INFLUENCE OF DELTA-6-DESATURASE ON HEPATIC MEMBRANE COMPOSITION IN OBESITY/INSULIN RESISTANCE: IMPLICATIONS FOR THE DEVELOPMENT OF CARDIOMETABOLIC SYNDROME

A Thesis Submitted to

The Department of Chemistry

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the degree of Master of Science

by Dina Safwat Nemr

under the supervision of Dr. Hassan Azzazy and Dr. Adam Chicco

FALL/2012
DEDICATION

To my family

whose patience and support gave me the strength to see this project to its completion and who provided a loving and nurturing environment throughout this experience.
ACKNOWLEDGMENTS

I would like to thank my advisor and mentor Dr. Hassan Azzazy for his continuous support, for creating exciting opportunities and new learning experiences throughout the three years of my study, and without whom none of this would have been possible.

I would also like to thank my co-advisor Dr. Adam Chicco for allowing me to conduct my thesis research at his laboratory at Colorado State University, and for being a patient, supportive and attentive mentor.

I thank the Chicco research group at Colorado State University, namely, PhD students Christopher Mulligan, Catherine Le and MS graduate Melissa Routh for their assistance and for making my stay a pleasant one.

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Last, but not least, I would like to thank the Chemistry Department and the American University in Cairo for their financial and academic support.
ABSTRACT

The American University in Cairo

Influence of Delta-6-desaturase on Hepatic Membrane Composition in Obesity/Insulin Resistance: Implications for the Development of Cardiometabolic Syndrome

Dina Safwat Nemr

Under the supervision of Dr. Hassan Azzazy and Dr. Adam Chicco

Obesity has become a worldwide phenomenon and has been accompanied by a parallel rise in the incidence of insulin resistance, type 2 diabetes, inflammation, dyslipidemia and cardiovascular disease. Together, these symptoms have been collectively referred to as “cardiometabolic disease” (CMD). Delta-6-desaturase catalyzes the rate limiting step in the conversion of linoleic acid (LA) into arachidonic acid (AA), which in turn is converted into pro-inflammatory eicosanoids. Increasing evidence suggests a link between D6D hyperactivity and the development of CMD, though this hypothesis remains to be tested experimentally.

We hypothesized that obesity and a high-fat diet, leading to the development of CMD, will be reversed/prevented by the pharmacological inhibition of D6D using the drug SC-26196. In mouse models of obesity resulting from leptin-deficiency (ob/ob) or a high-fat diet (in LDL receptor knockout mice) a detailed assessment of the acyl composition of serum and hepatic phospholipids was conducted to provide insight into the nature of phospholipid remodeling in disease and with D6D inhibition, as well as the potential interaction of D6D with phosphatidylethanolamine N-methyltransferase (PEMT), the enzyme which catalyzes the hepatic conversion of phosphatidylethanolamine (PE) into phosphatidylcholine (PC). The hepatic PC/PE ratio was used as a surrogate measure for PEMT activity. The extent of CMD was assessed by analyses of serum triglycerides, cholesterol, and macrophage chemoattractant protein-1, as well as hepatic free AA and eicosanoids.

Obesity and a high-fat diet resulted in elevated D6D activity, accompanied by manifestations of CMD, which were reversed with D6D inhibition. Though the mechanism of the interaction between D6D and PEMT remains unclear, the results suggest that it may be bidirectional, where D6D may influence PEMT activity, in addition to the reported effects of PEMT on D6D. Differences were observed between the ob/ob and LDLR-/- models in disease etiology, pathophysiology, and response to treatment. These differences should be considered when selecting a research model of CMD. In conclusion, the production of AA through D6D metabolism and the potential involvement of PEMT and other enzymes, such as PLA-2, appear to play an important role in the pathogenesis of CMD by complex interactions with multiple systems that merit further investigation.
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>AdoHcy</td>
<td>S-adenosylhomocysteine</td>
</tr>
<tr>
<td>AdoMet/SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>ALA</td>
<td>α-linolenic acid</td>
</tr>
<tr>
<td>AT</td>
<td>Acyl transferase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CDP-choline pathway</td>
<td>Cytidine diphosphate-choline/Kennedy pathway</td>
</tr>
<tr>
<td>CMD</td>
<td>Cardiometabolic disease</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme-A</td>
</tr>
<tr>
<td>Con</td>
<td>Control</td>
</tr>
<tr>
<td>Consc</td>
<td>Control + SC-26196</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclo-oxygenase</td>
</tr>
<tr>
<td>CSU</td>
<td>Colorado State University</td>
</tr>
<tr>
<td>CT</td>
<td>CTP:phosphocholine cytidylyltransferase</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450 mono-oxygenase/epoxidase</td>
</tr>
<tr>
<td>D5D</td>
<td>Delta-5-desaturase</td>
</tr>
<tr>
<td>D6D</td>
<td>Delta-6-desaturase</td>
</tr>
<tr>
<td>DGLA</td>
<td>Dihomo-γ-linolenic acid</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DHETEs</td>
<td>Dihydroxyeicosatrienoic acids</td>
</tr>
<tr>
<td>DiH2O</td>
<td>De-ionized water</td>
</tr>
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<td>EETs</td>
<td>Epoxyeicosatrienoic acids</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionization detector</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GLA</td>
<td>Gamma-linolenic acid</td>
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<tr>
<td>GLUT4</td>
<td>Glucose transporter type 4</td>
</tr>
<tr>
<td>GTT</td>
<td>Glucose tolerance test</td>
</tr>
<tr>
<td>Hcy</td>
<td>Homocysteine</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HETE</td>
<td>Hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>HPETE</td>
<td>Hydroperoxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HUFA</td>
<td>Highly unsaturated fatty acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IKKβ</td>
<td>Inhibitor of nuclear factor κB kinase-β</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin resistance</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>LA</td>
<td>Linoleic acid</td>
</tr>
<tr>
<td>LC/MS/MS</td>
<td>Liquid chromatography/mass spectrometry</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
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<td>LDLR&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>LDL-receptor-deficient mouse model</td>
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<td>LDLR-WDSC</td>
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<td>Lipoxygenase</td>
</tr>
<tr>
<td>LTs</td>
<td>Leukotrienes</td>
</tr>
<tr>
<td>MCP-1/CCL2</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MTP</td>
<td>Microsomal triglyceride-transfer protein</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acid</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NASH</td>
<td>Non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>Ob</td>
<td>Obese mice fed a regular chow diet</td>
</tr>
<tr>
<td>Ob/ob</td>
<td>Leptin-deficient mouse model of obesity</td>
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<tr>
<td>Obsc</td>
<td>Obese mice fed a regular chow diet + SC-26196</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PDK-1</td>
<td>Phosphoinositide-dependent protein kinase-1</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PEMT</td>
<td>Phosphatidylethanolamine N-methyltransferase</td>
</tr>
<tr>
<td>PGs</td>
<td>Prostaglandins</td>
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<td>PI3-K</td>
<td>Phosphatidylinositol 3-kinase</td>
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<td>PIP3</td>
<td>Phosphatidylinositol 3-phosphate</td>
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<td>PJNK</td>
<td>Phosphorylated Jun N-terminal kinase</td>
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<td>PKB/Akt</td>
<td>Protein kinase B</td>
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<td>PKC</td>
<td>Protein kinase C</td>
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<td>PLA-2</td>
<td>Phospholipase A-2</td>
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<td>PL</td>
<td>Phospholipid</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RU</td>
<td>Relative units</td>
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<tr>
<td>SFA</td>
<td>Saturated fatty acid</td>
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<tr>
<td>SH2</td>
<td>Src-homology-2 domain</td>
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<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<tr>
<td>SP1</td>
<td>Specificity protein-1</td>
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<td>Abbreviation</td>
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<tr>
<td>SREBP</td>
<td>Sterol regulatory element-binding protein</td>
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<td>Type 2 diabetes</td>
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<td>Very-low-density lipoprotein</td>
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<td>Western diet</td>
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<td>World Health Organization</td>
</tr>
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<td>WT</td>
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1.0 Introduction

1.1 Obesity and Cardiometabolic Disease

Obesity has been reaching epidemic proportions in developed countries. This is due not only to the quantity of food consumed, resulting in over-nutrition, but also due to the quality of food. The predominant “Western diet” (WD) in developed countries, being high in fat and sucrose, has been a leading cause of obesity. However, obesity has also extended to become a worldwide epidemic, where developing countries have also seen a rise in obesity and overweight in all age groups [1].

Obesity is associated with hypertrophy of adipocytes, resulting from the accumulation of lipid in the cells causing them to increase in size. This has been suggested to increase the production of proinflammatory signaling molecules which ultimately lead to tissue macrophage infiltration [2-3]. Obesity has also been associated with an accumulation of lipid in hepatocytes, causing hepatic steatosis which has also been associated with inflammation. This inflammation may be a direct result of the hepatic steatosis or an indirect result of the lipids and proinflammatory cytokines produced in the adipose tissue and transported to the liver via the portal circulation [2-3]. Both possibilities lead to the activation of Kupffer cells (hepatic macrophages), which has been found to increase with obesity. However, the site and source of inflammatory signaling remain unclear.

Regardless of its origin, chronic “low grade” inflammation has been implicated in the pathogenesis of insulin resistance (IR) and type 2 diabetes (T2D) through the activation of intracellular pathways that ultimately impair insulin signaling and glucose homeostasis [2-4]. In addition to releasing proinflammatory mediators, both the liver and adipose tissue also release pro-atherogenic mediators in response to inflammatory stimuli, further complicating the metabolic disease by promoting the formation of atherogenic plaques in vascular tissue, leading to atherosclerosis and cardiovascular disease (CVD). Consequently, the coincidence of IR and CVD is becoming increasingly prevalent in developed countries, leading many clinicians and scientists to describe these conditions as a single syndrome referred to as “cardiometabolic disease” (CMD).
1.2 Polyunsaturated Fatty Acids and Cardiometabolic Disease

Significant attention has been placed on the potential role of dietary polyunsaturated fatty acids (PUFAs) in modulating CMD risk [4-5]. Long chain n-3 PUFAs have been shown in some studies to have anti-inflammatory effects in patients with conditions such as rheumatoid arthritis, psoriasis, and Crohn’s disease, among others [4]. Still, research on the beneficial effects of n-3 PUFAs has generated conflicting results [4-5]. On the other hand, increased consumption of n-6 PUFAs, such as linoleic acid (18:2 n-6, LA), has been associated with negative health effects due to their conversion into pro-inflammatory eicosanoids [6-7]. The most abundant PUFA in the modern diet is LA. However, research on the effect of dietary LA on CMD risk has been similarly controversial, with studies demonstrating positive, negative and equivocal effects [6-10]. The delta-6-desaturase (D6D) enzyme catalyzes the rate limiting step in the conversion of LA into its highly unsaturated fatty acid (HUFA) derivative, arachidonic acid (AA), which in turn is converted into pro-inflammatory eicosanoids by the LOX and COX pathways. Recent epidemiological studies have suggested a link between D6D hyperactivity, promoting enhanced conversion of LA into AA, with the development of CMD [11-12]. However, this hypothesis remains to be tested experimentally.

1.3 D6D, PEMT and CMD

Support for this hypothesis comes from recent data, where oral administration of a D6D inhibitor compound to mouse models of obesity/IR (ob/ob mice) normalized glucose intolerance, reduced serum insulin and triglyceride levels, and reduced serum pro-inflammatory eicosanoids and hepatic macrophage infiltration, among other effects (A. Chicco; unpublished data; and [13]). Given the well-established role of inflammation in the pathogenesis of CMD, it is plausible that the effect of D6D inhibition on these parameters is responsible for its protective effects. However, preliminary evidence indicates that there may be other contributing mechanisms (A. Chicco; unpublished data).
In the present study, we hypothesized that obesity and the consumption of a WD inducing inflammation, IR, T2D, and CVD, will be reversed/prevented by the drug SC-26196 which inhibits D6D activity. A detailed assessment of the contents and acyl compositions of individual PLs was therefore conducted to provide insight into the nature of PL remodeling, as well as the potential interaction of D6D with phosphatidylethanolamine-methyl-transferase (PEMT), the enzyme which catalyzes the hepatic conversion of phosphatidylethanolamine (PE) into phosphatidylcholine (PC). Moreover, the potential link between D6D and PEMT was investigated by indirectly monitoring PEMT activity through determining the hepatic PC/PE ratios in disease and with D6D inhibition.

Previous studies have demonstrated a positive relationship between D6D and PEMT activity [14], though the manner by which the two enzymes interact remains unclear. PEMT preferentially utilizes PE that contains an AA or DHA molecule in the sn-2 position, generating a PC molecule containing AA or DHA [15]. It is therefore plausible that D6D regulates PEMT activity by generating the DHA and/or AA required for the synthesis of its substrate (PE-DHA/AA). We performed a comprehensive analysis of the distribution and composition of liver PLs, with particular focus on PE and PC and their acyl composition (DHA and AA content), in order to explore the possibility of a novel link between D6D activity, PEMT activity, and CMD risk. Figure 1 illustrates the hypothesized links between D6D, PEMT, and CMD.

1.4 Aims of the Present Study

The first aim of this study was to investigate whether disease risk in rodent models of CMD correlates with changes in D6D activity/inhibition, reflected by changes in serum PL FA composition, followed by characterizing the effect of disease and D6D inhibition on liver membrane composition. The mouse models assessed were ob/ob mice which are leptin deficient and C57B1/6 lean mice lacking LDL receptors (LDLR−/−).
In the ob/ob mouse study, the mice were fed a regular chow diet throughout the study, with the addition of the D6D inhibitor for the last four weeks of the study at 100 mg/kg body weight in treatment groups. In the LDLR−/− model, 2-month-old mice were fed a WD for 12 weeks. The D6D inhibitor (SC-26196) was added to the chow for the last four weeks at 100 mg/kg body weight. SC-26196 is a highly selective inhibitor of D6D with oral activity in mice [16].

The lipid analyses conducted in serum and total liver homogenates were (1) the fatty acyl composition of serum PLs extracted by TLC and further analyzed for FA content by GC; (2) total hepatic PL FA composition (TLC, GC); (3) the separation of individual PL classes (PE and PC) via normal-phase HPLC using UV (ultra-violet) detection (206 nm); (4) the fatty acid composition of each PL fraction collected from HPLC via GC; (5) and the hepatic ratio of PC to PE by a spectrophotometric phosphorous assay.

The second aim was to correlate the changes in serum and liver membrane composition with evidence for CMD risk. To examine the extent of cardiovascular disease risk (CVD), serum triglycerides and cholesterol were analyzed using commercially available assay kits. Hepatic inflammation was examined by measuring a panel of hepatic eicosanoids (e.g. HETEs and PGs) and free AA via LC/MS/MS; and systemic inflammation was examined by measuring serum Monocyte Chemoattractant Protein (MCP-1) using the ELISA method.
2.0 Literature Review

2.1 Obesity and Cardiometabolic Disease

Obesity and overweight are descriptions of weight ranges that exceed what is considered to be a normal, healthy weight, using the body mass index (BMI) of an individual’s weight in relation to their height [17]. A normal BMI is considered to be between 18.5 and 24.9 kg/m$^2$ [17-18], while an individual with a BMI $\geq$25 kg/m$^2$ is overweight and a BMI of $\geq$30 kg/m$^2$ is obese [17, 19].

According to the WHO [1], in 2008, 1.5 billion adults worldwide were overweight, 500 million of which were obese. Thus, more than one in 10 adults were found to be obese. In 2010, 35 million children in developing countries were overweight compared to 8 million in developed countries. In Egypt, the prevalence of obesity and overweight in 2010 was 76% in both males and females [20]. Worldwide mortality rates resulting from overweight/obesity amounted to 2.8 million in 2010 [18]. There has also been a parallel increase in diseases associated with obesity, such as type 2 diabetes (T2D), hyperinsulinemia/IR, hyperlipidemia, hypertension, and cardiovascular disease (CVD), collectively referred to as cardiometabolic disease (CMD). The hallmark of CMD is considered to be IR [21].

2.2 Adipose Tissue and Adipokines

Adipose tissue is the main site of lipid storage in the body composed of a tissue matrix containing adipocytes, preadipocytes, and vascular, nerve and immune cells, such as macrophages [2, 22]. There are two types of adipose tissue: the brown adipose tissue, responsible for thermogenesis, and the white adipose tissue, which may further be classified into subcutaneous and visceral/abdominal fat [2]. Aside from serving as an energy depot through the mobilization of lipids, adipose tissue, specifically white adipose tissue, also secretes substances of metabolic and immune function, including leptin, adiponectin, resistin, tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1(MCP-1), and other various cytokines termed “adipokines” [2, 22]. Adipose tissue is thus more than a site of energy storage, but
plays an active role in other body functions, including appetite, inflammation and insulin sensitivity.

2.3 Obesity and Inflammation

Obesity has been associated with a state of sub-acute/low grade inflammation, due to its secretion of adipokines, most of which are proinflammatory, as well as macrophage infiltration in the adipose and other tissues [2]. Adipose tissue expression of MCP-1, a chemo-attractant which attracts and recruits macrophages, increases in obesity. Both the adipocytes and recruited macrophages further secrete more cytokines, leading to a state of inflammation. On the other hand, the expression of adiponectin, which is also an adipokine, but one which may have a protective role against insulin resistance and atherosclerosis, is decreased in obesity [22]. Recently, significance has been placed on abdominal obesity being an indicator of CMD [2]. This may be due to better access of abdominal/visceral fat to the portal circulation, thus enabling it to have a more direct impact on the liver [2]. Moreover, abdominal/visceral fat along with macrophages also produce higher amounts of proinflammatory adipokines, such as TNF-α and interleukin-6 (IL-6), compared to subcutaneous fat [2]. Figure 2 summarizes the potential mechanism by which obesity may cause inflammation leading to IR and CVD [3].

Obesity also affects the liver, where accumulation of fat can lead to hepatic steatosis and non-alcoholic fatty liver disease (NAFLD) [2]. This is also accompanied by an increased expression of cytokines and macrophage infiltration in the liver, in addition to the transport of inflammatory substances to the liver via the portal circulation, leading to the activation of the Kupffer cells: the resident macrophages of the liver [2]. Together, these factors contribute to the promotion of hepatic inflammation and IR. In addition to its promotion of inflammation, obesity may also lead to endoplasmic reticular (ER) stress and oxidative stress with the release of reactive oxygen species (ROS) [23-24] all of which have also been implicated in the pathogenesis of IR.
2.4 Insulin Action and Signaling

Insulin is a key regulator of carbohydrate, lipid and protein metabolism. The major effects of insulin on carbohydrate metabolism and glucose blood concentration include increased glucose uptake and utilization by cells, and inhibition of glycogenolysis and stimulation of glycogen synthesis in the liver [25]. Insulin also plays a role in lipid metabolism by inhibiting lipolysis; in protein metabolism by stimulating protein synthesis; and in cell growth and differentiation [25].

