MATERNAL OBESITY AND FETAL METABOLIC PROGRAMMING:
IDENTIFYING A ROLE FOR MATERNAL INFLAMMATION AND FETAL LIPID EXPOSURE

by

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Maternal Obesity and Fetal Metabolic Programming: Identifying a Role for Maternal Inflammation and Fetal Lipid Exposure

Thesis directed by Professor Jacob Friedman.

ABSTRACT

Maternal obesity is associated with greater risk of pregnancy complications. Additionally, children born to overweight and diabetic mothers are often large for gestational age, and at increased risk for developing metabolic syndrome later in life. It is thought that the intrauterine environment might be central to these observed outcomes, however, the particular characteristics of this environment remain elusive.

The role of maternal lipids as a major contributor to fetal fat accretion is becoming increasingly recognized, but little is known about 1) the mechanisms regulating lipid transport across the placenta, and 2) how these lipids might impart persistent metabolic change on the fetus. Throughout the following work we address these questions in both human pregnancy and a high-fat diet mouse model of obese pregnancy. Our findings in humans suggest that placental growth and triglyceride hydrolase activity play an important role in regulating lipid flux to the developing fetus. In mice, our findings demonstrate how maternal obesity is complicated by increased maternal inflammation and insulin resistance, leading to placental overgrowth and increased lipid uptake. Further, these lipids appear to be transported to the fetus where they alter metabolic gene pathways and promote fetal
hepatic lipid droplet accumulation. Lastly, we demonstrate that manipulating maternal dietary fatty acid profiles can alter this trajectory using a transgenic mouse model engineered to convert omega-6 fatty acids to omega-3 fatty acids. These mothers demonstrated reduced markers of systemic inflammation and improved insulin sensitivity, despite equivalent weight gain to wild type obese mothers. Additionally, placental growth was normalized and placental and fetal liver lipid exposure reduced. This resulted in either amelioration or complete prevention of adverse metabolic programming outcomes in wild type adult offspring. These results not only help us understand the interplay between pregnancy, diet, and obesity in regards to maternal lipid metabolism and fetal lipid exposure, but also suggest a potential means of protecting the developing fetus. A targeted intervention at the level of maternal and placental lipid metabolism is thus an important frontier in the quest for reducing fetal lipid exposure and the development of metabolic syndrome in children.

The form and content of this abstract are approved. I recommend its publication.

Approved: Jacob Friedman
I dedicate this work to my parents, who always supported me in all my academic pursuits, and instilled in me a desire for lifelong learning.
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<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-Activated Protein Kinase</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginase</td>
</tr>
<tr>
<td>ASM</td>
<td>Acid-soluble metabolites</td>
</tr>
<tr>
<td>AT</td>
<td>Adipose tissue</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CD</td>
<td>Control diet</td>
</tr>
<tr>
<td>CCR2</td>
<td>Chemokine receptor 2</td>
</tr>
<tr>
<td>CPT-1</td>
<td>Carnitine palmitoyltransferase 1</td>
</tr>
<tr>
<td>DEXA</td>
<td>Dual energy X-ray absorptiometry</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexanoic acid</td>
</tr>
<tr>
<td>EFA</td>
<td>Essential fatty acid</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentanoic acid</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FABP</td>
<td>Fatty acid binding protein</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>FADS</td>
<td>Fatty acid desaturase</td>
</tr>
<tr>
<td>FBG</td>
<td>Fasting blood glucose</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>GA</td>
<td>Gestational age</td>
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</table>
GC-MS  Gas chromatography – mass spectrometry
GDM   Gestational diabetes mellitus
GH-V  Placental growth hormone
GPX   Glutathione peroxidase
HFD   High-fat diet
HOMA  Homeostatic model assessment
hPL   Human placental lactogen
IGF   Insulin-like growth factor
IL    Interleukin
iNOS  Inducible nitric oxide synthase
IR    Insulin resistance
ITT   Insulin tolerance test
IUGR  Intrauterine growth restriction
LGA   Large for gestational age
LPL   Lipoprotein lipase
LXR   Liver X-receptor
MCP-1 Monocyte chemotactant protein 1
MUFA  Monounsaturated fatty acid
NAFLD Non-alcoholic fatty liver disease
NASH  Non-alcoholic steatohepatitis
NEFA  Non-esterified fatty acid
OGTT  Oral glucose tolerance test
PEPCK  Phosphoenolpyruvate carboxykinase
PLA2   Phospholipase A2
PPAR   Peroxisome proliferator-activated receptor
PUFA   Polyunsaturated fatty acid
ROS    Reactive oxygen species
RXR    Retinoid X receptor
SCD    Steroyl-CoA desaturase
SEM    Standard error of mean
SGA    Small for gestational age
SOD    Superoxide dismutase
SREBP  Sterol regulatory element binding protein
SVF    Stromal-vascular fraction
TG     Triglyceride
TLR    Toll-like receptor
TNFα   Tumor necrosis factor α
TZD    Thiazolidinedione
VLDL   Very low-density lipoprotein
WT     Wild type
CHAPTER I

INTRODUCTION

1.1 Fetal Metabolic Programming

The emergence of adult metabolic disease ‘epidemics’ in children is an advancing public health concern, with childhood obesity, diabetes, cardiovascular disease and nonalcoholic fatty liver disease (NAFLD) all increasing at alarming rates. While this is partially due to consumption of calorie-dense, nutrient-low foods and sedentary behaviors, an emerging body of evidence also suggests that the ability to respond to metabolic challenges during postnatal life may be linked to environmental influences during fetal development [2-8].

