypothesis. Loss of ACC may explain the inability of CMPF-treated mice to accumulate hepatic triglyceride, even when maintained on a 60%kcal from fat high fat diet.

Differences in fat distribution and adipocyte size may be attributed to increases in circulating FGF21. Previous studies using recombinant FGF21 have shown that elevated FGF21 induces browning of white adipocytes, increasing energy expenditure and decreasing body weight (Fisher et al., 2012). This finding has been recapitulated in human clinical trials of FGF21, which have shown significant weight loss with minimal impact on food consumption and activity levels (Kharitonenkov and Adams, 2014). SRM-MS analysis of adipocytes following CMPF injection have minimal CMPF accumulation, suggests lack of a direct effect of CMPF in this tissue. This remains an avenue for future investigation.

The loss of glucose metabolic genes including the glucose transporter GLUT2 and glucokinase is characteristic of the effect of CMPF, and is also observed in islets isolated from CMPF-treated mice months following treatment. This may be a contributing factor to the induction of a fasting-like state due to reduced availability of glucose. Reduced glucose availability alters the ability of hepatocytes to store glycogen (Leturque et al., 2009), and induction of a “fasting-like” state by CMPF likely potentiates breakdown of the glycogen stores that are present. It may be important to characterize the effect of reduced hepatic glycogen on the metabolic health of CMPF-treated
mice. Characterization of chow-fed mice treated with CMPF using hyperinsulinemic euglycemia clamps demonstrated a significant reduction in both glucose appearance and disappearance rates, suggesting altered gluconeogenesis even under normal chow-fed conditions (Prentice et al., 2014). These mice may have an impaired response to fasting, particularly if the fasting response is stimulated under normal fed conditions.
Figure 44. Schematic Diagram Showing the Proposed Role of FGF21 in Reducing Hepatic Lipid Accumulation with CMPF Treatment
5.5 MATERIALS AND METHODS

5.5.1 CMPF Preparation

CMPF was purchased from Cayman Chemical (product number 10007133) and dissolved in 100% ethanol to a stock concentration of 100mM. CMPF was stored at 4°C. For injections, CMPF was dissolved in 100ul sterile saline within an insulin syringe for injection.

5.5.2 Intraperitoneal injection of CMPF and Tolerance Tests

Seven-week-old male CD1 mice were purchased from Charles River and allowed to acclimate for one week prior to the beginning of experiments. Mice were maintained on standard chow diet while being injected intraperitoneally (i.p.) with 6mg/kg CMPF or vehicle control at 24h intervals for seven days, as previously described (Prentice et al., 2014). 24 hours following the final injection, mice were switched to either a 60%kcal from fat HFD (HFD; OpenSource diets D12492, Research Diets Inc, USA) or a sucrose-matched control (OpenSource diets D12450J, Research Diets Inc, USA) for 4 weeks. Mice were also monitored weekly for individual body weights and whole-cage food consumption.

At the end of the follow-up period, mice were fasted overnight for 14h before an intraperitoneal glucose tolerance test (ipGTT). Mice were injected ip with 1g/kg sucrose. IpGTTs, and measurement of plasma insulin were performed as previously described (Allister et al., 2013). Blood (<25ul) was collected from the tail vein at 0 (fasting), 10, 20 and 30 minutes from quantification of plasma insulin and blood glucose. Blood glucose was also measured at 60 and 120 minutes post injection. IP insulin tolerance tests (ipITT) were performed following a 4h fast. 1.5IU/kg insulin was injected and blood glucose was measured at 0, 10, 20, 30, 45, 60 and 120 minutes. Plasma insulin was quantified by ELISA (Alpco Ultrasensitive Insulin ELISA). All experiments were approved by the Animal Care Committee at the University of Toronto and animals handled according to the Canadian Council of Animal Care guidelines.
5.5.3 SRM-MS