On a molecular level, insulin action on glucose regulation begins at the insulin receptor which lies on the surface of insulin-sensitive cells (predominantly adipose, liver and muscle tissue). The insulin receptor is a heterotetrameric trans-membrane tyrosine kinase receptor formed of two extracellular α-subunits and two intracellular/cytoplasmic β-subunits [25-28]. The two α-subunits are linked to each other and to the β-subunits through covalent disulfide bonds [26-28]. The α-subunits have an inhibitory effect on the tyrosine kinase activity of the β-subunits until insulin binds to the α-subunits [25]. Upon binding of circulating insulin to the α-subunits on the cell surface, a conformational change occurs in the α-subunits which enables the binding of adenosine triphosphate (ATP) to the β-subunits followed by the autophosphorylation of one β-subunit, which in turn trans-phosphorylates its neighboring β-subunit, thus activating the two β-subunits’ intracellular tyrosine kinase activity [25-28]. This leads to a series of downstream phosphorylation cascades, starting with the insulin receptor substrate (IRS) (Figure 3) [25].

There are four members of the IRS family: IRS1-4, the most significant of which in the regulation of metabolism are IRS-1 and IRS-2 [26]. However, insulin action does not seem to depend solely on IRS, as the absence of one or more members of the IRS family does not fully impede insulin action as does the absence of the insulin receptor, suggesting that there is redundant action of IRS proteins and perhaps other substrates in the signaling process at that level [26]. Phosphorylation of the β-subunits of the insulin receptor leads to the binding and autophosphorylation of IRS, which then acts as a docking protein for other substrates downstream, specifically those
containing Src-homology-2 (SH2) domains [25, 28-30], the most significant of which in the acute response to insulin is the phosphatidylinositol 3-kinase (PI3-K).

PI3-K is a heterodimeric enzyme formed of two subunits: a regulatory p85-subunit and a catalytic p110-subunit [25, 27]. PI3-K contains two SH2 domains which interact with tyrosine-phosphorylated motifs on the IRS [25]. Interaction with IRS activates PI3-K which catalyzes the production of phosphatidylinositol 3-phosphate (PIP3) [26] and recruits 3-phosphoinositide-dependent protein kinase-1 (PDK-1), a serine-threonine kinase. Multiple downstream substrate interactions lead to the translocation of glucose transporter type 4 (GLUT4) vesicles and their fusion with the plasma membrane, facilitating a 20 to 30-fold increase in cellular glucose uptake [27]. Among such interactions is the serine-threonine phosphorylation and activation of protein kinase B (PKB), also referred to as Akt, by PDK-1, followed by the phosphorylation of Akt substrate (AS160) which is catalyzed by Akt. PDK also activates the λ and ζ isoforms of protein kinase C (PKC), which then undergo autophosphorylation and conformational changes, and contribute to GLUT4 translocation [31].

While the involvement of PI3-K, PDK, Akt and PKC in the translocation of GLUT4 to the plasma membrane are well established, the exact mechanisms of GLUT4 translocation and the inhibition of its internalization are not yet fully understood [25].

2.5 Insulin Resistance and Inflammation

IR is a major contributor to the development of T2D and the metabolic syndrome, characterized by a decrease in insulin sensitivity, decreased glucose uptake by peripheral tissue, increased hepatic glucose production, and β-cell over-production of insulin, collectively leading to impaired glucose tolerance and hyperinsulinemia [22]. Many factors are involved in the development of IR, including obesity, inflammation, and dyslipidemia, in addition to and/or resulting from a sedentary lifestyle, diet and a genetic predisposition, among other factors [23].

As mentioned above, increased adiposity is accompanied by a state of low-grade inflammation due to an increase of cytokine production and macrophage infiltration.
The primary mechanism by which obesity and inflammation compromise insulin sensitivity appears to be downstream of insulin binding to the insulin receptor, with the activation of Jun N-terminal kinase (JNK), inhibitor of nuclear factor κB kinase (I KKβ), and nuclear factor κB (NF-κB) pathways of inflammation (Figure 4) [22]. Activation of JNK into PJNK by cytokines/adipokines leads to IR through its phosphorylation of serine residues on IRS proteins, especially IRS-1, thereby inhibiting their tyrosine kinase activity [2-3, 22-23, 32]. Activation of I KKβ by various stress and inflammatory stimuli leads to phosphorylation and proteosomal degradation of IκB, an endogenous inhibitor of NF-κB activity [3]. This leads to the activation of NF-κB which translocates to the nucleus and induces the expression of target genes [3], the products of which further promote gene expression of cytokines, adhesion molecules and oxidants, among others, in a feed-forward manner [33].

2.6 The Role of MCP-1 and Macrophages

The expansion of adipose tissue in obesity leads to the over-expression of MCP-1 by adipocytes as mentioned earlier. This over-expression seems to be in response to a high-fat diet, as well as to inflammatory stimuli, such as TNF-α, IL-6, and insulin, all of which are higher in obese states [34]. Other cells expressing MCP-1 include macrophages, endothelial cells, pancreatic β-cells, and skeletal and smooth muscle [34]. MCP-1, also referred to as CCL2, is a chemokine involved in the attraction of T-lymphocytes and circulating monocytes to sites of inflammation [34]. Over-expression of MCP-1 in adipose tissue is associated with hepatic steatosis and systemic inflammation leading to IR in the adipose tissue, liver and muscle [2]. In normal, lean individuals, resident macrophages are of the M2 type which has a protective role in adipose tissue [23, 32, 35]. On the other hand, obesity leads to tissue infiltration and the activation of proinflammatory M1 macrophages which are responsible for the release of many cytokines and inflammatory proteins [23, 32, 35]. It has been suggested that peroxisome proliferator-activated receptor γ (PPARγ) is involved in this switch in macrophage activation from the M2 to M1 state [32]. Both adipocytes and M1 macrophages release MCP-1 which serves to further attract more
macrophages to the site of inflammation, leading to a vicious cycle of inflammation and infiltration [23].

In the liver, MCP-1 activates the resident Kupffer cells [3], which may promote further hepatic inflammatory signaling. Studies conducted on the effect of Kupffer cell depletion on symptoms of CMD in obese might fed a high-fat diet, reported a decrease in hepatic triglyceride levels due to increased expression of PPAR-α which promotes FA oxidation, and a decrease in IL-1β, suggesting a role for IL-1β and Kupffer cell activation in the development of hepatic steatosis and IR [36]. Another study reported that the depletion of Kupffer cells prevented the development of hepatic steatosis and IR in rats fed a high-fat diet, due to a decrease in TNF-α secretion by Kupffer cells [37].

Other roles of MCP-1 include stimulation of endothelial cells to promote neovascularization of expanding adipose tissue [34], wound healing [34], migration of macrophages into sub-endothelial spaces (contributing to the formation of atheromas) [3], and increased expression of matrix metalloproteinases (MMPs) by activated macrophages, leading to the destabilization of atherosclerotic plaques [3]. TNF-α released by activated macrophages also influences adipose tissue metabolism, promoting lipolysis, thus increasing levels of circulating free fatty acids [38-39]. Taken together, there is evidence that macrophages play an important role in the pathogenesis of CMD.

2.7 The Role of Free Fatty Acids in Insulin Resistance

A link between circulating free fatty acids and IR has also been suggested. Obesity is associated with an increase in circulating free fatty acids which may impair cellular glucose uptake by interfering with insulin signaling phosphorylation cascades and glucose transport [40]. There are several proposed mechanisms of free fatty acid-induced IR. First, accumulation of diacylglycerol and long-chain acyl CoA in glucose-sensitive tissue such as muscle and liver leads to the activation of proinflammatory PKC θ [22]. Through its serine/threonine kinase activity, PKC θ directly interferes with IRS-1/2 tyrosine phosphorylation, PI3-K association with
IRS-1 [22, 41-42], and the activation of PKC \( \lambda/\zeta \) [42]. PKC \( \theta \) may also indirectly promote IR by activating NF-\( \kappa \)B [43]. Accumulation of free fatty acids in cells may also promote mitochondrial ROS production, ER stress, and oxidative stress [43-44] which in turn activate both the JNK and NF-\( \kappa \)B pathways [2]. Moreover, prolonged exposure of pancreatic \( \beta \)-cells to ROS has a toxic effect and leads to \( \beta \)-cell dysfunction, further exacerbating IR [43].

**2.8 Membrane Composition and Fatty Acids**

Cell membrane lipid bilayers are comprised of phospholipids (PLs). Each phospholipid is formed of a polar head group attached via a phosphate bond to a glycerol molecule to which two acyl chains (fatty acids) are attached. The acyl chains are esterified at the stereospecific positions sn-1 and sn-2 of the glycerol molecule, while the head group is at the sn-3 position. The phospholipids are arranged in a way where the head groups align non-covalently to form two polar surfaces: an outer extracellular surface and an inner surface facing the cytoplasm; while the acyl chains (tails) form the in-between layer arranged in a tail-to-tail fashion. The composition of PL head groups and fatty acid acyl chains are major determinants of membrane function and fluidity [45]. The fatty acids could be saturated (SFAs), monounsaturated (MUFAs) containing one double bond, and polyunsaturated fatty acids (PUFAs) containing two or more double bonds. Long-chain PUFAs with more than two double bonds, such as arachidonic acid (AA) and docosahexaenoic acid (DHA), are often referred to as highly unsaturated fatty acids (HUFAs).

Standard fatty acid nomenclature represents species by the number of carbons in the chain and the number of double bonds (Figure 5) [45]. For example, DHA can be represented as 22:6 n-3, where the number of carbons is 22, the number of double bonds is 6, and the position of the first double bond proximal to the methyl end is at carbon 3 in the chain.

Animals can synthesize SFAs and MUFAs de novo via the acetyl Coenzyme-A (CoA) pathway. However, they are unable to synthesize the PUFAs linoleic (18:2 n-6; LA) and \( \alpha \)-linolenic (18:3 n-3; ALA) acids, which must be obtained from the diet,
and are therefore referred to as essential fatty acids. Preformed HUFAs may be obtained from the diet through the ingestion of animal tissue [45], especially marine food [46], or formed endogenously from LA and ALA via desaturation and elongation pathways described in detail below.

The major fates of SFAs and MUFAs are storage in the form of triglycerides and oxidation as a source of energy with the release of carbon dioxide and water, whereas PUFAs are primarily incorporated into cell membrane PLs [45]. Changes in diet over the past decades have shown a remarkable shift in dietary PUFAs from a relatively balanced intake of LA and ALA (1-4:1) to a ratio of 15-16:1 [46]. This dramatic rise in n-6 PUFA intake has been attributed to a greater consumption of soybean oil [45], sunflower oil, and corn oil [46] in manufactured food, compared to a much lower consumption of dietary sources of n-3 PUFAs, such as green leafy vegetables [45] and canola and flaxseed oils [46].

### 2.9 PUFA Metabolism and Eicosanoids

The n-3 and n-6 PUFAs share several common metabolic pathways (Figure 6) [47]. Delta-6-desaturase (D6D) catalyzes the rate limiting step in the conversion of n-6 LA and n-3 ALA into γ-linolenic acid (GLA) and stearidonic acid, respectively, followed by their respective conversion catalyzed by elongase 5 into dihomo-γ-linolenic acid (DGLA) and eicosatrienoic acid and by delta-5-desaturase (D5D) into arachidonic acid (AA) and eicosapentaenoic acid (EPA), respectively. A further series of reactions involving elongases, D6D, and a final step of β-oxidation leads to the conversion of AA and EPA into docosapentaenoic acid and DHA, respectively. The n-3 and n-6 metabolic pathways compete over the elongase and desaturase enzymes mediating the pathways [47]. However, these enzymes exhibit a preference for the n-3 metabolic pathway [46], though an n-6 PUFA dominated diet would overcome this inherent preference [46]. As a result of this competition between the two pathways, supplementation or dietary excess of one would negatively affect the other [48]. For example, increased dietary ALA leads to the increase of n-3 PUFA derivatives and decreased levels of n-6 PUFA derivatives, especially AA [47].
Lipoxygenase (LOX) and cyclo-oxygenase (COX) enzymes catalyze the conversion of AA and EPA into a series of eicosanoids, such as prostaglandins (PGs), thromboxanes (TXs), and leukotrienes (LTs) [47]. Eicosanoids derived from AA are deemed pro-inflammatory (series-2 PGs and series-4 LTs), while those derived from EPA are anti-inflammatory (series-5 LTs and series-3 PGs and TXs) [47]. LOX and COX enzymes also catalyze the conversion of DGLA into series-1 PGs and LTs, which also exhibit anti-inflammatory effects [47]. Other anti-inflammatory and protective molecules, such as resolvins and protectins, are derived from further metabolism of EPA and DHA [47].

2.10 The Role of PUFAs in Cardiometabolic Disease

Based on the effects of PUFAs and their derivatives on inflammatory signaling, significant attention has been placed on their potential roles in modulating disease risk [4-5]. Long chain n-3 PUFAs have been shown in some studies to have anti-inflammatory effects in patients with conditions such as rheumatoid arthritis, psoriasis, and Crohn’s disease, among others [4]. The most studied dietary n-3 PUFAs are EPA and DHA, abundant in marine oils, and to a lesser extent, ALA. The most prominent effects of these PUFAs seem to be a decrease in serum triglycerides (TGs), low-density lipoprotein (LDL) and total cholesterol [47]. The mechanism of ALA effects is thought to be independent of that of DHA and EPA, though it is not fully understood [47]. Further reports on the effects of dietary EPA and DHA, ingested in the form of fish, fish oil, or dietary supplements, include the decrease in visceral and subcutaneous fat, hepatic fat storage, very-low-density lipoprotein (VLDL), blood pressure, and BMI, and an increase in high-density lipoprotein (HDL) [47].

With regards to cardiovascular health, ALA has been reported to have an inverse relation with the incidence of stroke and sudden cardiac death, but has little to no effect on overall CVD risk [49]. Likewise, EPA and DHA are reported to have a positive effect in preventing fatal CVD events, but have a less consistent effect on non-fatal events [49]. However, epidemiological studies show that consumption of fish at least one to three times per week decreases CVD risk by 40% compared to a
once-per-month or less consumption of fish [4]. As for inflammation and IR, n-3 HUFAs are reported to decrease activity of NF-κB directly, prevent macrophage tissue infiltration induced by obesity and a high-fat diet, and decrease JNK phosphorylation, thereby improving IR [4]. Interestingly, n-3 FAs have been reported to have a worsening effect on T2D and blood glucose levels at high doses, and no effect at low doses [5]. Research on the beneficial effects of n-3 PUFAs has generated conflicting results and full understanding of the involved mechanisms of action has not yet been reached [4-5].

Suggested mechanisms of n-3 FAs include the inhibition of toll-like receptors (TLR), especially TLR-2 and 4, by DHA [50]. TLRs are situated on the cell surface for the recognition of bacterial cell wall lipopolysaccharides, resulting in the activation of the NF-κB pathway [50]. TLRs are also stimulated by SFAs [50]. Additionally, EPA competes with AA for the COX and LOX enzymes, thereby creating a shift from the production of AA-derived proinflammatory eicosanoids to EPA-derived anti-inflammatory eicosanoids [4]. Both DHA and EPA are also suggested to have an inhibitory effect on phospholipase A-2 (PLA-2), which is responsible for mobilizing AA from cell membrane phospholipids [51]. Another possible mechanism by which n-3 HUFAs influence cell function is through the modulation of transcription factors, such as PPAR and sterol regulatory element-binding protein-1c (SREBP-1c) [4]. EPA activates genes dependent on PPAR-α which plays a role in FA oxidation [52], while DHA activates PPAR-γ which results in increased glucose clearance [53]. On the other hand, SREBP-1c is involved in hepatic lipogenesis and its expression is suppressed by both DHA and EPA [52]. Therefore, through these possible mechanisms, n-3 HUFAs may reduce inflammation, increase glucose clearance and FA oxidation, and decrease FA synthesis, thus improving insulin sensitivity.

On the other hand, increased consumption of n-6 PUFAs, such as linoleic acid (18:2 n-6, LA), has been associated with negative health effects due to their conversion into proinflammatory eicosanoids, such as prostaglandin E2 (PGE2), hydroxyeicosatetraenoic acids (HETEs), and LT-B4 [6-7]. The most abundant PUFA in the WD is LA. However, research on the effect of dietary LA on CMD risk has
been controversial, with studies demonstrating positive, negative and equivocal effects [6-10]. Some animal and in vitro studies show n-6 FAs to be proinflammatory and pro-thrombotic, while others report the exact opposite effects and suggest that n-6 FAs have an inverse relation to CVD risk and are thus cardio-protective [49]. Still others report that LA is anti-arrhythmic and may improve IR [49].

Based on the available evidence above, increasing attention has been placed on an “optimal” dietary n-6/n-3 PUFA ratio, an increase of which may be behind the harmful effects of the modern diet. Here too, however, there are conflicting results. For example, some studies show the increase in the n-6/n-3 ratio to have no effect on CVD risk [49], while others have suggested a link between the conversion of LA into AA and the development of CMD [11-12]. The reasoning behind this is that the substrate preferred by D6D is ALA, followed by LA and oleic acid (MUFA), provided that there is enough ALA in the diet [54]. Therefore, it is thought that the over-abundance of LA in the modern diet reduces the conversion of ALA into its protective n-3 PUFA derivatives by out-competing it at D6D [54]. As mentioned, the healthy n-6/n-3 PUFA ratio is viewed by some to be 1:4:1 [46], while others recommend replacing dietary SFAs and MUFAs with both n-6 and n-3 PUFAs, rather than favoring one PUFA over the other or placing emphasis on a target ratio between the two [49].

In order to make sense of the enormous amounts of conflicting information regarding the effects of PUFAs, some reasons for the inconsistencies have been proposed. In human studies, for example, the health status of the subjects can influence the results of PUFA intake [5]. Reports on subjects with IR show an improvement and even reversal of IR with n-3 PUFAs consumption, whereas subjects with T2D experience no effect or worsening of diabetes with n-3 PUFAs consumption [5]. On the other hand, it is difficult to examine the effect of n-3 PUFAs on IR in healthy subjects that already have normal responses to insulin [5]. The dose of PUFAs administered in a study, the duration of supplementation, the end-point of the study, and the composition of the supplement used vary widely across studies, likely contributing to the observed inconsistencies [5].
Geographical locations of the populations under study and their genetic profile may also be contributing factors, in addition to differences between human and different types of animal studies, and differences between *in vivo* and *in vitro* studies. In epidemiological studies, factors such as sample size lacking statistical power, the type of dietary assessment, and the use of serum FA composition as a tool for assessing dietary fat intake may give rise to inconsistencies [49]. For example, individual serum FAs are measured in relation to total FAs, while a change in the amount of one FA type may influence the amount of another [49]. This could affect the correlation between serum FAs and dietary FA intake [49]. Thus a standardization of methods and assessment tools is needed, in addition to the existing adjustments that are made for variations across studies.

**2.11 Delta-6-desaturase**

D6D is a 52.2 kDa membrane-bound microsomal enzyme which introduces a double bond at position delta-6 of a FA [55]. D6D catalyzes the rate limiting step in the conversion of LA into its HUFA derivative, AA, which in turn is converted into proinflammatory eicosanoids by the LOX and COX pathways in response to stimuli or cell injury. The same enzyme also catalyzes the conversion of ALA into its HUFA derivatives, EPA and DHA. These reactions occur primarily in the ER of the liver, as well as in the brain and other tissue [55-57]. It has been proposed that NADH-cytochrome b5 reductase transfers the electrons required by the catalytic site of D6D directly in the ER, as D6D contains a N-terminus region that is homologous to that of cytochrome b5 [55].