The Developmental Origins of Disease Hypothesis originally posited by Barker et. al. [9,10] has led to extensive research in the effects of fetal under-nutrition, low birth weight and development of chronic metabolic disease in the offspring. However, relatively less is known about the metabolic impact of fetal over-nutrition. Epidemiological studies have revealed strong statistical links between nutritional excess during pregnancy and later development of diseases such as obesity and Type 2 diabetes in adulthood. Most convincing are the studies in Pima Indians, which demonstrated that exposure to a diabetic intrauterine milieu during pregnancy can

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induce a 10-fold increase in the prevalence of diabetes by early adulthood, in comparison to children whose mothers did not develop diabetes until after delivery [11]. While this suggests that maternal hyperglycemia contributes to increased fetal growth and the development of metabolic disorders in offspring [12], recent work suggests that maternal pre gravid weight and serum triglyceride (TG) levels may be a better correlate of excessive fetal growth and adiposity [13-15]. These findings imply that other maternal metabolic factors traditionally associated with non-diabetic obese individuals – including hyperlipidemia, insulin resistance, hyper caloric diets, and low-grade systemic inflammation, may be contributing to the childhood obesity epidemic and its associated metabolic disorders.

The mechanisms whereby maternal obesity and nutrient excess in utero impart increased risk for future metabolic disease are poorly understood, but likely include changes in fetal nutrient exposure in combination with genetic, and epigenetic mechanisms. The in utero environment can substantially modify how the fetal genome is expressed, which may exert stimulatory or inhibitory effects on fetal growth and adiposity. Further, stresses in utero may impair long-term tissue function by altering developmental trajectories, or potentially causing irreversible damage to cells and cellular organelles. Throughout these chapters, we will examine how excess exposure to maternal lipids may impose such changes in fetal tissue function, and identify potential mechanisms for altering this programming trajectory.
1.2 Obesity and Insulin Resistance in Non-Pregnant Individuals

Obesity and pregnancy are independently associated with insulin resistance and inflammatory changes that may be exacerbated when combined with one another. In the non-pregnant state, hypertrophic adipose tissue stores result in reduced uptake and storage of fatty acids along with increased lipolysis, inflammatory cell infiltration and adipokine secretion [16, 17]. Markers of inflammation have been observed in both adipose tissue and liver of obese individuals and rodents [18], including tumor necrosis factor-α (TNF-α), chemokine receptor-2 (CCR2), monocyte chemotactant protein-1 (MCP-1), toll-like receptor-4 (TLR-4), and activated c-Jun N-Terminal Kinase (JNK). More than just a passive storage depot, adipocytes can synthesize, store, and secrete multiple pro-inflammatory cytokines, including interleukin-6 and 8 (IL-6 and IL-8), TNF-α, and MCP-1 [19-21], many of which play an important role in obesity-induced insulin resistance [22, 23]. However, the initiating steps in inflammation, and the mechanisms linking it to insulin resistance and increased lipolysis are still being investigated.

In both humans and animal models of obesity, adipocyte expansion and hypertrophy is associated with an accumulation of adipose tissue macrophages with a pro-inflammatory phenotype. In the obese state, macrophages appear to become polarized towards a more M1 phenotype, whereas the adipose tissue macrophage population in lean animals expresses greater amounts of M2 markers [24]. M1 macrophages are traditionally viewed as being more pro-inflammatory and are important for mounting an immune response via cytokines such as IL-1β, IL-6 and TNF-α. M2 macrophages, or ‘alternatively activated’ macrophages, are important for tissue
repair, remodeling and the resolution of inflammation, and are often characterized by their expression of IL-10 and Arginase. In inflamed, insulin-resistant adipose tissue, the anti-lipolytic effects of insulin are frequently diminished [25,26], resulting in elevated levels of free fatty acids (FFAs). This has given rise to the “expandability hypothesis,” which postulates that limitations in adipose tissue expandability may govern when lipids are stored in adipose tissue vs. supplied to other tissues in the body [27]. Inefficient storage of lipids in adipose tissue can suppress insulin sensitivity both locally and systemically. Locally, FFAs released by the adipocyte can produce a strong pro-inflammatory signal by binding Toll-like Receptors (TLRs) expressed on the cell surface of resident macrophages. TLR signaling induces Nuclear Factor kappaB (NF-κB) [28-31], a transcription factor that up-regulates pro-inflammatory cytokine production, leading to perpetual adipose tissue inflammation and increased insulin resistance. Systemically, pro-inflammatory cytokines and FFAs released by the adipose tissue can impact the liver, skeletal muscle, and brain, impairing whole-body insulin sensitivity, and promoting disease progression [32].