Briefly, mice were injected with 6mg/kg CMPF intraperitoneally, as described. Blood was collected 20 minutes, 2 hours, 24 hours, or 4 weeks following final injection. 20 minute and 2 hour blood samples were taken from separate mice due to the large volume (~60ul) required. CMPF standards (1 – 500 ng) and samples were spiked with 25 ng of CMPF-d5 (Cayman Chemical, USA) internal standard. A surrogate matrix of 4% BSA in PBS was used for standards. Plasma (20µL) and standards (20µL matrix surrogate) were diluted with 480 µL of ultrapure water. 20µL of 80% phosphoric acid was added, mixed by vortexing then 1.5 mL of ethyl acetate (EtOAc) was added. Samples were chilled on ice, and then centrifuged. The upper EtOAc layer was collected and re-extracted with another 1.5 mL of EtOAc. Samples were vortexed, chilled on ice, and centrifuged. The upper EtOAc layers were removed and combined with the previous extract. The combined EtOAc layers were dried and residues were reconstituted in 500 µL of acetonitrile and analyzed by LC-MS/MS using an Agilent 1200 HPLC with an API 4000 mass spectrometer (AB Sciex). For the liver samples, the extraction protocol is the same except that the liver was homogenized in 1:1 water:ethanol at a concentration of 100 mg/mL and the equivalent of 20 mg was extracted (200 µL). 300 µL water was added instead of 480µL. The curve range for the liver was 0.5–100 ng and the curve was extracted from control liver.

5.5.4 Fat Distribution Analysis by MRI

Mice were anesthetized using isofluorance and imaged by MRI. A heating pad through the imaging process maintained body temperature. 26 images were captured from each mouse, and fat distribution was assessed using TissueStudio where area of visceral and subcutaneous fat depots was quantified in each section for each animal. Total areas were summed and averaged within groups.

5.5.5 CLAMs

CLAM experiments were performed as previous described. Briefly, mice were housed individually in CLAMs with monitoring of food and water consumption, activity in x,y,and z planes, oxygen consumption and CO2 production were measured every 10 minutes over 48 hours. The cages were housed in a room with a standard 12 hour light-dark cycle. Mice were
provided with the same diet they were maintained on before entering the cages. The first 24 hours of data was removed from analysis while the mice were acclimated to the cages. Analysis was performed on the final 24 hours within the cages.

5.5.6 Quantification of Circulating Factors

Circulating leptin, adiponectin, FGF21, and triglyceride were measured in 14hr fasting samples obtained at the time of sacrifice. Total free fatty acids were quantified from 14hr fasting samples and following a 1hr refeed with ad libitum access to food. Assays were performed according to manufacturer protocols (BioVision, USA).

5.5.7 Western Blotting

To evaluate in vivo insulin signaling, mice were given a 1IU/kg insulin bolus by tail vein injection. Mice were sacrificed 10 minutes following injection, tissues isolated and flash frozen in liquid nitrogen. Liver and muscle tissues for analysis were ground in liquid nitrogen and lysed in RIPA buffer (Cell Signaling, USA) containing protease inhibitor cocktail (Roche, Canada) and stored at -20C prior to use. Lysates were spun at 12,000rpm and supernatant was evaluated for protein content by Bradford assay (BioRad, Canada). Equal amount of protein were then combined with sample buffer containing DTT and loaded onto a 4-15% SDS-PAGE gradient gel (BioRad, Canada) and run at increasing voltages of 50V for 30minutes, 75V for 30 minutes, and then 100V for 30 minutes. Proteins were then transferred onto PVDF membrane using a Turbo Blotter (BioRad, Canada). The membrane was probed with antibodies as listed in Table 15, and imaged using Kodak Imager 4000pro (Carestream, USA).