**2.12 D6D Regulation**

There are many factors that seem to contribute to D6D regulation. D6D is only fully active under severe dietary restriction of essential FAs, specifically n-3 and n-6 PUFAs, both series of which have a suppressive effect on D6D activity when supplied in adequate amounts in the diet [56]. On the other hand, under controlled dietary conditions, these suppressive effects of PUFAs seem to be strongly influenced by other components of the diet. For example, while LA and ALA had a dramatic
suppressive effect on D6D activity compared to a control animal group fed a high glucose/fat free diet, these unsaturated FAs had no effect on, and in some cases even increased, D6D activity when compared to a control group fed a high starch diet [58].

Regulation of the desaturase enzymes is thought to occur primarily at the transcriptional level, and is therefore greatly affected by the presence of transcription factors, such as the aforementioned SREBP-1c and PPAR transcription factors which increase D6D expression. On the other hand, the presence of high levels of HUFAs suppresses D6D activity indirectly by decreasing the activation of SREBP-1c through their destabilizing effects on SREBP-1c mRNA, decreasing the active nuclear form of SREBP-1c, thereby hindering it from activating target genes, as well as acting as competitive ligands [56]. Chronic deficiency of dietary n-3 PUFAs has been associated with up-regulation and increased activity of D6D [59]. HUFAs may also suppress D6D activity through product feedback inhibition.

Hormones and vitamins are also involved in the regulation of D6D activity and lipid homeostasis [58, 60]. Vitamin B6 has been reported to increase D6D activity [61]. Glucagon, ACTH, gluco- and mineralo-corticoids, and epinephrine suppress D6D activity; while insulin increases D6D activity and mRNA expression, evidenced by suppressed activity in insulin dependent diabetes associated with insulin deficiency [60], and high D6D activity in T2D and insulin resistant states due to hyperinsulinemia.

2.13 D6D Indices: an Indirect Measurement of D6D Activity

Directly measuring D6D activity in the ER of humans is complicated, not feasible and is ethically questionable as the target organ would be the liver in order to study mRNA expression of D6D and protein levels [62]. Instead, D6D activity has been measured across studies through the use of [product: precursor] PUFA ratios in serum and tissue PLs [63], since D6D catalyzes the rate limiting step in the conversion of LA and ALA to their HUFA derivatives. The D6D indices used in such studies have been [18:3 n-6/18:2 n-6] (LA/GLA) in serum [62, 64], [20:3 n-6/18:2 n-6] (DGLA/GLA) in PLs [62, 65], as well as [20:4 n-6/18:2 n-6] (AA/LA) and [20:5 n-
3/18:3 n-3 (EPA/ALA) [11]. High D6D indices and changes in serum and PL FA composition have been associated with the risk of IR [61], T2D [64], CVD [61, 66], and metabolic syndrome [61-62, 64], independent of factors such as smoking, physical activity and BMI [63].

2.14 Fads Genes and Polymorphisms

The genes encoding for D5D and D6D are fads1 and fads2, respectively, which lie in a head-to-head fashion in a gene cluster on human chromosome 11q12-11q13.1, separated by a 11 kb region [11, 67]. A third gene, fads3, lies within the same region, but the protein encoded by fads3 has not yet been identified [11].

Recent epidemiological studies have shown that single nucleotide polymorphisms (SNPs) in the fads2 gene encoding D6D that are associated with serum PUFA [precursor:product] ratios indicative of increased D6D activity (e.g. increased PL AA/LA ratio) are strongly linked to the development of inflammation [11], CVD [11, 68], and the metabolic syndrome [68]. It has also been shown that obesity, hyperinsulinemia and/or chronic consumption of a WD rich in sucrose and saturated fat each independently stimulates fads2 and/or D6D expression/activity in humans and animal models, resulting in an increase in AA/LA ratios in serum and tissue PLs [62, 69-70]. Despite this abundant evidence of increased D6D activity in CMD in humans and animals, its (patho)physiological role in this process remains unclear.

2.15 D6D Inhibition and Inflammation

The potential role of D6D in regulating inflammatory signaling through the production of AA and its derivatives has been previously suggested by animal studies [16]. The drug SC-26196 (Figure 7) is a selective D6D inhibitor (1000 times more selective to D6D compared to D5D and D9D) both in vivo and in vitro [57]. Effects of selective inhibition of D6D by the drug SC-26196 led to a decrease in AA synthesis, thus alleviating inflammation in a carrageenan paw edema mouse model, where the anti-inflammatory effect of D6D inhibition was evident by a 50% decrease in edema [16]. In another study, inhibition of D6D by SC-26196 prevented the conversion of LA into its downstream derivatives, resulting in an overall increase in
LA content and a decrease in AA [57]. The resulting decrease in AA inhibited colorectal tumorigenesis, an effect which was reversed by AA supplementation.

### 2.16 Arachidonic Acid Metabolism

In response to stimuli, such as cell injury or cytokine release, the AA incorporated into cell membrane PLs is liberated mainly via the hydrolysis action of PLA-2 enzymes [71-72], at the sn-2 acyl bond of PLs. However, free AA can also be generated through alternative pathways catalyzed by phospholipase C and D [72]. During inflammation, PLA-2 levels are increased at the transcriptional and post-translational levels, leading to an increase in free extracellular AA which is exchanged across cells and to which some cell types respond by generating even more free AA, raising AA levels from nanomolar to the hundred micromolar levels [72]. Being amphipathic, AA can bind to fatty acid binding proteins which further facilitate its uptake by cells after which it becomes trapped inside the cells by undergoing esterification through the action of coenzyme A synthetase [72]. AA then undergoes oxygenation via three main pathways catalyzed by COX, LOX, and cytochrome P450 mono-oxygenase/epoxidase (CYP) (Figure 8) [71].

#### 2.16.1 Cyclo-oxygenase (COX)

There are two isoforms of COX enzymes: COX-1 and COX-2. The COX-1 enzyme is encoded by a gene which is constitutively expressed by most cells, while COX-2 is encoded by an early response gene which is induced by stimuli, such as inflammatory events [71, 73]. However, in pancreatic β-cells, COX-2 seems to be the predominantly expressed COX enzyme [73]. The major products of the COX enzymes are prostaglandins (PGs) and thromboxanes (TXs). PGH2 is a product of COX action on AA and is the precursor of other PGs, such as PGD2, PGE2, PGF2, PGI2 and PGJ2 [74]. PGs and TXs seem to have opposing actions. For example, in the cardiovascular system (CVS), while TXs exert vasoconstrictive action and promote platelet aggregation, PGI2 causes vasodilation and inhibits aggregation [73]. In the kidney, PGI2, PGE2 and PGD2 all have vasodilatory actions on vascular smooth muscle cells [73]. Receptors of COX products are DP, EPs (EP1-4), IP, FP,
and TP [73]. The distribution of these receptors varies widely across all cells of the body, and binding to these receptors will illicit different patho/physiological responses [73]. Thus, when considering the actions of eicosanoids in general, it is important to note that these immunity-modulating molecules may have dual actions, both anti-inflammatory and proinflammatory, depending on receptor distribution, and the type of cell, its location and its function.

In pancreatic β-cells, the abundant receptor is EP3 and the predominant COX product is PGE2, which has been reported to have an inhibitory effect on insulin secretion [73]. Based on animal studies, this effect is believed to be induced by the action of IL-1β which activates COX-2 enzyme and the receptor EP3 through the NF-κB pathway, leading to a decrease in cAMP which results in a decrease in insulin secretion in response to high glucose levels [73]. On the other hand, this hypothesis has not been observed in humans, where PGE2 had no effect on insulin secretion. Thus, it is suggested that the inhibition of insulin secretion by COX-2 may be a result of increased availability of free AA in β-cells [73]. Nevertheless, hyperglycemia increases COX-2 expression in β-cells which also happens to correlate with plasma levels of leptin [73].

### 2.16.2 Lipoxygenase (LOX)

The second pathway by which AA is metabolized is through the LOX enzyme. There are several LOX isoforms: 5-LOX, 8-LOX, 12-LOX and 15-LOX, depending on the site of insertion of a hydroperoxy group by LOX on AA [71]. The products of the LOX pathways are hydroperoxyeicosatetraenoic acids (HPETEs), hydroxyeicosatetraenoic acids (HETEs), and lipoxins, among others. 5-HETEs are the precursors of LTA4 from which lipoxins, LTB4, and CysLTs (LTC4, LTD4, and LTE4) are generated [71]. The activation of the 5-LOX pathway, however, requires first the activity of 5-LOX-activating protein (FLAP) [75]. LOX receptors are BLT, CysLT, and LTE4 [73]. The main generators of LTs are inflammatory cells such as eosinophils, neutrophils, macrophages, mast cells, and monocytes, among others [71, 73]. LTs are relatively short-lived pro-inflammatory and chemotactic molecules which enhance secretion of cytokines, further attract inflammatory cells to sites of
inflammation and decrease insulin sensitivity in adipocytes [71, 73]. CysLTs promote contraction of smooth muscle, leading to vasoconstriction, while LTs affect vascular permeability and mostly oppose PGs action [71].

LOX products are major contributors to adipose tissue inflammation and IR, especially in obesity and high-fat diet [76]. The over-expression of 12/15-LOX enzymes leads to a rise in MCP-1 secretion which is involved in the development of IR [73]. Furthermore, inhibition of LOX lowers TGs and free fatty acids and improves IR and T2D in rats, while deletion of 12/15 LOX improves inflammation and IR induced by a high-fat diet in mice [73]. The products of 5-LOX have also been associated with IR. Disruption of Alox5, the gene encoding for 5-LOX, in an ApoE^{-/-} mouse model of hyperlipidemia resulted in a reduction of hepatic inflammation, macrophage tissue infiltration, NF-κB activation and cytokine release, in addition to upregulation of IRS-1 expression in adipose tissue, thereby improving insulin sensitivity [77].

2.16.3 Cytochrome P450 (CYP)

The third metabolic pathway is catalyzed by CYP, producing HETEs (16-20) and epoxyeicosatrienoic acids (EETs – 5,6; 8-9; 11,12; and 14,15) which are further hydrolyzed by soluble epoxide hydrolase to produce dihydroxyeicosatrienoic acids (DHETEs) [73]. The receptors of CYP products have not yet been identified [73]. 20-HETEs are vasoconstrictive and promote smooth muscle contraction, while EETs are vascular smooth muscle relaxants, have anti-inflammatory effects, and promote angiogenesis [73]. EETs have beneficial CVS effects but are short-lived as they are rapidly hydrolyzed into DHETEs which are less active molecules [73]. Thus, inhibitors of soluble epoxide hydrolase have been used to observe the beneficial effects of EETs [73].

2.17 Targeted Inhibition of Eicosanoid Production Pathways

High salicylate doses in ob/ob mice resulted in a reversal of symptoms of CMD, such as IR and dyslipidemia, due to the attenuation of inflammation which accompanies obesity in this model, confirming the implication of inflammation in the development
of disease [78]. One advantage of the SC-26196 D6D inhibitor drug is its upstream position in the metabolic pathways leading to eicosanoid production by inhibiting the production of AA itself rather than the downstream inhibition of AA metabolites.

Much research has been dedicated to selectively inhibiting specific enzymatic pathways leading to eicosanoids production or the action of individual eicosanoids in an effort to mitigate their inflammatory effects [74]. Results of these efforts have been controversial due to the vast array of eicosanoids and their ability to perform opposing actions depending on their location and cell type. Moreover, selectively blocking a pathway which would prevent the release of a proinflammatory eicosanoid may also prevent the release of an anti-inflammatory eicosanoid produced by the same pathway, depending on the disease [74]. The theory of “shunting” also arises when discussing selective targeting of eicosanoid production [79-80], where the inhibition of one eicosanoid synthesis pathway could lead to a diversion of AA metabolism into another pathway with potentially greater negative health effects [74]. For example, the inhibition of the COX pathway by Aspirin© leads to an increase in LTs production due to the diversion of AA to the LOX pathway, leading to smooth muscle contraction manifestations, such as asthma, and gastrointestinal complications [74]. Many COX-2 inhibitors have been withdrawn from the market due to their adverse cardiovascular effects. These effects have been attributed not only to the blockage of the anti-thrombogenic and anti-atherogenic effects of PGI2 in the process, but also to the possible diversion of AA to the COX-1 pathway, leading to an increased production of TXA2, and to the 5-LOX pathway, leading to an increased production of LTs, all of which increase the incidence of thrombogenesis and hypertension [74].

2.18 Interaction of PUFAs and Hepatic Phospholipid Metabolism

PC is a major PL composed of a glycerophosphocholine backbone to which two acyl chains are attached [15]. The length of these acyl chains, their position, and the presence and number of double bonds within them differ and create a wide variety of PL molecules [15]. SFAs, such as palmitic acid, typically occupy the sn-1 position of PC, while PUFAs, such as AA and DHA, occupy the sn-2 position [81].
2.18.1 Phosphatidylcholine Biosynthesis

The main pathway for PC biosynthesis is through the cytidine diphosphate-choline pathway (CDP-choline pathway, also referred to as the Kennedy pathway), where choline and ATP are required to produce PC through three enzymatic steps (Figure 9) [81]. About 30% of PC however is generated in the liver from PE via the enzyme PEMT [81]. PE is methylated sequentially through three steps catalyzed by PEMT which transfers methyl groups to PE from S-adenosylmethionine (AdoMet/SAM) [82] which is generated through the methionine cycle (Figure 10).

For each PC molecule synthesized through the PEMT pathway, three molecules of S-adenosylhomocysteine (AdoHcy) are generated which are further hydrolyzed in the liver into homocysteine (Hcy), a marker used to measure the risk of CVD [83]. The PC molecules synthesized via the CDP-choline pathway are different than those synthesized via the PEMT pathway in that the CDP-choline pathway synthesizes PC molecules containing medium chain, saturated FAs, such as 16:0/18:0, while the PEMT pathway synthesizes PC molecules with longer and unsaturated FA chains, such as 18:1/20:4 [15]. Thus, PEMT preferentially utilizes PE that contains AA or DHA in the sn-2 position, generating a PC molecule that also contains AA or DHA [15]. Therefore, it has been suggested that PC molecules rich in DHA can be used as a surrogate marker for in vivo PEMT activity, though factors such as dietary DHA may lead to inaccuracies and would require consideration [84]. The two pathways were found to have opposing effects on liver cell proliferation, where the CDP-choline pathway increases the proliferation rate of hepatoma cells, while the PEMT pathway has an inhibitory effect [15]. Furthermore, at least 8 major PC species are generated via the PEMT pathway, while only two or three are generated through the CDP-choline pathway [15]. The PEMT-generated PC also seems to undergo more active metabolism than the CDP-choline-generated PC [15].

PEMT is an ER-localized trans-membrane enzyme expressed mainly in the liver, although much smaller amounts are also expressed in the heart, adipocytes, testes and kidneys [82, 85]. Regulation of PEMT is dependent on the availability of substrates, such as AdoMet and PE; product feedback inhibition, such as AdoHcy; and
transcriptional regulation by estrogen, activating factor-1, hepatic nuclear factor and specificity protein 1 (SP1) transcription factors [82]. Estrogen acts as a positive regulator, where a higher expression of PEMT is observed in female mice than in males [82]. When fed a choline-deficient diet, pre-menopausal women are less likely to develop adverse symptoms, such as muscle and liver disease, compared to post-menopausal women and men [82]. On the other hand, while SP1 seems to act as a negative regulator of PEMT, it also acts as a positive regulator of CTP:phosphocholine cytidylyltransferase (CT), an enzyme which catalyzes the rate-limiting step in the CDP-choline mediated synthesis of PC [82]. This suggests a role for SP1 in the balance between the two pathways and the resulting PC species.

The significance of the PEMT pathway is not yet precisely understood [15] and has been a subject of debate. On the one hand, the PEMT and CDP-choline pathways have been deemed as non-interchangeable pathways that do not substitute for each other due to the different PC species generated by each pathway [15]. On the other hand, disruption of the PEMT gene resulted in mice which did not seem majorly affected when fed a choline-containing diet, an observation which was attributed to the possible increase of CT activity by ~60% [82]. When these mice were fed a choline-deficient diet, however, they developed severe liver failure within three days. These results show that the PEMT pathway may very well act as a substitute for the CDP-choline pathway, bearing evolutionary significance in choline-deficiency conditions such as starvation [82].

PC clearance occurs through biliary secretion, where complete secretion of the liver’s PL content into the bile is achieved within 24 hours [86]. Thus, it is the biliary secretion of PC which is believed to cause the severe liver failure and mortality in PEMT+/− mice fed a choline-deficient diet, resulting in an intolerable decrease in PC, with a PC/PE ratio falling below 1, the normal PC/PE ratio being ~1.8 [82]. This decrease in PC/PE ratio leads to protein leakage and apoptosis resulting from disturbances in membrane permeability [82, 86]. Steatosis and steatohepatitis are also manifestations of PEMT inactivity and a decreased PC/PE ratio, even when mice are fed a normal choline-containing diet [82].
2.18.2 PEMT and VLDL Assembly

In addition to modifying the PL composition of ER and plasma membranes (PC/PE ratio), the PEMT pathway is believed to be a major source of the PC used in the synthesis of VLDL. Recent evidence indicates that PEMT plays a pivotal role in hepatic VLDL synthesis and release, thereby potently influencing the development of hyperlipidemia and atherosclerosis [87]. High levels of VLDL (and LDL), as a result of increased secretion or decreased clearance, have been associated with increased risk of CVD [87]. PLs of newly synthesized VLDL molecules are rich in PE, whereas mature VLDL molecules are mostly composed of PC and to a lesser extent sphingomyelin [87]. The assembly of VLDL molecules starts in the liver with the production of apoB100 lipoprotein, which is translocated into the ER lumen, where triacylglycerols, cholesteryl esters and other lipoproteins are added to the apoB100 lipoprotein core by means of microsomal triglyceride-transfer protein (MTP), and continue to fuse with the new VLDL at later stages during maturity [86-88]. The hydrophobic triacylglycerols and cholesteryl esters are surrounded by a layer of cholesterol and PLs, the major component of which is PC which makes up ~60-80% (mol%) of total lipoprotein PLs [81]. Thus, changes in PC levels and membrane composition affect VLDL synthesis and secretion [81].

NAFLD is a pathological liver condition where lipids are deposited and accumulate in hepatocytes in the form of TGs in the absence of alcohol abuse (<10g/day) [83], as well as in the absence of viral, congenital and autoimmune factors [86]. The first stage of NAFLD is characterized by the development of steatosis (fatty liver), followed by the second stage in which inflammation is superimposed causing non-alcoholic steatohepatitis (NASH) and liver cell damage [83]. Chronic inflammation leads to the development of cirrhosis, liver failure and in some cases hepatocellular carcinoma [83]. NAFLD is considered the hepatic manifestation of the metabolic syndrome, and an independent risk factor for the development of T2D and CVD [83, 86]. The basic cause leading to hepatic fat deposition lies in an imbalance between lipid supply and disposal. There are three main sources of FAs: (1) dietary glucose and FAs; (2) de novo synthesis through lipogenesis; (3) and adipose tissue lipolysis
generating non-esterified FAs, which represent the major bulk of the lipid supply to the liver (~60%) [83, 86]. Dietary FAs are absorbed in the intestine, undergo assembly into chylomicrons, and are released as free FAs into the circulation after hydrolysis of ~80% of chylomicrons by lipoprotein lipases [83].