5.5.8 Quantification of Liver Triglyceride

Livers were isolated, embedded into TissueTek and frozen. Staining of sections was performed as previously described. For H&E staining, livers were weighed and fixed in 10% neutral buffered formalin at the time of sacrifice. Tissue processing and immunostaining for insulin have been described previously (Luu et al., 2013). Briefly, pancreata were dehydrated, cleared and embedded in paraffin. Paraffin sections were cut at 4um from the middle of the pancreas. Sections were then dried and deparaffinized for staining. Images of
each section were acquired using Aperio Imagescope at 40x magnification (Aperio Imagescope).

### 5.5.9 Fatty Acid Uptake and Oxidation

Fatty acid and glucose uptake and oxidation experiments were performed as previously described. Briefly, primary hepatocytes were isolated from 7-9 week old wild type mice and plated at a density of 400,000 cells per well in 12-well plates. Cells were allowed to attach in DMEM media supplemented with 10% FBS for 4 hours. Cells were then washed and cultured in Williams Solution without FBS. Cell were treated with vehicle or 200µM for CMPF starting at this time point for 18 hours. Compound C and AICAR were incubated for 4 hours prior to oxidation and uptake. CMPF was conjugated to fatty acid free BSA for 4 hours prior to starting treatment. At the end of the treatment period, cells were incubated with transport solution containing 20µM oleic acid and 0.5µCi/ml of 14C oleic acid, conjugated to BSA for 4 hours. Uptake experiments were performed at room temperature for 3 minutes, oxidation experiments were performed at 37°C for 90 minutes. Data was normalized to protein content determined by Bradford assay.

### 5.5.10 Gene Expression

Total RNA was extracted from isolated islets using the Qiagen RNeasy Plus mini kit (Hilden, Germany). Reverse transcription from total RNA and quantitative real time PCR (qPCR) analysis was performed as previously described (Basford et al., 2012). Reverse transcription was performed using a M-MLV kit according to manufacturers instructions (Sigma Aldrich, Canada). Primers were designed using Primer3 software (NCBI) and are listed in Table. Data were normalized to β actin mRNA. Microarray analysis was performed as previously described (Basford et al., 2012) using the Affymetrix Mouse 430 2.0 Gene Chip at the University Health Network microarray center (Toronto, Canada). Significant changes were defined as P<0.05.

### 5.5.11 FGF21KO Mice

Six to nine week old male FGF21KO mice were obtained from Taconic along with age-matched c57 controls. Mice were allowed to acclimate for one week prior to the beginning
of the treatment period and randomized to chow and HFD control and CMPF treatment groups. Mice were injected with 6mg/kg/day CMPF for seven days and changed to HFD and matched sucrose control as described above. Mice were maintained on the diet for six weeks prior to evaluation. FGF21 deletion was confirmed by PCR.

### 5.5.12 Statistics

Statistical significance was assessed using either the Student’s t-test, or a two-way ANOVA for repeated measures followed by a Bonferroni post-test comparison where required. $P<0.05$ was considered significant. All data is mean ± SEM unless otherwise specified.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
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</thead>
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<tr>
<td>B-Actin</td>
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<td>CCCTGGCTGCCTCAACAC</td>
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<tr>
<td>Glut2</td>
<td>CCTTGGCCTTGTGTTCT</td>
<td>TTGTACAGCAGCTTTGCGTG</td>
</tr>
<tr>
<td>GCK</td>
<td>GAGATGGATGTGGTGCAAT</td>
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</tr>
<tr>
<td>Ucp2</td>
<td>CAGCCAGGCCCAGTACC</td>
<td>CAATGCGGACGGAGGCAAAGC</td>
</tr>
<tr>
<td>Acc1</td>
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<td>AGGATCTACCAAGCCACAT</td>
</tr>
<tr>
<td>Acc2</td>
<td>ACCTGCAGAGAAGGTGAG</td>
<td>GTGTTTTGAAACGCACCTC</td>
</tr>
<tr>
<td>Srebp-1c</td>
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<td>GGCACTGGCTCTCTTGAT</td>
</tr>
<tr>
<td>Pparg</td>
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<td>GCTGACGTGCTCTGTGACGA</td>
</tr>
</tbody>
</table>
### Table 19. Primers Used for Western Blotting

<table>
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<th>Antibody</th>
<th>Western Dilution</th>
<th>Supplier</th>
<th>Cat. Number</th>
</tr>
</thead>
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<tr>
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<td>Cell Signaling</td>
<td>3662</td>
</tr>
<tr>
<td>Rabbit α pACC</td>
<td>1:1000</td>
<td>Cell Signaling</td>
<td>11818</td>
</tr>
<tr>
<td>Rabbit α AMPKα</td>
<td>1:1000</td>
<td>Cell Signaling</td>
<td>2603</td>
</tr>
<tr>
<td>Rabbit α pAMPK</td>
<td>1:1000</td>
<td>Cell Signaling</td>
<td>2535</td>
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<td>Rabbit α Akt2</td>
<td>1:1000</td>
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<td>Rabbit α pAkt2 (Ser473)</td>
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<td>Cell Signaling</td>
<td>8599</td>
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</table>
5.6 CONCLUDING REMARKS

In this chapter we broaden our investigation of the effect of CMPF from a primary focus on the pancreatic beta cell to the liver. We demonstrate that CMPF has a direct effect on the liver, enhancing fatty acid uptake and oxidation to prevent the development of steatosis and hepatic insulin resistance, even with exposure to a 60% kcal from fat high fat diet (HFD). This effect is mediated by the induction of FGF21, which acts to inhibit expression of Acetyl-CoA Carboxylase (ACC) enzymes and prevent feedback inhibition of beta-oxidation and triglyceride synthesis and storage. Genetic deletion of FGF21 almost completely inhibits the effect of CMPF, and mice develop steatosis to a similar degree as HFD FGF21KO controls. This is an interesting finding given the apparent direct effect of CMPF on driving preferential beta-oxidation in the beta cell. This may be attributed to the difference in preferred substrates between the liver and beta cell. While the beta cell is designed to exquisitely sense and respond to glucose as its primary fuel source, the hepatocyte primarily relies on beta-oxidation and stores glucose as glycogen for release under fasting conditions to prevent hypoglycemia. The mechanism of CMPF-induced preferential fatty acid oxidation in the beta cell may therefore already be activated in the hepatocyte, thus CMPF is not effective under conditions where FGF21 is eliminated. This remains an avenue for future investigation.

An important factor in the interpretation of the liver studies is the mode of CMPF delivery by intraperitoneal injection, as CMPF will have a large first pass clearance through the liver. Therefore, concentrations of CMPF seen by the liver may be supraphysiological and the observed effect represent pharmacological activity rather than a physiological function. Preliminary studies using subcutaneous injection of CMPF have demonstrated lower circulating levels with the same 6mg/kg dosage and a similar effect on preventing the development of fatty liver as the intraperitoneal route, suggesting that increased lipolysis is associated with systemic increase in CMPF. Further studies should explore alternate modes of CMPF delivery, including chronic administration through osmotic pump, to ensure that the observed effect is not indirectly due to the route of administration.

The mechanism underlying induction of FGF21 expression also remains unanswered. Trial studies investigating direct inhibition of ACC or activation of PGC1a by CMPF have yielded negative results, suggesting that CMPF is not an allosteric regulator of these enzymes. This is
consistent with CMPF being eliminated from tissues and circulation within 24 hours of the final injection. It is possible that a metabolite of CMPF has this function, however the identity of this metabolite must be determined before this can be tested. Work is currently underway to test analogs of CMPF in this way.