Membrane composition of PC has been implicated as a leading factor in the disruption of VLDL secretion and clearance, since assembly of VLDL occurs in the ER and is therefore likely to reflect the same changes in microsomal membrane PL composition [87]. Significant decreases in the PC/PE hepatic ratio lead to loss of membrane integrity and leakage of proinflammatory cytokines into hepatocytes, causing NASH [81]. On the other hand, decreased VLDL secretion and increased clearance have cardioprotective effects, explained by the lower availability of circulating TGs which would prevent myocardial lipid accumulation [81]. These effects were observed in PEMT-deficient mice which, despite developing NAFLD, were protected from CVD [81, 89]. Inhibition of PEMT activity prevents the generation of three Hcy molecules for every PC molecule synthesized. A 3 μmol/L rise in plasma Hcy has been associated with a 10% increase in myocardial infarction risk and a 20% increase in stroke risk [87]. The decrease in Hcy production by PEMT inhibition was also found to coincide with reduced atherosclerotic risk [87].

PEMT is suggested to be a predictor of NAFLD, as PEMT genetic mutations in humans that result in compromised PEMT activity are more often found in individuals with NAFLD than in healthy ones [81]. Both the CDP-choline and PEMT pathways are required for normal VLDL synthesis and secretion, as deficiency in either PEMT or CT results in ~50% lower apoB lipoprotein secretion [81]. The effect of PEMT inhibition however was only observed when PEMT+/− mice were challenged with a high-fat diet [81]. A decrease in the amount of PC in the monolayer surrounding VLDL enhances its degradation [81], as this surface layer interacts with receptors, lipases and transport proteins [87]. Interestingly, PE, which is typically more concentrated on the inner side of membranes [86], seems to be preferentially hydrolyzed in the liver and plasma compared to PC [87]. Thus, a decrease in the PC/PE ratio increases the catabolism and clearance of VLDL particles with relatively
high PE content, as well as the degradation of apoB lipoprotein by receptor-mediated degradation [87].

As mentioned, the PC species synthesized via the methylation of PE via PEMT are rich in unsaturated FAs, such as DHA and AA [15, 82]. During VLDL assembly, the utilization of some FAs is preferred over others. For example, oleic acid (18:1 n9) increases secretion of VLDL, while EPA (20:5 n3) reduces the assembly and secretion of VLDL [88]. Moreover, TGs derived from EPA (20:5 n3) were not well-secreted and accumulated in the cytosol, while those derived from oleic acid (18:1 n9) were efficiently assembled into VLDL and secreted [88]. DHA also impairs the secretion of apoB lipoprotein by increasing its oxidation and degradation [88]. However, the precise mechanism by which different FAs affect VLDL secretion is not yet clearly understood [88].

2.19 Potential Link Between D6D And PEMT

A recent study identified a link between PEMT and D6D enzymes, though the manner by which these two pathways influence each other is not yet clear [14, 90]. Eritadenine (Lentinus edodes) is an edible mushroom popular in Japan which has hypocholesterolemic effects [90]. Eritadenine was found to have an inhibitory effect on PEMT in mice, decreasing production of PC by >90% and increasing PE in the microsome, even after addition of choline in the diet [90]. Interestingly, eritadenine also inhibited D6D, leading to a decrease in LA derivatives, such as AA [90]. It is plausible that D6D regulates PEMT activity by generating the DHA or AA required for the synthesis of its substrate (PE-DHA or AA). However, this has not yet been demonstrated experimentally.

Another study observed that methionine restriction in mice decreased both DHA and AA membrane content and increased LA content [91]. Since PE conversion into PC by PEMT is dependent on the donation of methyl groups from the methionine cycle by AdoMet in its conversion into homocysteine, methionine restriction would thus decrease the supply of methyl groups and in turn the production of PE-derived PC. These findings further support the link between PEMT and D6D, as the altered PEMT
pathway appears to have an inhibitory effect on D6D, evidenced by the decrease in D6D indices (AA/LA ratio).

2.20 Concluding Summary

Obesity and the consumption of a high-fat diet are associated with adipose tissue inflammation, proinflammatory cytokine release, steatosis and proinflammatory eicosanoid production, leading to inflammation and CMD, as well as altered membrane FA composition. D6D catalyzes the conversion of n-3 and n-6 PUFAs into their HUFA derivatives, including DHA, EPA and AA. These HUFAs may be incorporated into membrane PLs or further metabolized to produce eicosanoids which function as immune modulators. The activity of D6D is typically measured using [product:precursor] ratios, referred to as D6D indices, such as AA/LA and DGLA/LA ratios. Increases in these indices have been associated with IR and inflammation, while inhibition of D6D has the reverse effects. On the other hand, inhibition of D6D appears to increase hepatic lipid accumulation, an effect which may be due to an underlying link with PEMT. Altered PC production affects normal VLDL synthesis and secretion in the liver, leading to steatosis. The link between the two pathways may lie in the observation that the PC species produced via the PEMT pathway are exceptionally rich in HUFAs, such as DHA and AA, which are produced via the D6D pathway.
3.0 Materials and Methods

3.1 Mouse Models and Management of Colonies

3.1.1 Ob/Ob Mice

Manipulations of the ob gene have rendered a mouse model of obesity (ob/ob) which has been extensively used in research [78, 92-96]. The ob gene encodes for the hormone leptin and is expressed mainly in the adipose tissue and to a lesser extent in the gastric epithelium and placenta [97]. In both humans and mice, there is a direct relationship between plasma leptin levels and adipose tissue mass [97]. Leptin deficiency seems to induce “perceived starvation” in mice manifested in the form of hypothermia, hyperphagia, infertility, and decreased immune function, activity and energy expenditure [97-98]. Additionally, these ob/ob mice develop hyperinsulinemia, hyperglycemia, and hyperlipidemia, with increased serum cholesterol levels and blood lipid profile (LDL and HDL), decreased VLDL secretion by the liver, and mild hypertriglyceridemia [98]. Together with the availability of food, these factors lead to the development of many of the characteristics of the metabolic syndrome, such as obesity, IR, and T2D [97]. The onset of obesity and hyperinsulinemia is observed within two weeks after the birth of ob/ob mice, while hyperglycemia is observed in the fourth week and glucose levels peak during the third and fifth months, after which these levels begin to normalize with advanced age [98]. Ob/ob mice are three times more obese compared to wild-types, with a fat content that is five-fold higher [97]. While these mice may serve as good models for NAFLD due to increased hepatic lipid storage, they may not be as useful in studying NASH, due to their inhibited immune responses as a result of leptin deficiency, which would reduce the incidence of inflammation [98].

3.1.2 Ob/Ob Mice Diet and Phenotyping

C57BL/6J control and ob/ob mice were obtained from the Jackson Laboratory and housed in the Laboratory Animal Resource Facility at Colorado State University (CSU, Fort Collins, Colorado, USA). The mice were fed a standard chow diet before the onset of the study and until the age of 4 months. At 4 months of age, the mice
were weighed and monitored, and glucose tolerance tests (GTTs) were performed. The mice were then sorted into one of four treatment groups: control (con), control + SC-26196 (consc), obese (ob), and obese + SC-26196 (obsc).

In the con and ob groups, the mice were fed *ad libitum* a standard chow diet, while those in the consc and obsc groups were fed *ad libitum* chow containing 100 mg/kg SC-26196 for the last 4 weeks of the study. During the 4-week duration of the study, the food intake and body weights were monitored and recorded on a daily basis. On week 4 of the study, post-treatment GTTs were performed on all groups, after which the mice were sacrificed at the end of week 4.

### 3.1.3 LDL-R Knockout (LDLR\(^{-/-}\)) Mice

While the ob/ob mouse models are useful for studying general obesity, the LDL-receptor knockout mice may provide a more clinically relevant model when fed a high-fat diet. These C57B1/6, lean mice lacking LDL receptors develop modest dyslipidemia and atherosclerosis when fed a normal chow diet [99]. However, when fed a WD, which is high in fat and sucrose, they develop marked hyperlipidemia, with elevated plasma cholesterol and atherosclerosis [99]. Human mutation in the LDLR gene leads to the development of familial hypercholesterolemia [99]. Mice lacking the LDL receptor show similar lipid profile abnormalities as in humans, where cholesterol is mainly present in the LDL fraction as opposed to the HDL fraction [99-100]. In LDLR\(^{-/-}\) mice, there is an increase in the secretion of lipoproteins, such as apoB [81], while their degradation is impaired, leading to a decrease in lipoprotein clearance [100]. Thus, in addition to serving as useful models for hypercholesterolemia and atherosclerosis, and contrary to the ob/ob models, LDLR\(^{-/-}\) mice are also useful in studying NASH [100], since their immune responses are not impaired.

### 3.1.4 LDLR\(^{-/-}\) Mice Diet and Phenotyping

LDLR\(^{-/-}\) mice were obtained from the Jackson Laboratory and housed in the Laboratory Animal Resource Facility at CSU (Fort Collins, Colorado, USA). Two-month-old mice were fed a WD for 12 weeks. The mice were then sorted into one of
two treatment groups: LDLR-WD and LDLR-WDSC. SC-26196 was added to the chow for the last 4 weeks at 100 mg/kg body weight.

The Western diet basal mix (TD.09346 79% Basal Mix – Fat Omitted) was obtained from Harlan Laboratories. This diet formula is designed as a 79% basal mix to allow for the controlled addition of fat. The high-fat content of the WD was created by the addition of 21% lard to the basal mix.

3.2 SC-26196 D6D Inhibitor

SC-26196 is a highly selective D6D inhibitor which is orally active in mice [101]. SC-26196 was synthesized by Biofine International, Inc. (Washington, USA). Control-SC, obsc, and LDLR-WDSC groups of both mouse models were fed ad libitum chow containing 100 mg/kg SC-26196 for the last 4 weeks of the study.

3.3 Experimental Design

The first aim of this study was to investigate whether disease risk in rodent models of CMD correlates with D6D hyperactivity/inhibition. The total serum PLs were extracted by two methods: TLC and a high-throughput extraction method, the latter proving more efficient. The extracted PL samples were then further analyzed using GC to determine their FA composition and D6D indices (AA/LA and DGLA/LA ratios) as a measure of D6D activity. This was followed by the aim to characterize the effect of disease and D6D inhibition on liver membrane composition in order to investigate whether the changes observed in the serum were reflective of underlying changes in the liver, and whether D6D hyperactivity may be involved in the development of hepatic inflammation and IR through a greater production of AA. Thus, total hepatic membrane PL extraction and GC analysis were also performed for liver homogenates.

To investigate the potential link between D6D and PEMT, the hepatic PC/PE ratio was determined, as well as the FA content of each PL fraction: PE and PC. This was done first by the separation of the hepatic PL fractions using HPLC (UV detector). The PE and PC fractions were collected and analyzed using GC for their FA content.
In a separate HPLC run, the PE and PC fractions of liver homogenates were collected and split between the bicinchoninic acid (BCA) protein assay and the phosphorous assay. The phosphorous assay involves the destructive digestion of the PLs for the spectrophotometric quantitation of the released lipid phosphates, and thereby the quantitation of the PLs in each sample. The livers of the ob/ob and LDLR−/− mice are infiltrated with fat due to the presence of NAFLD. The BCA protein assay was therefore conducted in order to overcome the influence of the NAFLD by normalizing the results of the phosphorous assay which are expressed in pg/mg liver tissue to pg/mg of protein.

The second aim was to correlate these changes in serum and liver membrane composition with evidence for CMD risk. To examine the extent of CVD risk, serum triglycerides and cholesterol were analyzed using commercially available assay kits. Hepatic inflammation was examined by measuring a panel of hepatic eicosanoids (e.g. HETEs and PGs) and free AA via LC/MS/MS; and systemic inflammation was examined by measuring serum MCP-1 using the ELISA method.

### 3.4 Thin Layer Chromatography (TLC) Phospholipid Extraction

This modified TLC method [102] was performed to extract PLs from mouse serum and liver homogenate samples for further analysis using GC. Layers of 0.5 mm silica gel G on glass plates (20 x 20 cm) were used as the stationary phase of TLC for the separation of lipid classes in serum and liver homogenate samples. The mobile phase consisted of hexane:diethyl ether:formic acid at a volume ratio of 80:20:2. The samples were spotted onto their labeled location at the bottom of the plate, which was then placed in a glass chamber containing the mobile phase and under a fumehood. The mobile phase ascends by capillarity with the elution first of cholesterol esters, followed by triacylglycerols, free fatty acids, cholesterol, 1,3-diacylglycerols, 1,2-diacylglycerols, monoacylglycerols, and phospholipids last. Thus, when the mobile phase reaches the top of the plate, the phospholipids remain at the location where samples were added at the bottom. The silica gel was scraped off the plate at each sample location into separate tubes and re-suspended in 0.5 mL hexane. This was followed by the addition of 0.5 N potassium hydroxide in methanol for the separation.
of FAs, and the addition of 3 mL 12-14% BF3 in methanol for methylation of FAs into methyl esters for GC analysis. The samples were then placed on a heat plate at 100°C for 30 min, left to cool, followed by the addition of 0.5 mL hexane and 1 mL deionized water (diH2O) for separation of the phases (top layer is hexane containing the lipids). The top layer was aspirated and transferred to 2 mL GC vials, dried out under nitrogen flow and re-suspended in 100 µL hexane.

3.5 High-Throughput Phospholipid Extraction Method

This modified alternate PL extraction method was also used and found to be more efficient than the TLC method [103]. In an eppendorf tube, 600 µL methanol (4°C) were added to 50 µL serum samples. For liver samples, ~20 mg liver tissue were homogenized in 800 µL methanol in a glass homogenizer, transferred to an eppendorf tube and vortexed for ~30 s. The tube was then centrifuged at 900g (~3200 rpm) for 5 min. In a glass tube for each sample, 25 µL sodium methoxide solution were added, followed by addition of the methanol supernatant from the centrifuge tube. The tubes were vortexed for ~30 s to allow selective synthesis of methyl esters from glycerophospholipids fatty acids, allowing a reaction time of 3 min. The reaction was stopped by the addition of 75 µL methanolic HCl to each tube. Fatty acid methyl esters were extracted by the addition of 700 µL hexane to each tube and vortexed for ~30 s. The upper layer of hexane (containing fatty acid methyl esters) was transferred using a glass pipette into a 2 mL GC vial. Extraction was repeated with an additional 700 µL hexane and the extracts were combined and dried down under nitrogen flow. The serum samples were re-suspended in 50 µL hexane, and 2 µL were injected in the GC with a 15:1 split ratio, while the liver samples were re-suspended in 250 µL hexane, and 2 µL were injected in the GC with a 15:1 split ratio.

3.6 High Performance Liquid Chromatography (HPLC)

A modified HPLC method [104] was performed in order to separate the PE and PC PL classes from total PLs in mouse liver homogenate samples.
3.6.1 Tissue Preparation

Tissues were prepared for HPLC using a modified Folch method [105]. The liver tissue samples stored in a -80°C freezer were transferred in liquid nitrogen and quickly cut using a sterile scalpel and weighed (~20 mg). The liver tissue samples were homogenized manually using glass homogenizers in 1.5 mL 2:1 chloroform:methanol and mixed by vortex. The volume of 2:1 chloroform:methanol was then brought up to 4 mL in the homogenizer. Homogenization and vortex steps were repeated until samples were visibly homogenous, and poured through a 2V grade qualitative 12.5 cm Whatman filter into a clean, labeled 10 mL glass tube. In the new tube, the volume of 2:1 chloroform:methanol was brought up again to maintain the volume at 4 mL. In order to separate the two phases of the filtrate, 1 mL diH2O (or 0.88% potassium chloride) were added and mixed by vortex. Samples were then centrifuged at 2500 rpm for 10 min. The top layer containing the methanol with the non-lipid portion was aspirated and safely discarded. The lower layer containing the chloroform and lipid portion was dried down under nitrogen, re-suspended in 0.5 mL hexane, and mixed by vortex for 20 s. The sample was transferred to HPLC vials, dried down again under nitrogen flow, and re-suspended in 100 μL hexane. The HPLC mobile phases composition and ratios are shown in Table 1.

3.6.2 Separation of PE and PC Phospholipid Classes

PE and PC phospholipid classes were separated by normal-phase HPLC (HP1100) and an Agilent Zorbax Rx-Sil, 4.6 X 250 mm, 5-micron column. The column was washed by running solvents A and B through the column for ~6 min, after which the solvents were set to start at 100% solvent A, and to increase from solvent B to A from 0% to 100% over 6 min at a flow rate of 1.5 mL/min and a UV-detection of 206 nm. Solvent B was held at 100% for 5 min after which solvent A was brought up again to 100% by 12 min and reached equilibration by 18 min. Standards (obtained from Avanti Polar lipids) and samples were injected at a preset volume (~40-60 μL). Based on the elution time of the standards, the individual phospholipid fractions (peaks) were collected (PE elutes at ~ 7 min and PC at ~ 12 min). The collected phospholipid fractions were then analyzed for FA content using GC.
3.7 Gas Chromatography (GC)

Total serum and liver PLs, as well as PE and PC PL fractions collected via HPLC, were further analyzed using GC for the determination of their FA composition. The extracted PL samples/fractions were dried down under nitrogen flow, re-suspended in 0.5 mL hexane and mixed by vortex for 20 s. This was followed by the addition of 2 mL 12% boron trifluoride (BF₃) in methanol and mixing by vortex for 20 s. Samples were placed on a heat plate at 100°C for 30 min and left to cool, after which 1mL diH₂O and 0.5 mL hexane were added and mixed by vortex for 20 s. The top layer was aspirated and transferred to 2 mL GC vials. Samples were dried down under nitrogen and re-suspended in 80-100 μL hexane.

FA analysis was performed by an Agilent 6890 Series GC with FID, using an Agilent DB-225 30 m X 0.25 mm X 0.25 μm column. GC Standards (Table 1; purchased from Nu-Chek Prep, Inc., Minnesota, USA) and samples were injected at a preset volume (4 µL) with a 1:1 split ratio.

3.8 BCA Protein Assay

The BCA protein assay was performed simultaneously with the phosphorous assay (following section: 3.8 Phosphorous Assay) by splitting each PE and PC PL fraction sample obtained via HPLC between the two assays. This was done in order to overcome the influence of the NAFLD which is present in the obese mouse livers by normalizing the results of the phosphorous assay which are expressed in pg/mg liver tissue to pg/mg of protein.

Hepatic tissue samples were cut and weighed (~15-30 mg), and 1 mL methanol was added to each sample in a glass homogenizer. Tissues were homogenized using glass on glass tissue grinder mortar and pestle on ice, avoiding the formation of foam or air bubbles. The total volume was measured and recorded (Total), along with the starting mass of each tissue sample. Out of the total volume, 10 μL were drawn out and transferred to eppendorf tubes, 2 mL chloroform were added and the solution was mixed by vortex.
Protein concentrations were measured by BCA assay (Thermo Sci, Catalog #23225). BSA (bovine serum albumin) standards (1-8) were prepared prior to protein assay by performing 1:1 serial dilutions BSA:diH2O and stored at 4°C for further use. Upon use for BCA protein assay, the BCA standards were vortexed and a volume of 10 µL was transferred in triplicates to a 96-well plate. The samples were vortexed and a volume of 10 µL from each sample was transferred in triplicates to the appropriate wells. BCA reagents were prepared according to manufacturer’s instructions by mixing 50:1 Reagent A:Reagent B. In each well, 200 µL of the BCA reagents mixture were transferred using a multichannel pipettor. The plate was then covered and incubated in an oven at 37°C for 30 min. Protein quantification was performed using a plate spectrophotometer set at 562 nm and the BCA protein quantification setting on the SoftMaxPro software.

3.9 Phosphorous Assay

In order to estimate the hepatic PC/PE ratio for the investigation into the potential link between D6D and PEMT, a modified lipid phosphorous assay was performed [106]. The assay involved the destructive digestion of the PLs for the spectrophotometric quantitation of the released lipid phosphates.

The phospholipid fractions collected via HPLC were dried down under nitrogen flow. To each dried down tube, 50 µL perchloric acid were added, mixed by vortex, covered with lids, placed on a heat plate at ≥160°C, and left overnight. Ammonium molybdate (0.25 g/10 mL or 0.075 g/3 mL) and L-ascorbic acid (1 g/10 mL or 0.3 g/3 mL) solutions were prepared.

The next day, samples were removed from the heat plate, allowed to cool, and the lids were removed under a fume hood. To each sample tube, 275 µL diH2O were added, followed by the addition of 41.7 µL ammonium molybdate, and 41.7 µL L-ascorbic acid. After each addition to the sample tubes, the contents were mixed by vortex for 20 s. Samples were placed on a heat plate at 100°C for 5 min.
The standard curve was created by preparing a stock solution of KH$_2$PO$_4$ (0.1 µg/mL). Dilutions of the stock solution for the creation of a standard curve were performed as follows: KH$_2$PO$_4$/diH$_2$O (µL): 0/275, 5/270, 15/260, 25/250, 40/235.

To each standard tube, 50 µL perchloric acid were added, followed by 41.7 µL ammonium molybdate and 41.7 µL L-ascorbic acid. After each addition to the standard tubes, contents were mixed by vortex for 20 s. Standards were placed on heat plate at 100°C for 5 min. The samples and standards were removed from the heat plate and left to cool.

Plate spectrophotometer: In a 96-well plate, 100 µL of samples and standards were transferred to the appropriate wells. Absorbance was read using the SoftMaxPro software at 800 nm.

The results expressed in pg/mg liver tissue were then normalized to the results obtained from the BCA protein assay and were then expressed as pg/mg protein in order to overcome the influence of the NAFLD which is present in the obese mouse livers.

3.10.1 MCP-1 ELISA Assay

To investigate the extent of systemic inflammation in the disease model and the effect of D6D inhibition, serum MCP-1 levels were quantified using Mouse CCL2 (MCP-1) ELISA Ready-SET-Go! (eBioscience, Catalog #88-7391). Mouse serum was collected after animal sacrifice and stored at -80°C as aliquots. Serum aliquots were thawed on ice prior to analysis and diluted 1:10 in diH$_2$O. The colorimetric assay was performed per manufacturer’s instructions.

Wash buffer was prepared as 1 x PBS (pH 7.4) with 0.05% Tween-20, and the stop solution as 2N H$_2$SO$_4$. The ELISA plate (96-well, Corning Costar 9018) was coated with 100 µL/well capture antibody in coating buffer (1:250; 48 µL:12 mL) and incubated overnight at 4°C. The next day, wells were aspirated cautiously so as not to scrape the antibody off the well surface, and washed by transferring ~250 µL/well
wash buffer into each well, soaking for 1 min. The plate was flipped and blotted on absorbent paper, the paper was renewed, and blotting continued until paper underneath was dry. This wash process was repeated 5 times. One part 5X assay diluent was diluted with 4 parts diH₂O, and the wells were blocked with 200 μL/well of the 1X assay diluent for 1 h at room temperature. Aspiration and washing steps were repeated as before.

Standards were diluted using the 1X assay diluent. Into the appropriate wells, 100 μL of standards were transferred. The top standards were made by 2-fold serial dilutions for standard curve. Into the appropriate wells, 100 μL of samples were transferred and the plate was sealed and incubated at room temperature for 4 h. Aspiration and wash steps were repeated as before. The detection antibody at a volume of 100 μL/well was diluted in 1X assay diluent. The plate was sealed and incubated at room temperature for 1 h. Aspiration and wash steps were repeated as before. 100 μL/well of Avidin-HRP were diluted in 1X assay diluent (1:250). The plate was sealed and incubated at room temperature for 30 min. Aspiration and wash steps were repeated as before but for 7 times and wells were soaked in wash buffer for 2 min before aspiration. 100 μL/well of substrate solution were added to each well and the plate was incubated at room temperature for 15 min. 50 μL of stop solution were added to each well. The plate was read at 450 nm and 570 nm. The values of the 570 nm reading were subtracted from the 450 nm reading.

3.10.2 Triglycerides Assay

To assess the extent of CVD risk serum triglyceride levels were quantified using the EnzyChrom™ Triglyceride Assay Kit (Cat# ETGA-200). Mouse serum was collected during animal sacrifice and stored at -80°C as aliquots. Serum aliquots were thawed on ice and diluted 1:10 in diH₂O. Triglyceride levels were quantified using the colorimetric assay per manufacturer protocol.

Kit components were equilibrated at room temperature. Enzyme Mix and Lipase were stored at 4°C. Standards were diluted as shown in Table 3.
Into a 96-well plate, 10 μL of the diluted standards were transferred into wells. The serum samples were diluted 5-fold in diH2O and 10 μL of each were transferred to wells.

The working reagent for each was prepared as follows: 100 μL assay buffer were mixed with 2 μL enzyme mix, 5 μL lipase, 1 μL ATP and 1 μL dye. Into each well, 100 μL of the working reagent were transferred with standards and samples. The plate was incubated at room temperature for 30 min and placed in the plate reader, shaken to mix, and the optical density (OD) was read at 570 nm.

3.10.3 Cholesterol Assay

To assess the extent of CVD risk, as well as the potential link between D6D and PEMT, quantitative colorimetric determinations of HDL and LDL/VLDL were performed using EnzyChrom™ HDL and LDL/VLDL Assay Kit (BioAssay Systems #EHDL-100). This assay is based on the separation of HDL and LDL/VLDL and the determination of cholesterol concentrations using cholesterol esterase/dehydrogenase. The reaction involves the reduction of NAD to NADH, the OD of which is directly proportionate to the cholesterol concentration at 340 nm.

Serum volumes of 20 μL were transferred to eppendorf tubes, 20 μL of precipitation reagent were added to each tube and the mixtures were vortexed and centrifuged at 9,500 x g for 5 min. In a new, clean tube labeled “HDL”, 24 μL of the supernatant were transferred and 96 μL of the assay buffer were added. The remaining supernatant was removed from the pellet, to which 40 μL phosphate buffered saline (PBS) were added and mixed by repeated pipetting. In another clean tube labeled “LDL/VLDL”, 24 μL of the mixture were transferred and 96 μL of the assay buffer were added. In a third tube labeled “Total”, 12 μL of the serum sample were mixed with 108 μL of the assay buffer.

In a tube labeled “Standard”, 12 μL of 300 mg/dL cholesterol were transferred and mixed with 108 μL of the Assay Buffer. In a clear 96-well plate, 50 μL of assay buffer (blank), 50 μL of standard, 50 μL of “Total”, 50 μL of “HDL”, and 50 μL of “LDL/VLDL” were transferred in duplicates into wells.
To each well, 60 μL of working reagent (50 μL assay buffer + 18 μL NAD solution + 1 μL enzyme mix for each well) were transferred rapidly using a multichannel pipettor. The plate was tapped and shaken by the spectrophotometer to mix well contents and incubated for 30 min at room temperature. The values were read at 340 nm OD.

### 3.11 LC/MS/MS

To investigate whether the liberation of free AA from membrane PLs and its metabolism into proinflammatory eicosanoids may be driving the development of CMD, hepatic free AA and eicosanoids were analyzed by a LC/MS/MS method adapted from Gijon et al [107] (at Dr. Robert Murphy’s Laboratory, Colorado University, Denver, Colorado, USA). A mixture of dueterated standards was added to the liver homogenate samples in 70% methanol, allowing for the analysis of over 30 eicosanoids. The dueterated standards included [d4]LTB4, [d8]5-HETE, [d4]TXB2, [d4]PGE2, and [d8]AA. Dilution of the samples was done with water to reach a final concentration of methanol of <15% and extracted using a solid-phase extraction cartridge (Strata C18-E, 100 mg/mL; Phenomenex, Torrance, CA). The eluted samples in methanol (1 mL) were then dried down and re-suspended in 40 μL HPLC solvent A, consisting of 8.3 mM acetic acid (using ammonium hydroxide to reach pH 5.7) and 20 μL HPLC solvent B consisting of acetonitrile-methanol (65:35, v/v). Into a C18 HPLC column (Columbus 150 × 1 mm, 5 μm; Phenomenex), 25 μL of each sample were injected and the flow rate was set at 50 μL/min, starting at 25% solvent B, gradually increasing to 85% in 24 min, and to 100% in 26 min, held at 100% for 12 min. The HPLC eluates were then analyzed by a triple quadrupole mass spectrometer (Sciex API 3000; PE-Sciex, Thornhill, Ontario, Canada). The mass spectrometry analysis was performed against validated standards in the negative ion mode.
4.0 Results

4.1 Ob/Ob Mouse Model Study

4.1.1 Total Serum PL FA Composition

Total serum PL FA composition was analyzed using GC following extraction of serum PLs of control (con), ob/ob (ob), and ob/ob + SC-26196 (obsc) groups (Figure 11). A total of 15 samples were analyzed: 3 con, 6 ob, and 6 obsc. Figure 12 shows total serum PL FA composition in the same groups, highlighting the PL content of LA (18:2), AA (20:4) and DHA (22:6) FAs.

A significant decrease occurred in the PL content of LA in the ob group vs. control (p-value < 0.01). Addition of the drug SC-26196 caused a significant increase in LA in the obsc group compared to ob (p-value < 0.01), restoring LA to levels that are equal or slightly greater than those in the control group. Both AA and DHA levels increased significantly in the ob compared to the control group (p-value < 0.01). This effect was reversed in the presence of the drug, where AA and DHA levels in obsc mice dropped to even lower levels than those in the control group (p-value < 0.01).

D6D indices show increased D6D activity in ob vs. control groups (p-value < 0.01), evidenced by an increase in both AA/LA and DGLA/LA ratios (Figures 13 and 14, respectively). These ratios dropped in the mice administered SC-26196 (p-value < 0.01), indicating D6D inhibition.

4.1.2 Total Liver PL FA Composition

Total liver PL FA composition was analyzed using GC following extraction of hepatic PLs of control (con), control + SC-26196 (consc), ob/ob (ob), and ob/ob + SC-26196 (obsc) groups (Figure 15). A total of 14 samples were analyzed: 3 con, 3 consc, 4 ob, and 4 obsc. Figure 16 shows the total liver PL FA composition in the same groups, highlighting the PL content of LA (18:2), AA (20:4) and DHA (22:6) FAs.
In the ob group, LA levels significantly decreased compared to those of the control group (p-value < 0.01). In the obsc group, LA levels increased compared to the ob group (p-value < 0.05), while AA and DHA remained almost constant.

Most notably, hepatic AA and DHA PL content decreased in the ob group compared to control (p-value < 0.01), contrary to expectations, while those in the obsc group remained fairly constant compared to ob (p-value > 0.05). There were no significant changes in consc vs. control (p-value > 0.05).

D6D indices (AA/LA and DGLA/LA ratios, Figures 17 and 18, respectively) decreased in the consc group compared to control, indicating a decrease in D6D activity with the drug (p-value < 0.05). While there were no significant changes in D6D indices in the ob group vs. control (p-value > 0.05), there was a decrease in both the obsc group vs. ob, and the consc group vs. control (p-value < 0.05), indicating D6D inhibition in both groups administered the drug.

### 4.1.3 Hepatic Free AA and Eicosanoids

Hepatic content of free AA, PGD-2, 5-HETE, 12-HETE and 15-HETE eicosanoids were analyzed using LC/MS/MS. A total of 6 samples were analyzed: 2 control, 2 ob, and 2 obsc. Free AA levels expressed in ng/mg tissue (Figure 19) showed no significant changes in the ob livers vs. control (p-value > 0.05), though a greater sample size may strengthen the statistical significance of its tendency to decrease. Free AA decreased in the presence of the D6D inhibitor (obsc vs. ob and control, p-value < 0.05).

The detected hepatic eicosanoids were PGD2, 5-HETE, 12-HETE and 15-HETE eicosanoids in livers of control, ob and obsc groups expressed in pg/mg tissue (Figure 20) and relative to control (100%) (Figure 21). With the exception of 5-HETEs decreasing in ob vs. control, there were no significant changes in eicosanoids across all groups (p-value > 0.05), though there was a tendency to decrease in the ob vs. control groups and a further decrease with the drug. A greater sample size is needed to confirm such changes.
4.1.4 The Hepatic PC/PE Ratio

The hepatic PC/PE ratio was determined by HPLC separation and extraction of PL classes, followed by determination of phosphorous content of each PL fraction by phosphorous assay. PC and PE were extracted from a total of 26 samples: 9 con, 9 ob, and 8 obsc. Figure 22 shows hepatic PC/PE ratio in control, consc, ob and obsc groups expressed in pg/mg protein.

Results show an increase in PC/PE ratio in the ob vs. control group (p-value < 0.001), indicating elevated activity of PEMT conversion of PE to PC. Administration of the D6D inhibitor (obsc) led to a decrease in the PC/PE ratio (p-value < 0.05), indicating a decrease in PEMT activity.

4.1.5 PE and PC FA Composition

Hepatic PE and PC PL classes were separated using HPLC and further analyzed using GC for FA content. PC and PE were extracted from 25 samples: 5 con, 12 ob, and 8 obsc samples. Figure 23 shows hepatic PE FA composition in con, ob, and obsc groups, and Figure 24 shows the same results, highlighting the PE content of LA (18:2), AA (20:4) and DHA (22:6) FAs.

Changes in LA content in PE across groups did not vary significantly in ob vs. control groups (p-value > 0.05), but showed a decrease in obsc vs. ob groups (p-value < 0.05). AA decreased in ob vs. control (p-value < 0.05) but showed no significant change with the drug (p-value > 0.05), while DHA showed no significant variations across all groups (p-value > 0.05). Changes in the PE AA/LA and DGLA/LA ratios (Figures 25 and 26, respectively) across groups also did not vary significantly.

Figure 27 shows hepatic PC FA composition in con, ob, and obsc groups, and Figure 28 shows the same results, highlighting the PC content of LA (18:2), AA (20:4) and DHA (22:6) FAs. The LA content of PC in the ob group showed a highly significant decrease compared to control (p-value < 0.001), and a drastic increase in the obsc group compared to both ob and control groups (p-value < 0.001), highly indicating elevated D6D activity in the ob group and decreased D6D activity with the drug in
the obs group. AA levels showed no significant difference in the ob group compared to control (p-value > 0.05), while DHA remained the same. However, both AA and DHA further decreased significantly with D6D inhibition (p-value < 0.001), still indicating a decrease in D6D activity.

D6D indices show increased D6D activity in ob vs. control groups (p-value < 0.001), evidenced by an increase in both AA/LA and DGLA/LA ratios (Figures 29 and 30, respectively). These ratios dropped in the obsc mice administered SC-26196 (p-value < 0.001), indicating D6D inhibition.

4.2 LDLR-/- Mouse Model Study

4.2.1 Total Serum PL FA Composition

The total serum PL FA composition was analyzed using GC following extraction of serum PLs of treatment groups: LDLR-WD (LDLR-/- mice fed a WD), and LDLR-WDSC (LDLR-/- mice fed a WD with addition of SC-26196). A total of 9 samples were analyzed: 3 con, 3 LDLR-WD, and 3 LDLR-WDSC.

Figure 31 shows the total serum PL FA composition in control (con), LDLR-WD, and LDLR-WD + SC-26196 (LDLR-WDSC) groups. Figure 32 shows the same results, highlighting the serum PL content of LA (18:2), AA (20:4) and DHA (22:6) FAs.

Similar to the ob/ob serum FA content, the results showed a decrease in serum LA content in the LDLR-WD group vs. control (p-value < 0.01), with no significant change in the LDLR-WDSC group vs. LDLR-WD (p-value > 0.05). The serum AA content increased in the LDLR-WD group vs. control (p-value < 0.001) and decreased with D6D inhibition (p-value < 0.05). DHA decreased in the LDLR-WD group vs. control (p-value < 0.001) and further decreased in the LDLR-WDSC group compared to LDLR-WD (p-value < 0.01).

Serum AA/LA and DGLA/LA ratios (Figures 33 and 34, respectively) increased significantly in the LDLR-WD group vs. control, indicating elevated D6D activity, and decreased with D6D inhibition in the LDLR-WDSC group (p-value < 0.05).
4.2.2 Total Liver PL FA Composition

Total liver PL FA composition was analyzed using GC following extraction of hepatic PLs of control (con), control + SC-26196 (consc), LDLR-WD, and LDLR-WD + SC-26196 (LDLR-WDSC) groups. A total of 14 samples were analyzed: 3 con, 3 consc, 5 LDLR-WD, and 3 LDLR-WDSC. Figure 35 shows the total liver PL FA composition in con, consc, LDLR-WD, and LDLR-WDSC groups. Figure 36 shows the same results, highlighting the hepatic PL content of LA (18:2), AA (20:4) and DHA (22:6) FAs.

In disease (LDLR-WD), LA decreased significantly (p-value < 0.05), indicating elevated D6D activity, but showed no significant changes with the drug (p-value > 0.05). AA decreased in disease vs. control (p-value < 0.05), but showed no significant changes with the drug (p-value > 0.05). Consc showed no significant changes across groups (p-value > 0.05). The serum AA/LA and DGLA/LA ratios (Figures 37 and 38, respectively) did not show significant changes across treatment groups.

4.2.3 Hepatic Free AA and Eicosanoids

The hepatic content of free AA, PGD2, 5-HETE, 12-HETE and 15-HETE eicosanoids were analyzed using LC/MS/MS. A total of 6 samples were analyzed: 2 con, 2 LDLR-WD, and 2 LDLR-WDSC. Free AA levels expressed in ng/mg tissue (Figure 39) showed a significant increase in LDLR-WD livers vs. control (p-value < 0.05), and a possible decrease in the presence of the D6D inhibitor (p-value 0.06). However, a greater sample size is needed to lend statistical significance to this possible decrease.

PGD-2, 5-HETE, 12-HETE and 15-HETE eicosanoids expressed in pg/mg tissue (Figure 40) and relative to control (100%) (Figure 41) showed no significant changes across groups, with the exception of 12-HETEs, which decreased significantly (p-value < 0.05) in the LDLR-WD group vs. control.
4.2.4 The Hepatic PC/PE Ratio

The hepatic PC/PE ratio was determined by HPLC separation and extraction of PL classes, followed by the determination of the phosphorous content of each class by phosphorous assay. PC and PE were extracted from a total of 16 samples: 9 con, 5 LDLR-WD, and 2 LDLR-WDSC.