Interestingly, while CMPF has the effect of both preventing steatosis, as discussed in this chapter, and reversing steatosis, as discussed in Chapter 3, this appears to occur through different mechanisms. FGF21 is not induced in obese mice that are treated with CMPF. When isolated hepatocytes are loaded with fatty acids prior to CMPF treatment, they maintain the same phenotype of enhanced fatty acid uptake and oxidation. This suggests that the primary function of CMPF to drive preferential beta-oxidation remains intact, and somehow the effect of FGF21 overpowers this effect in lean animals. It is possible that obese animals do not observe the induction of a “fasting-like” state due to the abundant nutrient supply in the liver of obese animals. It may be necessary to follow these animals longer term to observe induction of FGF21 expression and potentiation of a lean liver. This obese model may provide further insight into the mechanism of action of CMPF in the liver in the acute setting to understand how fatty acid oxidation is potentiated in a tissue that is already heavily reliant on fatty acid substrates.

Finally, the mechanism of long-term potentiation may be due to epigenetic changes induced by CMPF in both the liver and the beta cell. Chronic changes in gene expression, particularly GLUT2 and glucokinase indicate that epigenetic changes may be made to the promoter regions of these genes to regulate their expression. This is a valid explanation for how the effects of CMPF can potentiate for so long after CMPF is eliminated, and is an important avenue for future research.
Chapter 6- Discussion
6.1 DISCUSSION

Overall, our findings suggest that the furan fatty acid metabolite 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) may play a causal role in the development of gestational (GDM) and type 2 diabetes (T2DM). Analysis of human plasma from diverse populations has revealed that CMPF is significantly elevated in overt diabetes, and that a rapid elevation in CMPF is associated with acceleration of diabetes development. Interestingly, CMPF is modestly elevated in individuals who are classified as prediabetic, with either impaired glucose tolerance (IGT) or impaired fasting glucose (IFG) and this modest elevation is maintained long-term as the prediabetic status continues. This indicates that there may be a threshold concentration of CMPF that is required to induce beta cell dysfunction. Alternately, a modest level of CMPF could be protective against the development of overt diabetes, particularly due to its effect on improving liver insulin sensitivity.

The cause of elevated CMPF remains unknown, though increasing evidence is suggesting it is due to impairment in clearance rather than increased generation. This is supported by the diversity of the populations we have examined and by the timing of elevation in CMPF that we have been able to determine. We have evaluated plasma CMPF concentrations in populations of both males and females with diverse age, race, BMI, and geographical location. Our populations have included young pregnant women from Toronto, middle-aged men enrolled in cardiac trials from Indianapolis, Indiana, obese pregnant women in northern California, and older men and women from Shanghai, China. The differences in lifestyle and diet of these populations are significant, though the elevations in plasma CMPF concentrations appear to be quite consistent, suggesting an alternate underlying mechanism other than environmental exposure. We propose that this mechanism is likely related to impaired function of the organic anion transporters (OATs) in the kidneys of these individuals. OAT expression is regulated by sex hormone expression profiles, with high levels of androgens resulting in decreased expression of OAT3, the primary transporter of CMPF (Breljak et al., 2013; Deguchi et al., 2005). Multiple studies have determined that altered androgen levels in both males and females is associated with significantly increased risk of T2D (Navarro et al., 2015). Additionally, kidney dysfunction is a prominent complication of diabetes and differences in filtration are apparent early in disease progression. It would be interesting to examine changes in circulating CMPF as they correlate with differences in OAT transporter expression. For example, women with polycystic ovarian syndrome (PCOS)
or entering menopause when androgen levels are changing (Nisenblat and Norman, 2009) may have elevated levels of CMPF, putting them at increased risk of diabetes development. Studies have demonstrated that menopausal women who do not undergo hormone replacement therapy have a significantly greater risk of developing T2DM compared to women with estrogen supplementation, supporting this hypothesis (Rossi et al., 2004).