The hepatic PC/PE ratio expressed in pg/mg protein (Figure 42) showed no significant variations across groups, except for an increase in the PC/PE ratio of LDLR-WD livers compared to control (p-value < 0.05).

4.2.5 PE and PC FA Composition

The hepatic PE and PC PL classes were separated using HPLC and further analyzed using GC for FA content. PC and PE were extracted from a total of 12 samples: 5 con, 4 LDLR-WD, and 3 LDLR-WDSC.

Figure 43 shows the hepatic PE FA composition in con, LDLR-WD, and LDLR-WDSC groups. Figure 44 shows the same results, highlighting the hepatic PE content of LA (18:2), AA (20:4) and DHA (22:6) FAs. The hepatic PE FA composition was more reflective of changes in D6D activity compared to the same analysis in the ob/ob study. This was mainly due to the changes in LA content, which decreased in LDLR-WD vs. control (p-value < 0.001), and increased with the drug (p-value < 0.01). There were no significant changes in the AA content across groups (p-value > 0.05), while DHA decreased in the LDLR-WD group vs. control (p-value < 0.05).

The hepatic PE AA/LA and DGLA/LA ratios (Figures 45 and 46, respectively) increased in the LDLR-WD group vs. control, indicating elevated D6D activity, and decreased with the drug, indicating D6D inhibition (p-value < 0.01).

Figure 47 shows the hepatic PC FA composition in con, LDLR-WD, and LDLR-WDSC groups. Figure 48 shows the same results, highlighting the hepatic PC content of LA (18:2), AA (20:4) and DHA (22:6) FAs. The PC content of LA decreased in the LDLR-WD group vs. control (p-value < 0.01), suggesting elevated D6D activity, and increased with D6D inhibition (p-value < 0.001). AA did not change significantly.
in LDLR-WD vs. control (p-value > 0.05), but decreased with the drug (LDLR-WDSC) (p-value < 0.05). DHA decreased in LDLR-WD vs. control and further decreased in LDLR-WDSC (p-value < 0.05).

The hepatic PC AA/LA and DGLA/LA ratios (Figures 49 and 50, respectively) increased in the LDLR-WD group vs. control (p-value < 0.01), indicating elevated D6D activity, and decreased in LDLR-WDSC (p-value < 0.05), indicating D6D inhibition.

4.2.6 Serum MCP-1 Analysis by ELISA

To investigate the presence of systemic inflammation in the disease group (LDLR-WD) and the effect of D6D inhibition (LDLR-WDSC), serum MCP-1 levels were quantified using Mouse CCL2 (MCP-1) ELISA Ready-SET-Go! A total of 10 serum samples were analyzed: 2 control, 3 consc, 3 LDLR-WD, and 2 LDLR-WDSC. Figure 52 shows serum MCP-1 levels in control, consc, LDLR-WD, and LDLR-WDSC groups.

Results showed a slight increase in MCP-1 levels in the LDLR-WD group vs. control (p-value < 0.05), suggesting a possible increase in systemic inflammation in the disease state. MCP-1 levels showed no significant changes with the drug (p-value 0.07), though a greater sample size may confirm the possible alleviation of inflammation with D6D inhibition. Results were also suggestive of a slight decrease in MCP-1 in the consc group compared to control (p-value 0.06).

4.2.7 Serum Triglycerides Assay

To investigate the extent of CVD risk in the disease group (LDLR-WD) and effect of D6D inhibition (consc and LDLR-WDSC), serum triglyceride levels were quantified using the EnzyChromTM Triglyceride colorimetric assay. A total of 7 samples were analyzed: 3 con, 1 consc, 1 LDLR-WD, and 2 LDLR-WDSC. Sample size was small due to depletion of serum samples. Figure 54 shows serum triglyceride levels in control, control + SC-26196 (consc), LDLR-WD and LDLR-WDSC groups. The results showed an expected decrease in serum triglycerides in the groups administered
the D6D inhibitor (consc and LDLR-WDSC), and a drastic increase in the LDLR-WD group. However, a greater sample size is needed to obtain statistical significance of these results.

4.2.8 Serum Cholesterol Assay

To investigate the extent of CVD risk in disease (LDLR-WD) and the effect of D6D inhibition (LDLR-WDSC), as well as the potential link between D6D and PEMT, Total, HDL and LDL/VLDL Cholesterol were quantified using a colorimetric assay. A total of 2 samples were analyzed: 1 LDLR-WD and 1 LDLR-WDSC, performed in duplicates. Sample size was small due to depletion of available serum samples. Figure 55 shows Total Cholesterol, HDL, and LDL/VLDL cholesterol in LDLR-WD and LDLR-WDSC groups. Results show much higher levels of total cholesterol in the LDLR-WD group compared to LDLR-WDSC (p-value < 0.01). HDL cholesterol remained the same in both groups, while LDL/VLDL cholesterol was high in the LDLR-WD group and decreased with D6D inhibition (p-value < 0.01).
5.0 Discussion

D6D catalyzes the rate limiting step in the conversion of LA into its HUFA derivative, AA, and the conversion of ALA into its HUFA derivatives, EPA and DHA. Inflammatory stimuli and cell injury lead to an increase in PL liberation of AA, which is converted via COX, LOX and CYP enzymatic pathways into eicosanoids, such as PGs, TXs, and HETEs. These immunity-modulating molecules may have anti- or pro-inflammatory effects, depending on their type, site and receptor distribution.

D6D activity is measured indirectly in serum and tissue membranes through the use of [product: precursor] PUFA ratios, the most common of which has been the AA/LA ratio [11, 57, 101]. The D6D enzyme is encoded by the fads2 gene. Obesity, hyperinsulinemia, and the consumption of a WD each independently stimulates an increase in D6D expression and/or activity, leading to an increase in AA/LA serum and tissue PL ratios. Recent epidemiological studies have also shown that SNPs in the human fads2 gene associated with high D6D indices are strongly linked to the development of inflammation, CVD and the metabolic syndrome [11, 64, 68].

The link between D6D activity and inflammation has been directly supported by animal studies. Inhibition of D6D in a carrageenan paw edema mouse model led to a decrease in tissue AA/LA ratios accompanied by a decrease in inflammation evidenced by a 50% alleviation of paw edema [101]. In another study, D6D inhibition also led to a decrease in tissue AA/LA ratios accompanied by a decrease in colorectal tumorigenesis [57]. However, despite this abundant evidence of increased D6D activity in disease both in humans and animals, its (patho)physiological role in this process remains unclear.

5.1 Ob/Ob Mouse Model Study

A commonly investigated model of obesity is the ob/ob mouse model which is leptin-deficient due to manipulations of the ob gene. Leptin deficiency in this model causes “perceived starvation” leading to obesity as a result of hyperphagia and reduced
physical activity and energy expenditure. These mice develop characteristics of CMD in the form of obesity, hyperinsulinemia, hyperglycemia, and NAFLD.

Preliminary data showed an elevation of serum AA/LA ratio, as well as marked glucose intolerance and hyperinsulinemia, in ob/ob mice versus lean C57B1/6 mice (control) fed a regular chow diet (A. Chicco; unpublished data). Given the apparent role of D6D activity in CMD, it became of interest to further evaluate its role via pharmacological inhibition using the experimental D6D inhibitor compound SC-26196 [101]. C57B1/6 lean mice (con) and ob/ob mice were fed a standard chow diet throughout the course of the study with the addition of SC-26196 to the diet for the last 4 weeks in the obsc and consc groups, after which the animals were sacrificed at 5-months of age.

Glucose tolerance tests (GTTs) were conducted prior to treatment and post-treatment with SC-26196 [13]. Pre-treatment GTTs showed no differences between the control and consc groups and between the obese and obsc groups, the latter groups showing elevated blood glucose levels compared to the control group. Post-treatment GTTs resulted in significantly higher blood glucose levels in the ob group vs. control (265 ± 22.0 mg/dL vs 164 ± 14.8 mg/dL, respectively). In the obsc group, the blood glucose levels were lower compared to the ob group 2 hours post-injection (198 ± 13.5 mg/dL vs 265 ± 22.0 mg/dL, respectively) (Figure 56). Thus, the glucose intolerance which was developed in obesity was reversed by D6D inhibition, supporting the link between D6D activity and the development of IR.

5.1.1 Total Serum PL FA Composition

GC analysis of serum PL FA composition showed a marked decrease in PL LA content in disease (ob group), which was completely restored by D6D inhibition in the obsc group (Figure 12). Both AA and DHA content increased in disease and decreased with D6D inhibition. Thus, in the ob mice, D6D activity was elevated in disease, evidenced by an increase in D6D indices (AA/LA and DGLA/LA ratios). The addition of the drug SC-26196 inhibited D6D activity, evidenced by a drop in the D6D index ratios (Figures 13 and 14). Thus, in this mouse model, the obesity
induced by hyperphagia and low energy expenditure resulting from leptin deficiency led to manifestations of CMD, such as glucose intolerance and hyperinsulinemia, and was accompanied with or caused by an increase in D6D activity. These effects were reversed with D6D inhibition.

It is therefore possible that the changes in serum AA/LA ratios observed in human and animal studies, in addition to those observed in control vs. disease models (A. Chicco; unpublished data) and in pre- and post-D6D inhibitor treatment groups, are a reflection of an underlying hepatic alteration in PL composition and AA pools. Hepatic inflammation has been implicated in the development of glucose intolerance in models of obesity and CMD, which may be a result of elevations in hepatic AA production due to increased D6D activity. This study thus aimed to characterize the effect of disease and D6D inhibition on liver PL composition in mouse models of CMD.

5.1.2 Total Liver PL FA Composition

Total liver PL FA composition was analyzed using GC in control and ob/ob mouse groups and treatment groups, consc and obsc (Figures 15 and 16). The hepatic PL content of LA across all groups showed the most significant changes, decreasing in ob vs. control, and increasing with the addition of the drug (obsc). However, changes seemed to differ than those observed in serum FA analyses with regards to AA and DHA contents across groups. Most notably, hepatic AA and DHA PL content decreased in the ob group compared to control, contrary to expectations, while in the obsc group, they remained fairly constant compared to the ob group.

However, the predominant FA in the chow diet is LA (n-6 PUFA) (1.71%), and the ratio of n-6/n-3 PUFAs is 17.1:1. It has been reported that a “healthy” n-6/n-3 PUFA ratio is 1-4:1 [46]. Although there is a reported preference of D6D to metabolize ALA over LA, when the n-6/n-3 ratio exceeds “healthy” limits, this drives the enzymatic reaction towards LA metabolism due to substrate competition [54]. Therefore, despite elevation of D6D activity, indicated by an increase in serum D6D indices, the decrease in DHA in the disease model may have resulted from a higher LA supply
and metabolism. Though this would be expected to result in elevated AA in the ob group, the opposite effect was observed.

Importantly, D6D indices (AA/LA and DGLA/LA) decreased in both treatment groups (Figures 17 and 18). The expected changes in LA content across the groups indicate that indeed D6D was activated in the obese mice and inhibited in the groups administered the drug. The liver is constantly metabolizing and exporting lipids, for example, through the incorporation of FAs into membrane PLs, as well as secreting them in the form of lipoproteins into the bloodstream. The decrease in AA in the ob group vs. control may be due to an increase in AA secretion on PLs and/or lipoproteins, such as VLDL. This is supported by the observation that AA and DHA levels were elevated in the ob serum PLs compared to control.

5.1.3 Hepatic Free AA and Eicosanoids

The implication of free hepatic AA and its proinflammatory eicosanoid derivatives in the development of hepatic inflammation and glucose intolerance was investigated by LC/MS/MS analysis of control, ob and obsc livers. Dueterated standards were added to homogenized liver samples which allow for the detection of over 30 eicosanoids. Separation by HPLC was followed by analysis using a triple quadrupole mass spectrometer in the negative ion mode. The panel of detected hepatic eicosanoids was composed of PGD-2, an AA derivative produced via the COX pathway, and 5-HETEs, 12-HETEs, 15-HETEs, produced through the LOX pathways. These LOX products have been associated with IR, inflammation and hyperlipidemia [76-77].

There were no significant changes in hepatic free AA in the ob group compared to control (Figure 19). However, administration of the D6D inhibitor (obsc) led to a significant decrease in free AA, suggesting that there may be a decrease in the liberation of AA and production of proinflammatory eicosanoids with D6D inhibition. With the exception of 5-HETEs decreasing in ob vs. control, there were no significant changes in eicosanoids across all groups (Figures 20 and 21). The possible tendency of eicosanoids to decrease in disease (p-value > 0.05) requires a greater sample size to obtain statistical significance. However, these possible changes
may be attributed to the presence of marked steatosis in the ob/ob mouse livers. Since all levels were expressed in ng or pg per mg of tissue, the replacement of healthy hepatic tissue by fatty infiltration which occurs in this model may account for the possible trend of lower free AA and eicosanoids levels observed in these livers compared to those of control mice.

5.1.4 Potential Link Between D6D and PEMT

Regulation of PEMT is dependent on the availability of substrates, such as AdoMet and PE; product feedback inhibition, such as AdoHcy; and transcriptional regulation by estrogen, activating factor-1, hepatic nuclear factor and specificity protein 1 (SP1) transcription factors [82]. Estrogen acts as a positive regulator, where a higher expression of PEMT is observed in female mice than in males [82]. On the other hand, while SP1 seems to act as a negative regulator of PEMT, it also acts as a positive regulator of CTP:phosphocholine cytidylyltransferase (CT), an enzyme which catalyzes the rate-limiting step in the CDP-choline mediated synthesis of PC [82]. This suggests a role for SP1 in the balance between the two pathways and the resulting PC species.

Recent studies on the effects of eritadenine, a popular edible mushroom in Japan, showed an inhibitory effect on PEMT, leading to a decrease in PC production by >90% and an increase in microsomal PE [90]. Interestingly, the decrease in PC/PE ratio by eritadenine was accompanied by D6D inhibition, evidenced by a decrease in the AA/LA ratio. Another study showing that dietary methionine restriction inhibits PEMT activity also reported a decrease in AA/LA ratio, suggesting that D6D activity was affected [91]. Furthermore, studies on PEMT-deficient mice showed that, when fed a high-fat diet, these mice did not develop the obesity and IR that PEMT+/+ mice did under the same conditions [89], mimicking the effect of the D6D inhibitor. These studies have thus indicated the possibility of a link between PEMT and D6D, though the manner by which these two enzymatic pathways interact is not yet clear.

The effect of D6D inhibition on PEMT activity was indirectly examined in this study by measuring the hepatic PC/PE ratio and the FA content of PC and PE PL fractions.
Separation of individual PL classes was performed using HPLC by which PC and PE were collected and further analyzed using GC for FA content. The PC/PE ratio was measured by means of a spectrophotometric assay of the phosphorous content of each PL class.

5.1.5 The Hepatic PC/PE Ratio

As hypothesized, results showed an increase in hepatic PC/PE ratio in the ob groups in which D6D activity was elevated (Figure 22). Administration of the D6D inhibitor indeed affected PEMT activity, leading to a decrease in hepatic PC/PE ratio. Livers of normal C57B1/6 mice administered the D6D inhibitor (consc) also showed a decrease in PC/PE ratio compared to controls. These results support the hypothesis that a link does exist between D6D and PEMT activity. Moreover, it suggests that this interaction may be bidirectional, such that D6D activity influences PEMT activity, in addition to the aforementioned published effects of PEMT on D6D.

5.1.6 PE and PC FA Composition

To further investigate the link between D6D activity and PEMT, the FA content of individual hepatic PLs was analyzed. The FA content of PE did not significantly reflect D6D activity, as LA did not vary in ob vs. control groups, though it modestly increased with D6D inhibition (Figure 24). AA decreased in ob vs. control but showed no significant change with the drug, while DHA showed no significant variations across all groups. Changes in D6D indices across groups also did not vary significantly, largely due to the low changes in LA (Figures 25 and 26).

On the other hand, the LA content of PC in the ob group showed a highly significant decrease compared to control, and an increase in the obsc group compared to both ob and control groups, highly indicating elevated D6D activity in the ob group and decreased D6D activity with the drug (Figure 28). AA levels showed no significant difference in the ob group compared to control, while DHA remained the same. However, both AA and DHA further decreased significantly with D6D inhibition, indicating a decrease in D6D activity. The PC AA/LA and DGLA/LA ratios (Figures
29 and 30, respectively) both significantly increased in the ob group and decreased with the drug.

PE is the second most abundant PL following PC and is synthesized from ethanolamine and phosphatidylserine. PE undergoes three methylation steps in its conversion into PC by PEMT, though the exact sequence of events during these three steps is not yet clear. However, during these methylation steps, and even after PC synthesis, other enzymes come into play and there is continued remodeling of FA composition. For example, PLA-2 deacylation cleaves LA and stearic acid (18:0) off, while acyl transferase (AT) reacylation adds FAs such as AA and oleic acid (18:1n9) to PC during the synthesis of PC from PE and its remodeling [108]. This may account for the differences observed in the FA content of PE and PC, where the PC FA content is more reflective of D6D activity than PE, due to the continuous remodeling and redistribution of FAs from PE onto PC.

5.1.7 Ob/Ob Mouse Model: Concluding Summary

Glucose intolerance in this model was reversed by the addition of a D6D inhibitor in the chow, supporting the hypothesis that increased D6D activity in obese states increases the risk of CMD [13]. To investigate the manner by which D6D activity contributes to CMD risk and the effect of direct D6D inhibition by the drug SC-26196, serum and hepatic D6D indices and PL FA content, as well as free hepatic AA and eicosanoids were investigated. Obesity in this model showed elevated D6D indices in the serum which were reversed with D6D inhibition. In the liver, LA was most indicative of D6D activity, increasing in obesity and decreasing with D6D inhibition. However, hepatic PL content of AA and DHA appeared to decrease in obesity, contrary to expectations. This may be accounted for by the presence of NAFLD, the continuous export of lipids by the liver, and the influence of other enzymatic pathways, such as PLA-2 and AT. From these results, however, it is apparent that D6D activity is elevated in states of obesity, leading to manifestations of CMD, which were reversed by D6D inhibition. Studies have indicated a possible link between D6D and PEMT [90-91]. This link was supported by evidence for an elevation of D6D activity in obesity that was accompanied by an increase in the
PC/PE ratio reflective of increased PEMT activity, and a decrease with the pharmacological inhibition of D6D by SC-26196. The nature and pathophysiological implications of this interaction in the context of CMD warrants further investigation.

5.2 LDLR−/− Mouse Model Study

Since leptin deficiency is not a common abnormality in humans, the LDL-Receptor knockout (LDLR−/−) mice have been reported to provide a more clinically relevant model of obesity and hyperlipidemia [99-100]. When fed a high-fat diet, such as the WD which is high in fat and sucrose, these mice develop marked hyperlipidemia, with elevated plasma cholesterol and atherosclerosis [99]. Based on the results obtained from the ob/ob model and the putative involvement of D6D in the pathogenesis of atherosclerosis [109], it became of interest to investigate the same parameters in mice lacking the LDL-receptor (LDLR−/−) to test the effects of a WD on D6D activity, as well as D6D inhibition by SC-26196 in this model of CMD.

Two-month-old mice were fed a high-fat, high-cholesterol WD for 12 weeks. The mice were sorted into one of two treatment groups: LDLR-WD and LDLR-WDSC. SC-26196 was added to the chow for the last 4 weeks in the LDLR-WDSC group at 100 mg/kg body weight, after which the animals were sacrificed at 5 months of age. The WD consisted of a fat-omitted basal mix (79%), with the addition of 21% lard.

GTTs were conducted at the onset of the study and pre- and post-treatment with SC-26196. In the LDLR-WD group, results showed glucose intolerance, which was ameliorated with the D6D inhibitor (A. Chicco; unpublished data). Fasting insulin levels were markedly high in the LDLR-WD group, as were serum LDL, VLDL and triglycerides, all of which were lowered by the drug. Furthermore, brachiocephalic artery hematoxylin and eosin (H&E) stained sections showed a prevention/reversal of atherosclerosis with the D6D inhibitor (A. Chicco; unpublished data). These results indicate that the LDLR-WD mice develop CMD and that D6D inhibition reverses disease in this model as it does in the ob/ob model.
5.2.1 Total Serum PL FA Composition

The effects of disease in this model and D6D inhibition on serum PL composition were analyzed via GC following PL extraction (Figures 31 and 32). Similar to the ob/ob model, serum PL LA content decreased in the CMD model fed a WD (LDLR-WD), while AA increased compared to the control group. The increase in AA in the disease group was reversed with D6D inhibition.

As expected, DHA content decreased in the LDLR-WD group vs. control and decreased further with the drug. This is due to the WD being overwhelmingly predominant in SFAs and n-6 PUFAs, likely leading to both over-activity of D6D, as well as an abundance of LA, shifting PUFA metabolism to favor the n-6 metabolic pathway due to substrate competition [62, 69-70]. Therefore, in the disease state (LDLR-WD), DHA content would decrease despite elevated D6D activity, indicated by the drastic decrease in LA serum content. D6D activity was also reflected in a significant increase in its indices (AA/LA and DGLA/LA ratios) in disease, indicating elevated D6D activity, and a decrease with D6D inhibition (Figures 33 and 34).

5.2.2 Total Liver PL FA Composition

Since the serum FA composition in both models (ob/ob and LDLR−/−) were similar, it was expected that the liver would also show similar results. As expected, in the disease model (LDLR-WD), LA decreased significantly, indicating elevated D6D activity (Figure 36), though no significant changes were observed with D6D inhibition. AA decreased in disease vs. control, but showed no significant changes with the drug. It is unclear why AA composition trends in such manner in this model, though one explanation may be that there is an increase in AA hydrolysis from PLs. The control group administered the drug (consc) showed no significant changes across all groups. D6D indices showed no significant changes across groups (Figures 37 and 38), with the exception of the consc group which showed the most remarkable decrease in indices, indicating D6D inhibition.
5.2.3 Hepatic Free AA and Eicosanoids

Analysis of hepatic free AA (Figure 39) showed an increase in AA levels in LDLR-WD livers vs. control, and a possible decrease in the presence of the D6D inhibitor, supporting the hypothesis that in this disease model, there may be increased hydrolysis of AA from PLs, leading to higher levels of free AA.

Only 12-HETEs significantly decreased in LDLR-WD compared to control, while 5-HETEs remained constant (Figures 40 and 41). D6D inhibition led to changes of low significance, trending towards a decrease in PGD-2, 12-HETEs and 15-HETEs, while 5-HETEs also remained constant.

As in the ob/ob livers, hepatic steatosis is also present in the LDLR⁺⁻ livers, leading to a replacement of healthy hepatic tissue with fat. This may account for the low changes in eicosanoid levels in the LDLR-WD group vs. control. However, D6D inhibition did not seem to affect the production of 5-HETEs, possibly due to the involvement of 5-LOX-activating protein (FLAP) which is required first to activate the 5-LOX pathway [75], in addition to the conversion of 5-HETEs into leukotrienes, which are less stable and rarely reach tissue concentrations that are detectable in liver tissue.

On the other hand, free AA levels still increased in disease vs. control, despite the presence of steatosis and contrary to ob/ob results. It is plausible that conversion of LA to AA in this model, as well as AA hydrolysis, is much greater than in the ob/ob model which allowed it to surpass control levels despite the presence of less hepatic tissue.

5.2.4 The Hepatic PC/PE Ratio

A study reported that LDLR⁺⁻/PEMT⁻⁻ mice were resistant to developing atherosclerosis and CVD compared to LDLR⁺⁻/PEMT⁺⁺ [87], showing the same phenotypic effect observed with the pharmacological inhibition of D6D. Furthermore, studies on PEMT⁻⁻ mice fed a regular chow diet showed greatly reduced hepatic AA/LA and DGLA/LA ratios (Figures 57 and 58, respectively) compared to wild
type (WT) mice (A. Chicco; unpublished data), further supporting the hypothesis that PEMT and D6D are co-regulated by some mechanism. Whether inhibition of D6D alters PEMT activity in this model is unclear. Thus, in this study, the hepatic PC/PE ratio was examined as a surrogate measure of PEMT activity in the disease state, where D6D activity is elevated, and in the presence of the SC-26196 drug, where D6D activity is inhibited.

With the exception of the LDLR-WD group in which the PC/PE ratio increased in line with hypothesis, the hepatic PC/PE analysis revealed almost no variations across treatment groups (Figure 42), contrary to expectations and to the results obtained in the ob/ob model. This may be explained by the inability of LDLR−/− mouse livers to uptake LDL from the serum due to the absence of the LDLR-receptor. This leads to marked hyperlipidemia, which in turn disrupts lipoprotein and possibly PL secretion, causing immature VLDL to accumulate in the liver. This disruption of lipoprotein uptake and release may be altering PEMT activity via feedback inhibition, preventing PEMT from converting more PE into PC. Thus, the composition and ratio of the “trapped” PE and PC in the liver would not change, despite changes in D6D activity across groups. This finding also serves to highlight that there are significant differences between the ob/ob and LDLR−/− models in their disease etiology, pathophysiology, and their response to treatment. These differences should be considered when selecting a research model of CMD.

5.2.5 PE and PC FA Composition

Hepatic PE FA composition (Figure 44) was more reflective of changes in D6D activity than in the ob/ob model, due to significant changes in LA, which decreased in LDLR-WD vs. control, and increased with the drug. AA showed no significant change in LDLR-WD vs. control, while DHA decreased in LDLR-WD vs. control. Contrary to the ob/ob model, where D6D indices of PE did not show significant variations across groups, both AA/LA and DGLA/LA ratios (Figures 45 and 46, respectively) in the LDLR−/− model increased in the LDLR-WD group and decreased with administration of the drug. This is primarily due to the significant changes in LA across groups. This supports the hypothesis that disruption in lipid uptake and
secretion in the LDLR model alters PEMT activity via negative feedback inhibition, preventing further conversion of PE into PC, and therefore the redistribution of FAs onto PC. Thus PE would maintain its FA composition longer and be more reflective of D6D activity.

The hepatic PC content of LA decreased in the LDLR-WD group, also suggesting elevated D6D activity, while AA remained the same compared to control (Figure 48). Since PEMT seems to prefer the conversion of PE molecules with AA content [15], and assuming PEMT activity is compromised due to the hypothesized feedback inhibition resulting from altered VLDL secretion and uptake, the PC content of AA in this model may not change as a result of reduced PC synthesis from AA-containing PE.

D6D inhibition led to an increase in PC content of LA and a significant decrease in AA, dramatically decreasing the AA/LA ratio (Figure 49). This may indicate that hydrolysis of LA and reacylation of its D6D product AA occurs more readily in PC vs. PE, particularly since PC secretion is inhibited in this model.

5.2.6 LDLR\textsuperscript{−/−} Mouse Model Study: Concluding Summary

LDLR\textsuperscript{−/−} mice are characterized by developing marked hyperlipidemia and atherosclerosis when fed a high-fat diet, as well as developing obesity, IR and NAFLD. Serum FA composition analyses in this model were similar to those observed in the ob/ob model, where D6D indices were elevated in disease and reversed by the drug due to D6D inhibition. In the liver, total hepatic FA content of LA was most suggestive of changes in D6D activity, decreasing with disease and showing a possible trend towards an increase with D6D inhibition. As in the ob/ob model, AA appeared to decrease with disease/high-fat diet. However, in this model, the reduction in AA content cannot be explained by hepatic export of lipoproteins, as VLDL secretion is greatly compromised. Nevertheless, NAFLD and the activity of other enzymes, such as PLA-2 and AT, may be contributing to these changes in hepatic AA content. Perhaps the most striking difference between the two models is in the PC/PE ratio, which showed almost no variation across treatment groups in the
LDLR−/− model, whereas in the ob/ob model, the PC/PE ratio was elevated in disease and decreased with D6D inhibition in concordance with D6D indices. This may be due to feedback inhibition of PEMT resulting from marked hyperlipidemia which alters hepatic lipid secretion, despite elevation in D6D activity in disease.

5.3 Correlation with Inflammation, CVD Risk, and PEMT Activity

5.3.1 Serum MCP-1

To investigate the state of systemic inflammation in the disease model and the effect of D6D inhibition, serum MCP-1 was quantified by ELISA method. MCP-1 is a chemokine released by adipocytes and macrophages, among other cells, which attracts and recruits T-lymphocytes and circulating monocytes, which in turn produce inflammatory molecules, such as cytokines. Over-expression of MCP-1 has been observed in response to obesity, inflammation and the consumption of a high-fat diet, and has been associated with hepatic steatosis and IR [2]. Serum MCP-1 was examined in groups of control mice, control mice administered the D6D inhibitor (consc), LDLR−/− mice on a WD (LDLR-WD), and LDLR−/− mice on a WD with the addition of the D6D inhibitor (LDLR-WDSC).

Results showed a modest increase in MCP-1 levels in the LDLR-WD group vs. control, indicating a possible increase in systemic inflammation in the disease state (Figure 52). However, MCP-1 levels showed no significant changes in thr groups administered the drug. These results suggest that systemic inflammation may be increased in CMD, though there may also be other inflammatory mechanisms involved in this process. A greater sample size is required to obtain more statistically significant results in order to observe whether D6D inhibition leads to alleviation of inflammation and a decrease in serum MCP-1 levels.

5.3.2 Serum Triglycerides and Cholesterol

Serum triglyceride and cholesterol levels were investigated via spectrophotometric assay of control, consc, LDLR-WD and LDLR-WDSC groups. Serum triglycerides were markedly high in the LDLR-WD group compared to control, and decreased to
control levels with D6D inhibition (Figure 54). Total cholesterol levels were significantly higher in the LDLR-WD group compared to LDLR-WDSC (Figure 55). HDL cholesterol remained equal in both groups, while LDL/VLDL cholesterol was higher in the LDLR-WD group. In both assays, however, sample sizes were small due to depletion of available serum samples. Thus, a greater sample size is required to obtain more conclusive and significant results.

LDLR\textsuperscript{−/−} mice serve as a model of hyperlipidemia due to LDL-receptor deficiency which impairs lipoprotein uptake by the liver. VLDL secretion and clearance are affected by PC biosynthesis by PEMT and hepatic PC/PE ratios, where a significant decrease in the PC/PE ratio leads to degradation of VLDL, as well as membrane disruption and leakage [81]. While decreased VLDL secretion has been shown to have cardio-protective effects due to lower levels of plasma TGs, the altered secretion leads to the development of NAFLD [81]. This has also been observed in PEMT\textsuperscript{−/−} mice [81, 89]. PEMT\textsuperscript{−/−}/LDLR\textsuperscript{−/−} mice fed a high-fat diet were found to have altered VLDL secretion, and a decrease in plasma VLDL/LDL and atherosclerotic lesions [87]. Plasma TGs were decreased by ~70% and cholesterol by ~56% compared to PEMT\textsuperscript{+/+}/LDLR\textsuperscript{−/−} mice [82]. The PEMT-deficient mice were protected against obesity and IR, but developed NAFLD. In line with the results in this study, no alterations in HDL levels were observed in the PEMT\textsuperscript{−/−}/LDLR\textsuperscript{−/−} compared to the PEMT\textsuperscript{+/+}/LDLR\textsuperscript{−/−} mice. In humans, PEMT is suggested to be a predictor of NAFLD, as PEMT genetic mutations in humans that result in compromised PEMT activity are more often found in individuals with NAFLD [81].

These findings may support the link between D6D and PEMT activities, since inhibition of D6D activity by SC-26196 led to the same effects observed in PEMT\textsuperscript{−/−} mice. Thus, despite the unexpected lack of variation in PC/PE ratios across the control, LDLR-WD and LDLR-WDSC groups, which cast a shadow on the link between D6D and PEMT, the serum lipid profile findings, though non-conclusive, suggested the possibility that D6D inhibition had the same effects observed in PEMT\textsuperscript{−/−} mice, and that D6D inhibition may be inhibiting PEMT activity.
5.4 Differences Between the Ob/Ob and LDLR $^/-$ Models

Significant differences were observed between the ob/ob and LDLR $^/-$ models in their disease etiology, pathophysiology, and their response to treatment. In the ob/ob mouse model fed a regular chow diet, obesity was induced as a result of hyperphagia, low physical activity and low energy expenditure. In the LDLR $^/-$ model, obesity was induced as a result of altered lipid uptake from the circulation by the liver due to deficiency in the LDL-receptor, resulting in an accumulation of lipids in the circulation, adipose tissue, as well as in the liver. Thus, D6D hyperactivity and inhibition and its potential influence on PEMT reflected differently on the two models.

For example, the hepatic PE FA composition in the ob/ob model showed no significant changes with D6D hyperactivity or inhibition, which may be due to the continuous remodeling and redistribution of FAs from PE onto PC, the FA composition of which reflected the changes D6D activity. On the other hand, the hepatic PE FA composition and D6D indices in the LDLR $^/-$ model showed more significant changes reflective of D6D hyperactivity and inhibition. Since lipid uptake is altered in this model, leading to an accumulation of lipids in the circulation, which may alter hepatic production and secretion of lipids, causing immature VLDL to accumulate in the liver, this may have an inhibitory effect on PEMT activity through product feedback inhibition. Thus, the “trapped” PE and PC in the liver maintain their FA composition longer with minimal remodeling or redistribution of FAs from PE onto PC. This hypothesis was also supported by the PC/PE ratio which did not vary significantly across groups in the LDLR $^/-$ model, except in the LDLR-WD group in which the PC/PE ratio increased. In the ob/ob model, the PC/PE ratio increased in the disease group, accompanied by an increase in D6D indices, and decreased with D6D inhibition.

Another difference between the two models was in the levels of hepatic free AA, which were higher in the LDLR-WD group, indicating an increase in AA hydrolysis and liberation from hepatic membranes, while in the ob/ob model, there were no significant changes in free AA levels. The high free AA levels in the liver may
indicate a higher degree of inflammation in the LDLR−/− model, but also suggests that there may be differences in the activity of other enzymes in these two models, such as PLA-2 and AT, which are not yet fully understood.

Furthermore, while the ob/ob mice may serve as good models for NAFLD due to increased hepatic lipid storage, they may not be as useful in studying NASH, due to their inhibited immune responses as a result of leptin deficiency, which would reduce the incidence of inflammation [98]. For example, serum MCP-1 levels showed no significant differences across groups and TNF-α levels were not detected in the ob/ob model [13], while in the LDLR−/− model there was a modest increase in serum MCP-1 levels in disease, suggesting a possible increase in systemic inflammation in disease.

The LDLR−/− model may also provide a more clinically relevant model when fed a high-fat diet [99-100], as leptin deficiency is a rare condition in humans. There is an increase in the secretion of lipoproteins, such as apoB [81], in LDLR−/− mice, while lipoprotein degradation is impaired, leading to a decrease in lipoprotein clearance [100]. Thus, in addition to serving as useful models for hypercholesterolemia and atherosclerosis, and contrary to the ob/ob models, LDLR−/− mice are also useful in studying NASH [100], since their immune responses are not impaired. However, it should be taken into consideration that the congestion of lipids in the circulation and other tissues may alter the normal or expected (patho)physiology with regards to lipid metabolism and homeostasis in the LDLR−/− model. These differences should thus be considered when selecting a research model of CMD.
6.0 Conclusion and Future Prospects

In agreement with clinical and animal studies in the literature, obesity and a high-fat diet result in elevated D6D activity, which may contribute to the development of CMD and its pathogenesis via the increased production of AA, which is further metabolized into proinflammatory eicosanoids, or by other mechanisms, such as its link with PEMT activity. Though the mechanism of the interaction between D6D and PEMT remains unclear, the results of this study suggest that it may be bidirectional, where D6D may influence PEMT activity, in addition to the reported effects of PEMT on D6D activity.

This study demonstrates that D6D inhibition via the pharmacological action of SC-26196 has broad effects on PL metabolism and membrane composition, in addition to its effects on AA production, all of which appear to play an important role in the pathogenesis of CMD by complex interactions with multiple systems that merit further investigation.

Increased AA production and AA-derived eicosanoids have been implicated in the development of inflammatory diseases, CMD and cancer [71, 73, 101, 110]. Much effort has been dedicated to the selective inhibition of eicosanoid pathways or individual eicosanoid species to alleviate inflammation and reverse disease [74]. An advantage of the SC-26196 D6D inhibitor drug is its upstream position in the metabolic pathways leading to eicosanoid production by inhibiting the production of AA itself rather than the downstream inhibition of AA metabolites. Thus, targeted inhibition of D6D has been suggested to be of therapeutic value [57, 101].

Future investigations should include quantitation of inflammatory proteins, such as PJNK, TNF-α, and liver F4/80 (macrophages cell-surface antigen), as well as of insulin signaling molecules, such as IRS and PIRS, by means of immunoblotting and/or gene expression, to help determine the mechanisms involved in the development of inflammation and IR in states of D6D hyperactivity vs. inhibition. Radioactive labeling and in vivo tracing of dietary LA may also help to identify the fate of its derivatives. For example, in this study, despite reductions in PL LA in
disease models, indicating increased D6D activity, and elevations in LA with D6D inhibition, the utilization and fate of LA derivatives, especially AA, were not clear and require further investigation. Furthermore, immunoblotting for apoB lipoprotein in the liver may be useful in further studying the effect of D6D on PEMT activity and hepatic VLDL release.

Large families of PLAs and AT enzymes mediate remodeling of PLs with differing FA and PL substrate specificities that are only minimally understood. Therefore, further study will be required to determine how these enzymes interact with D6D and PEMT to influence the FA composition and fates of PE and PC in health and disease.