Our studies in rodent models have shown that CMPF is causally related to beta cell dysfunction, independent of insulin resistant status. Elevation of circulating CMPF levels to those found in diabetic patients impaired glucose-stimulated insulin secretion in vivo, ex vivo, and directly following in vitro treatment. This impairment is associated with both direct reduction in glucose metabolism, in favor of preferential fatty acid metabolism, as well as with the generation of reactive oxygen species (ROS), which alters transcription factor activity and expression of genes required for insulin biosynthesis, glucose sensing, and glucose metabolism. Under conditions where islets are in a compensatory state when exposed to CMPF, such as diet-induced obesity or genetic obesity in the leptin-deficient ob/ob mouse, CMPF appears to shunt the excess glucose toward alternate pathways, resulting in high levels of advanced glycation end products (AGEs) and impaired insulin granule processing, accelerating diabetes development.

The progression from normal glucose tolerance to prediabetes occurs according to a fairly predictable series of events including the induction of insulin resistance, enhanced insulin output both under basal and glucose-stimulated conditions, and a gradual loss of 1st phase insulin secretion resulting in the prediabetic state (Weir and Bonner-Weir, 2004). Importantly, this prediabetic status with mild alterations in glucose tolerance can be maintained for years with only minimal impact on overall health (Bertram and Vos, 2010). The risk of transition from prediabetes to overt diabetes occurs is about 10% annually, and when this occurs, the evolution is extremely rapid, with fasting blood glucose levels changing from 7-8mmol/l to 17-20mmol/l within weeks (Weir and Bonner-Weir, 2004). This development is not associated with sudden changes in BMI or insulin resistance, but instead due to a “tipping point” in beta cell function with complete failure of compensation. Many argue that this tipping point is simply due to exhaustion of the beta cell following chronic exposure to glucolipotoxic conditions, ER or oxidative stress (Poitout et al., 2010; Robertson, 2004). However, this model is not consistent with acute forms of diabetes, such as gestational diabetes that occurs only weeks following induction of insulin resistance (Buchanan and Xiang, 2005b). Therefore, there may be some
independent factor that is the causal agent underlying this acceleration to beta cell failure. Our studies in a human population suggest that a rapid elevation in CMPF may cause this tipping point in beta cell dysfunction. The rapid elevation of CMPF in models of diabetes progression accelerates diabetes development and produces a beta cell phenotype that closely resembles the human condition including reduced glucose sensitivity (Mari et al., 2010), loss of first phase insulin secretion (Cheng et al., 2013), elevation in proinsulin content and secretion (Grill et al., 2002; Sempoux et al., 2001) and AGE formation (Singh et al., 2001). and disproportionate loss of large islets (Kilimnik et al., 2011). Thus, elevated circulating CMPF is a major risk factor for the development of beta cell failure in the progression of diabetes.

The activity of CMPF in the beta cell can be prevented with blockage of the CMPF transporter organic anion transporter 3 (OAT3). Using pharmacological inhibition or genetic deletion, the effects of CMPF can be completely blocked in vitro, suggesting that the activity of CMPF is dependent on its entry into the cell. Once inside, CMPF, or a metabolite of CMPF, increases ROS levels and acts to impair glucose metabolism. The identity of possible metabolites remains to be elucidated. Preliminary studies using untargeted mass spectrometry have revealed a subset of potential candidate molecules, primarily with a larger molecular weight than the parent ion of CMPF itself. This suggests that the active form of CMPF may be an ester of the compound. This may also explain the long-lasting phenotype of CMPF. If CMPF is esterified to a membrane, it would be undetectable as CMPF in our targeted mass spectrometry approach looking for residual CMPF in tissues weeks following treatment, and remain in the cells to have metabolic activity. Through collaborations with Eli Lilly and Company we are now testing putative forms of CMPF, including multiple ester compounds, that may be generated in vivo and function as the active compound. This remains an exciting avenue of future research.