A third model currently under investigation is the fads2-overexpressor, a transgenic mouse model in which the fads2 gene encoding for D6D is over-expressed, resulting in elevated D6D activity in comparison to non-transgenic WT mice (A. Chicco; unpublished data). This model serves to further examine the role of D6D activity in CMD and to monitor the development of the metabolic syndrome. Preliminary data in this model fed a WD indicate the presence of glucose intolerance compared to WT mice fed the same diet. Analyses of serum and hepatic PL composition, D6D indices and hepatic PC/PE ratio in these models may shed new light or further support the hypotheses of this study.
## Tables

### Table 1. HPLC mobile phases

<table>
<thead>
<tr>
<th>Solvent A</th>
<th>Hexane</th>
<th>Isopropanol</th>
<th>0.3 mM potassium acetate (pH 7.0)</th>
<th>Acetic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>42.4</td>
<td>56.6</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>300ml</td>
<td>127.2</td>
<td>169.8</td>
<td>3</td>
<td>0.03</td>
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<td>Isopropanol</td>
<td>5 mM potassium acetate (pH 7.0)</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>100%</td>
<td>38.6</td>
<td>51.4</td>
<td>10</td>
<td>0.1</td>
</tr>
<tr>
<td>300ml</td>
<td>115.5</td>
<td>154.5</td>
<td>30</td>
<td>0.3</td>
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### Table 2. Shorthand designation of fatty acids and their trivial names

<table>
<thead>
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<th>Shorthand designation</th>
<th>Trivial Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>Palmitic acid</td>
</tr>
<tr>
<td>16:1 (n-7)</td>
<td>Palmitoleic acid</td>
</tr>
<tr>
<td>18:0</td>
<td>Stearic acid</td>
</tr>
<tr>
<td>18:1 (n-9)</td>
<td>Oleic acid</td>
</tr>
<tr>
<td>18:1 (n-7)</td>
<td>Vaccenic acid</td>
</tr>
<tr>
<td>18:2 (n-6)</td>
<td>Linoleic acid (LA)</td>
</tr>
<tr>
<td>18:3 (n-3)</td>
<td>α-linolenic acid (ALA)</td>
</tr>
<tr>
<td>20:3 (n-6)</td>
<td>Dihomo-γ-linolenic acid (DGLA)</td>
</tr>
<tr>
<td>20:4 (n-6)</td>
<td>Arachidonic acid (AA)</td>
</tr>
<tr>
<td>20:5 (n-3)</td>
<td>Eicosapentaenoic acid (EPA)</td>
</tr>
<tr>
<td>22:5 (n-3)</td>
<td>Docosapentaenoic acid</td>
</tr>
<tr>
<td>22:6 (n-3)</td>
<td>Docosahexaenoic acid (DHA)</td>
</tr>
</tbody>
</table>
Table 3. Triglycerides assay standards dilution

<table>
<thead>
<tr>
<th>No.</th>
<th>Standard + diH₂O</th>
<th>Vol (μL)</th>
<th>Triglyceride (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 μl + 990 μl</td>
<td>1000</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>6 μl + 994 μl</td>
<td>1000</td>
<td>0.6</td>
</tr>
<tr>
<td>3</td>
<td>3 μl + 997 μl</td>
<td>1000</td>
<td>0.3</td>
</tr>
<tr>
<td>4</td>
<td>0 μl + 1000 μl</td>
<td>1000</td>
<td>0</td>
</tr>
</tbody>
</table>
**Figure 1. Hypothesized links between D6D, PEMT, and CMD.** D6D catalyzes the rate limiting step in the conversion of LA into AA and ALA into DHA. The WD is predominant in LA, and the AA is converted into pro-inflammatory eicosanoids via the LOX and COX pathways, leading to inflammation and development of CMD. The AA and DHA produced via the D6D pathway are incorporated onto PE, which, via PEMT, is converted into PC. The methyl group required for the conversion of PE to PC is supplied by the methionine-homocysteine cycle. The resulting PC-DHA/AA molecules are incorporated into cell membranes, secreted into the bile, and represent a main component of lipoproteins, such as VLDL. Factors which increase D6D activity/expression include the consumption of a WD, hyperinsulinemia, obesity, and some SNPs in the *fads2* gene. Inhibition of D6D can be achieved by the pharmacological action of SC-26196.

AA: arachidonic acid; AdoHcy: S-adenosylhomocysteine; AdoMet: S-adenosylmethionine; ALA: alpha-linolenic acid; CMD: cardiometabolic disease;
COX: cyclo-oxygenase enzyme; D6D: delta-6-desaturase; DHA: docosahexaenoic acid; Hcy: homocysteine; LA: linoleic acid; LOX: lipoxygenase enzyme; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PEMT: phosphatidylethanolamine N-methyltransferase; SNPs: single nucleotide polymorphisms; WD: Western diet.
Figure 2. Pathophysiology linking obesity with inflammation and CMD.
Increased adiposity results in the release of inflammatory cytokines and chemokines which induce local inflammation and IR. Through the portal circulation, these inflammatory mediators and free FAs are transported from the adipose tissue to the liver, leading to the development of fatty liver, hepatic inflammation and IR. Both the adipose tissue and the liver release proinflammatory and pro-atherogenic mediators into the systemic circulation, ultimately leading to the development of systemic IR and CVD due to hyperlipidemia and atherosclerosis [3].
**Figure 3. Insulin signaling** (adapted from Saltiel, A.R. et al., 2001 [25]). Glucose binds to the insulin receptor which undergoes autophosphorylation, initiating a series of phosphorylation cascades which include IRS, PI3-K, and Akt proteins, ultimately leading to the translocation of GLUT4 to the cell membrane and cellular uptake of glucose.

Akt: protein kinase B (PKB); IRS: insulin receptor substrate; PI3-K: phosphoinositide 3-kinase; PKC: protein kinase C; PP1: protein phosphatase 1; PTP1β: protein tyrosine phosphatase 1β.
Figure 4. Link between metabolic stress, inflammatory signaling and insulin signaling pathways [22]. Nutrient overload leads increased adiposity and the release of cytokines, chemokines and free fatty acids by the adipose tissue and macrophages. These molecules activate serine-threonine kinase pathways, which lead to the inactivation of IRS proteins, thereby altering insulin signaling. Activated JNK and IKKβ also activate transcription factors, increasing expression of inflammatory genes. The metabolic stress induced by increased adiposity also activates JNK and IKKβ through oxidative and ER stress.

Akt: protein kinase B; DAG: diacylglycerol; ER: endoplasmic reticulum; FFAs: free fatty acids; IKKβ: inhibitor of nuclear factor κB kinase-β; IL: interleukin; IR: insulin receptor; IRS: insulin receptor substrate; JNK: Jun N-terminal kinase; TNF-α: tumor necrosis factor-α; MCP-1: monocyte chemoattractant protein-1; PKC θ: protein kinase C- θ.
Figure 5. Triglyceride and phospholipid structure and fatty acid nomenclature [45]. The triglyceride is composed of the fully saturated stearic acid (18:0) in the sn-1 position, the mono-unsaturated oleic acid (18:1 n-9) in the sn-2 position, showing the location of the double bond at C9 from the methyl end of the acyl chain; and the poly-unsaturated linoleic acid (18:2 n-6) at the sn-3 position, showing the location of its two double bonds, the first being at C6 from the methyl end of the acyl chain. The phospholipid is composed of the saturated palmitic acid (16:0) in the sn-1 position and poly-unsaturated docosahexaenoic acid (22:6 n-3), showing the positions of its six double bonds the first of which is at C3 from the methyl end of the acyl chain.
LA (18:2 n-6) and ALA (18:3 n-3) are essential FAs which cannot be synthesized by the body and are supplied in the diet. The first step in both pathways is the rate limiting step catalyzed by the same D6D enzyme. The most significant derivatives of both pathways include AA (20:4 n-6), EPA (20:5 n-3) and DHA (22:6 n-3), all of which are further metabolized via COX and LOX pathways into eicosanoids. The eicosanoids derived from AA are considered proinflammatory, while those derived from EPA and DHA are considered anti-inflammatory.

5-HPEPE: 5-hydroperoxyeicosapentaenoic acid; 5-HPETE: 5-hydroperoxyeicosatetraenoic acid; AA: arachidonic acid; ALA: α-linolenic acid; COX: cyclo-oxygenase; DHA: docosahexaenoic acid; Elovl: elongase; EPA: eicosapentaenoic acid; LA: linoleic acid; LOX: Lipoxygenase; LT: leukotriene; PG: prostaglandin; TX: thromboxane.
Figure 7. Chemical structure of the delta-6-desaturase inhibitor SC-26196 [101].
Figure 8. Biosynthesis of eicosanoids from arachidonic acid [71]. Stress and inflammatory stimuli lead to the hydrolysis of AA from cell membranes by PLA-2. The free AA is then further metabolized via COX, LOX and CYP pathways into eicosanoids. Products of the COX-1 and COX-2 pathways include PGs, while those of the 5-, 12- and 15-LOX enzymes include HETEs. CYP products include HETEs and epoxides.

AA: arachidonic acid; COX: cyclo-oxygenase; HETEs: hydroxyeicosatetraenoic acids; HPETEs: Hydroperoxyeicosatetraenoic acid; LO: lipoxygenase; LT: leukotriene; LX: lipoxin; PGs: prostaglandins; PLA-2: phospholipase A-2; TX: thromboxane.
Figure 9. Biosynthesis of phosphatidylcholine via the CDP-choline and PEMT pathways [81]. The PEMT pathway involves the conversion of PE into PC via three methylation steps. The methyl donor is AdoMet derived from the methionine cycle. AdoHcy: S-adenosylhomocysteine; AdoMet: S-adenosylmethionine; CDP-choline: cytidine diphosphate-choline; CK: choline kinase; CPT: choline phosphotransferase; CT: CTP:phosphocholine cytidylyltransferase; DAG: diacylglycerol; PC: phosphatidylcholine; PDME: phosphatidyldimethylethanolamine; PE: phosphatidylethanolamine; PEMT: phosphatidylethanolamine N-methyl transferase; PMME: phosphatidylmonomethylethanolamine.
Figure 10. The conversion of PE into PC via PEMT and its link with the methionine cycle. Each AdoMet molecule derived from methionine donates one methyl group. PEMT utilizes three methyl groups from three AdoMet molecules in order to convert PE into PC.

Figure 11. Total serum PL FA composition in control (con), ob/ob (ob), and ob/ob + SC-26196 (obsc) groups (n = 15: 3 con, 6 ob, 6 obsc)

Figure 12. Total serum PL FA composition in control, ob, and obsc groups, highlighting PL content of LA (18:2), AA (20:4) and DHA (22:6) FAs (n = 15: 3 con, 6 ob, 6 obsc)
Figure 13. The serum AA/LA ratio in control, ob, and obsc groups, as a measure of D6D activity (n = 15: 3 con, 6 ob, 6 obsc)

Figure 14. The serum DGLA/LA ratio in control, ob, and obsc groups, as a measure of D6D activity (n = 15: 3 con, 6 ob, 6 obsc)
Figure 15. Total liver PL FA composition in control, consc, ob, and obsc groups (n = 14: 3 con, 3 consc, 4 ob, and 4 obsc)

Figure 16. Total liver PL FA composition in control, consc, ob, and obsc groups, highlighting PL content of LA (18:2), AA (20:4) and DHA (22:6) FAs (n = 14: 3 con, 3 consc, 4 ob, and 4 obsc)
Figure 17. The hepatic AA/LA ratio in control, consc, ob, and obsc groups, as a measure of D6D activity (n = 14: 3 con, 3 consc, 4 ob, and 4 obsc)

Figure 18. The hepatic DGLA/LA ratio in control, consc, ob, and obsc groups, as a measure of D6D activity (n = 14: 3 con, 3 consc, 4 ob, and 4 obsc)
Figure 19. Free AA levels in livers of control, ob and obsc groups expressed in ng/mg liver tissue (n = 6: 2 con, 2 ob, 2 obs)

Figure 20. PGD2, 5-HETE, 12-HETE and 15-HETE eicosanoids in livers of control, ob and obsc groups expressed in pg/mg liver tissue (n = 6: 2 con, 2 ob, 2 obsc)
Figure 21. PGD2, 5-HETE, 12-HETE and 15-HETE eicosanoids in livers of control, ob and obsc groups expressed relative to control (100%), (n = 6: 2 con, 2 ob, 2 obsc)

Figure 22. The hepatic PC/PE ratio in control, consc, ob and obsc groups expressed in pg/mg protein (n = 26: 9 con, 9 ob, 8 obsc)
Figure 23. The hepatic PE FA composition in control, ob, and obsc groups (n = 25: 5 con, 12 ob, 8 obsc)

```
Figure 24. The hepatic PE FA composition in control, ob, and obsc groups, highlighting PE content of LA (18:2), AA (20:4) and DHA (22:6) FAs (n = 25: 5 con, 12 ob, 8 obsc)
```
Figure 25. The hepatic PE AA/LA ratio in control, ob, and obsc groups, as a measure of D6D activity (n = 25: 5 con, 12 ob, 8 obsc)

Figure 26. The hepatic PE DGLA/LA ratio in control, ob, and obsc groups, as a measure of D6D activity (n = 25: 5 con, 12 ob, 8 obsc)
Figure 27. The hepatic PC FA composition in control, ob, and obsc groups (n = 25: 5 con, 12 ob, 8 obsc)

Figure 28. The hepatic PC FA composition in control, ob, and obsc groups, highlighting PC content of LA (18:2), AA (20:4) and DHA (22:6) FAs (n = 25: 5 con, 12 ob, 8 obsc)
Figure 29. The hepatic PC AA/LA ratio in control, ob, and obsc groups, as a measure of D6D activity (n = 25: 5 con, 12 ob, 8 obsc)

Figure 30. The hepatic PC DGLA/LA ratio in control, ob, and obsc groups, as a measure of D6D activity (n = 25: 5 con, 12 ob, 8 obsc)
Figure 31. Total serum PL FA composition in control, LDLR-WD, and LDLR-WD + SC-26196 (LDLR-WDSC) groups (n = 9: 3 con, 3 LDLR-WD, and 3 LDLR-WDSC)

Figure 32. Total serum PL FA composition in control, LDLR-WD, and LDLR-WDSC groups, highlighting PL content of LA (18:2), AA (20:4) and DHA (22:6) FAs (n = 9: 3 con, 3 LDLR-WD, and 3 LDLR-WDSC)
Figure 33. The serum AA/LA ratio in control, LDLR-WD, and LDLR-WDSC groups, as a measure of D6D activity (n = 9: 3 con, 3 LDLR-WD, and 3 LDLR-WDSC)

Figure 34. The serum DGLA/LA ratio in control, LDLR-WD, and LDLR-WDSC groups, as a measure of D6D activity (n = 9: 3 con, 3 LDLR-WD, and 3 LDLR-WDSC)
Figure 35. Total liver PL FA composition in control, consc, LDLR-WD, and LDLR-WDSC groups (n = 14: 3 con, 3 consc, 5 LDLR-WD, 3 LDLR-WDSC)

Figure 36. Total liver PL FA composition in control, consc, LDLR-WD, and LDLR-WDSC groups, highlighting PL content of LA (18:2), AA (20:4) and DHA (22:6) FAs (n = 14: 3 con, 3 consc, 5 LDLR-WD, 3 LDLR-WDSC)
Figure 37. The total liver AA/LA ratio in control, consc, LDLR-WD, and LDLR-WDSC groups, as a measure of D6D activity (n = 14: 3 con, 3 consc, 5 LDLR-WD, 3 LDLR-WDSC)

Figure 38. The total liver DGLA/LA ratio in control, consc, LDLR-WD, and LDLR-WDSC groups, as a measure of D6D activity (n = 14: 3 con, 3 consc, 5 LDLR-WD, 3 LDLR-WDSC)
Figure 39. Free AA levels in livers of control, LDLR-WD and LDLR-WDSC groups expressed in ng/mg liver tissue (n = 6: 2 con, 2 LDLR-WD, 2 LDLR-WDSC)

Figure 40. PGD2, 5-HETE, 12-HETE and 15-HETE eicosanoids in livers of control, LDLR-WD and LDLR-WDSC groups expressed in pg/mg liver tissue (n = 6: 2 con, 2 LDLR-WD, 2 LDLR-WDSC)
Figure 41. PGD2, 5-HETE, 12-HETE and 15-HETE eicosanoids in livers of control, LDLR-WD and LDLR-WDSC groups expressed relative to control (100%) (n = 6: 2 con, 2 LDLR-WD, 2 LDLR-WDSC)

Figure 42. The hepatic PC/PE ratio in control, consc, LDLR-WD and LDLR-WDSC groups expressed in pg/mg protein (n = 16: 9 con, 5 LDLR-WD, 2 LDLR-WDSC)
Figure 43. The hepatic PE FA composition in control, LDLR-WD, and LDLR-WDSC groups (n = 12: 5 con, 4 LDLR-WD, 3 LDLR-WDSC)

Figure 44. The hepatic PE FA composition in control, LDLR-WD, and LDLR-WDSC groups, highlighting PE content of LA (18:2), AA (20:4) and DHA (22:6) FAs (n = 12: 5 con, 4 LDLR-WD, 3 LDLR-WDSC)
Figure 45. The hepatic PE AA/LA ratio in control, LDLR-WD, and LDLR-WDSC groups as a measure of D6D activity (n = 12: 5 con, 4 LDLR-WD, 3 LDLR-WDSC)

Figure 46. The hepatic PE DGLA/LA ratio in control, LDLR-WD, and LDLR-WDSC groups as a measure of D6D activity (n = 12: 5 con, 4 LDLR-WD, 3 LDLR-WDSC)
Figure 47. The hepatic PC FA composition in control, LDLR-WD, and LDLR-WDSC groups (n = 12: 5 con, 4 LDLR-WD, 3 LDLR-WDSC)

Figure 48. The hepatic PC FA composition in control, LDLR-WD, and LDLR-WDSC groups, highlighting PC content of LA (18:2), AA (20:4) and DHA (22:6) FAs (n = 12: 5 con, 4 LDLR-WD, 3 LDLR-WDSC)
Figure 49. The hepatic PC AA/LA ratio in control, LDLR-WD, and LDLR-WDSC groups as a measure of D6D activity (n = 12: 5 con, 4 LDLR-WD, 3 LDLR-WDSC)

Figure 50. The hepatic PC DGLA/LA ratio in control, LDLR-WD, and LDLR-WDSC groups as a measure of D6D activity (n = 12: 5 con, 4 LDLR-WD, 3 LDLR-WDSC)
Figure 51. Serum MCP-1 standard curve

Figure 52. Serum MCP-1 levels in control, consc, LDLR-WD, and LDLR-WDSC groups, expressed relative to control (100\%). (n = 10: 2 con, 3 consc, 3 LDLR-WD, 2 LDLR-WDSC)
Figure 53. Serum triglycerides standard curve

Figure 54. Serum triglyceride levels in control, consc, LDLR-WD and LDLR-WDSC groups, expressed relative to control (100%), (n = 7: 3 con, 1 consc, 1 LDLR-WD, 2 LDLR-WDSC)
Figure 55. Total cholesterol, HDL, and LDL/VLDL cholesterol in serum of LDLR-WD and LDLR-WDSC groups (n = 2: 1 LDLR-WD, 1 LDLR-WDSC – in duplicates).

Figure 56. Post-treatment blood glucose levels at 2-hours post-injection of glucose [13].
Figure 57. The AA/LA ratio in livers of wild type (WT) and PEMT−/− mice as a measure of D6D activity (A. Chicco; unpublished data).

Figure 58. The DGLA/LA ratio in livers of wild type (WT) and PEMT−/− mice as a measure of D6D activity (A. Chicco; unpublished data).
References


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