The underlying mechanism of CMPF appears to be related to the induction of metabolic inflexibility, an impaired ability to switch between fatty acid and carbohydrate substrates. This induction appears to occur very acutely, following only one dose in vivo or 24 hour treatment in vitro. Interestingly, this switch appears to be reversible if fatty acid utilization is inhibited, as in the case of 1hr etomoxir treatment prior to glucose-stimulated insulin secretion. This indicates that there is likely no difference in protein expression or activity that is solely responsible for this phenotype. Rather, we believe that CMPF enhances fatty acid oxidation through a yet unknown pathway, and that it is this increase in beta-oxidation and respective increased in acetyl-CoA and
fatty acid-CoA that is inhibiting glucose metabolism, as has been extensively described in the literature (Hue and Taegtmeyer, 2009).

While this metabolic inflexibility phenotype appears to be extremely detrimental from the perspective of beta cell function, it is paradoxically beneficial for hepatic health and insulin sensitivity. Non-alcoholic fatty liver disease (NAFLD) is a condition of excessive fat deposition in the liver caused by excessive fatty acids that exceed the storage capacity of the adipose tissue as well as the metabolic capacity of the liver (Birkenfeld and Shulman, 2014). The excessive diacylglyceride content of the liver alters insulin receptor signaling, causing insulin resistance and contributing the metabolic syndrome, prediabetes, and overt diabetes. In some cases, NAFLD can progress to more severe forms of liver disease including Non-Alcoholic Steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma (Angulo, 2002; Bayard et al., 2006). CMPF treatment appears to prevent NASH through enhancing the capacity of hepatocytes to metabolize fatty acids, preventing lipogenesis and storage of lipids as triacylglyceride. This also acts to protect against the development of insulin resistance due to maintenance of insulin receptor signaling. Thus, if the activity of CMPF on enhancing fatty acid oxidation could be targeted specifically to the liver, it may represent an exciting therapeutic for NAFLD and insulin resistance.

Interestingly, the effect of CMPF on preventing the development of steatosis is also maintained long-term, after CMPF is no longer in circulation. This capacity is maintained through induction of FGF21 very rapidly following CMPF treatment in lean animals. FGF21 may act on tissues other than the liver to protect against further impairment in insulin signaling (Fisher et al., 2012; Galman et al., 2008). Several pharmaceutical companies are currently investigating FGF21 as a drug for weight control and insulin sensitization (Kharitonenkov and Adams, 2014). Transgenic rodent models constitutively overexpressing FGF21 are leaner, have increased insulin sensitivity, extended lifespans, and increased brown adipose tissue, which is linked to increased thermogenesis and weight loss, making this an exciting therapeutic option (Kharitonenkov and Adams, 2014; Zhang et al., 2012). Clinical trials with recombinant FGF21 have shown promising results in terms of reducing appetite and subsequently body weight, though data on promoting insulin sensitivity in liver and adipose tissue has been more inconclusive (Gaich et al., 2013). Thus, while CMPF may be very specific in the tissues it is able to enter and effect due to the expression profile of OAT3, it may activate other factors which can enter circulation and modify...
whole-body metabolism. The effect of CMPF on other metabolic tissues including muscle and adipose tissue, including the possibility of increased browning of adipose tissue, is currently under investigation.

Recent work in models of obesity through high fat diet (HFD) feeding, or genetic deletion of leptin, has revealed that CMPF is also capable of completely eliminating steatosis in these models after only two weeks of treatment. Unexpectedly, this activity is independent of FGF21. However, the robust induction of beta-oxidation remains a consistent finding with CMPF treatment. This result gives us important insight into mechanism of induction of FGF21 in lean animals. FGF21 is a stress hormone, elevated in periods of extended fasting (Galman et al., 2008). It acts to promote fatty acid oxidation to enhance survival under these conditions, where glucose supply is limited and circulating free fatty acids are elevated. In lean animals, the induction of fatty acid oxidation by CMPF combined with limited glucose concentrations due to reduced expression of glucose transporters and a low supply of stored fatty acids may exacerbate these fasting signals, eliciting the expression stress-response hormones such as FGF21. FGF21 can then activate a feedback loop by enhancing beta-oxidation and altering gene expression, including reductions in acetyl-CoA carboxylase (ACC) enzymes that act as rate-limiting steps for beta-oxidation and lipogenesis, to potentiate this cycle (Abu-Elheiga et al., 2001). In obese conditions, however, glucose and fatty acid supplies are abundant in the steatotic liver in the forms of glycogen and triglyceride. When CMPF is elevated and activates fatty acid oxidation, there is plenty of substrate available, thus an extreme starving response is not activated. It is possible that this pathway would be activated in a prolonged protocol after all of the substrates have been utilized, but this remains to be determined.

There is complex crosstalk between the beta cell and liver to regulate metabolic homeostasis. When insulin is secreted from the beta cell, it enters the portal vein and moves directly into the liver where it has both metabolic actions and is cleared from circulation. Approximately 60% of secreted insulin is cleared by the liver, making the concentration of insulin seen by the liver significantly higher than any other tissue in the body (Kotronen et al., 2007). Once in the liver, insulin acts to stimulate synthesis of glycogen and lipids, and halt lipolysis and gluconeogenesis (Birkenfeld and Shulman, 2014; Kotronen et al., 2008). Thus, alterations in circulating insulin can act to modify development of NAFLD. Loss of insulin signaling in the liver caused by genetic deletion of the liver insulin receptor (LIRKO), for example, prevents the development of
steatosis due to a loss of insulin-mediated activation of SREBP-1c, the primary mediator of lipogenesis (Haas et al., 2012). Conversely, induction of hepatic insulin resistance feeds back to stimulate beta cell compensation to increase insulin production. Islets of the LIRKO mouse are extremely hypertrophic, with an increase in insulin area of 2-3 fold compared to controls (Michael et al., 2000). Thus modulation of either insulin secretion or hepatic insulin sensitivity can have significant consequences on the opposing tissue type. This has important implications in the study of CMPF, particularly in the context of insulin resistance.

While it is clear that CMPF has a direct effect on both the beta cell and the hepatocyte based on *in vitro* assays, the impact on whole-body glucose tolerance and insulin sensitivity is somewhat more complex. It can be argued that the prevention or elimination of steatosis in obese animals is caused by reduced insulin secretion from the beta cell. Conversely, reduced insulin secretion and alterations in beta cell mass may be due to reduced insulin demand associated with improved hepatic insulin sensitivity. Evidence suggests that the defect in insulin secretion is independent of the hepatic phenotype, as reduced glucose-stimulated insulin secretion is present in lean chow-fed animals where there is no difference in insulin sensitivity. It is also unlikely that reduced insulin secretion is responsible for the improvements in steatosis, due to the fact that fasting and random insulin levels are not significantly different compared to controls, and the defect is only apparent under glucose challenge testing. However, these are simply observations and do not provide conclusive evidence one way or the other. Further research is required to elucidate the directionality of this relationship under insulin resistance conditions. Use of LIRKO mice to establish hepatic insulin resistance may be one model to elucidate this interaction.

In conclusion, the discovery of CMPF as being elevated in human diabetic patients has opened the door to many exciting avenues of future research. CMPF may play a causal role in accelerating beta cell failure in the development of human diabetes, and thus may represent a drug target for the prevention of diabetes. The elevation in CMPF in prediabetic and diabetic individuals also makes it an interesting candidate biomarker for the prediction of diabetes risk. The evaluation of CMPF levels by a simple ELISA assay makes this technology highly translatable to the clinic. Finally, the role of CMPF in improving hepatic insulin sensitivity and ameliorating steatosis is a novel concept, particularly due to the long-term effects of CMPF. If specificity can be established to target CMPF delivery to the liver and protect the beta cell,
CMPF could be used to prevent and treat NAFLD and NASH, and possibly improve insulin sensitivity to prevent against diabetes development.
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metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme 29, 301-307.