Preventing the initial infiltration of macrophages into adipose tissue, or the activation of resident adipose tissue macrophages, appears to be an important step in reducing the downstream consequences of obesity. For instance, targeted deletion of specific inflammatory genes in bone marrow-derived macrophages disrupts the link between dietary/genetic obesity and insulin resistance [33,34]. Additionally, there is good evidence that weight loss [35,36], diet and exercise [37] and treatment with insulin sensitizing drugs [38] can reduce macrophage infiltration of adipose tissue and decrease
expression of inflammatory markers, which leads to an overall improvement in whole-body insulin sensitivity due to the reduction of pro-inflammatory cytokines and FFAs released systemically. Thus, prevention of obesity-linked inflammation may be an important therapeutic target to reduce the negative impact of obesity on maternal metabolism, and ultimately control the type and level of nutrients available for fetal growth and overall development.

1.3 Maternal Obesity, Fuel Switching, and Fetal Nutrient Supply

Two-thirds of women in the United States are currently overweight or obese at the time of conception [39-41]. Not surprisingly, the increasing prevalence of obesity in pregnant women has led to the suggestion that maternal obesity alone may be a more significant factor than maternal diabetes in perpetuating the overall obesity epidemic [42]. Infants born to obese and/or diabetic mothers are often large for gestational age (LGA, defined as ≥90th percentile for gestational age), demonstrate increased adiposity at birth, and are at increased risk for developing obesity and metabolic syndrome in later life [2,3,43]. While gestational diabetes (GDM) is a known risk factor for LGA and macrosomic births (>4000 grams), the majority of LGA infants are born to mothers with normal glucose levels. In fact, maternal hyperglycemia only accounts for 25% of the differences in birth weight in multivariate models [44,45], which suggests that other factors in addition to maternal-fetal glucose may be important.

Recent work has demonstrated that maternal pre-pregnancy BMI and TG levels also play a significant role in mediating excessive fetal growth [13-15,46-48]. In a
prospective study of offspring born to women with either normal glucose tolerance or GDM, maternal BMI was the strongest perinatal predictor for both overweight at 8 years of age and percentage body fat [3]. Additionally, in multiple cohorts of GDM women with well controlled glucose levels, elevated maternal fasting serum TG and FFAs were independently associated with increased birth weight and neonatal adiposity [15,37].

During pregnancy, maternal metabolism undergoes profound adjustments to meet the nutrient needs of the developing fetus. Early in gestation (the first and early second trimester), maternal insulin sensitivity can actually increase modestly [49], leading to increased maternal adipose tissue lipid storage. During this time period, pregnant women are in an anabolic state and accumulate fat as a result of enhanced lipogenesis and increased adipose tissue lipoprotein lipase (LPL) activity, which hydrolyzes circulating TG for tissue uptake and storage [49,50], resulting in a 3.5-6.0 kilogram increase in fat stores [51]. Lean women increase their fat stores more than obese women per kilogram body weight, which is likely due to higher insulin sensitivity in early pregnancy, promoting lipogenesis and storage [50,52]. However, excess gestational weight gain remains a problem in overweight and obese women [53,54], which may have important implications for excessive fetal growth.

From mid to late gestation, maternal lipid metabolism switches from an anabolic to a catabolic state, concomitant with increasing maternal insulin resistance [55,56]. TG stored in adipocyte lipid droplets are hydrolyzed into FFAs through lipolysis [57], which is initiated by hormone sensitive lipase and inhibited by insulin. The insulin resistant state of the third trimester is reflected by a decrease in adipose tissue LPL activity and
accelerated lipolysis, leading to high levels of circulating FFAs and glycerol, and a marked increase in hepatic very-low-density lipoprotein-triglyceride (VLDL-TG) synthesis, which is further stimulated by the high estrogen levels of pregnancy [58]. The signals responsible for this metabolic switch, from lipid storage in early pregnancy to lipid mobilization in late gestation, are not well understood; however placental hormones that increase with advancing gestation and are known to induce maternal insulin resistance, such as human placental lactogen (hPL) and placental growth hormone (GH-V), may play a major role [59-61].

The high levels of TG in maternal circulation with advancing gestation may create a steep concentration gradient across the placenta, which accelerates their transport and deposition in fetal tissues [62]. Therefore, the hypertriglyceridemia facilitated by the insulin resistance of obese and diabetic mothers are potential factors to enhance substrate availability to the fetus. Interestingly, in women with well controlled GDM, third trimester FFAs and TG were better predictors of neonatal fat mass than glucose [15]. This corresponds with data on placental gene expression, which shows that 67% of differentially expressed genes in women with Gestational or Type I Diabetes were related to lipid transport pathways, and only 9% to glucose transport pathways [63]. In term human trophoblasts, insulin and fatty acids have been shown to enhance the expression of adipophilin, which is associated with cellular lipid droplets and implicated in cellular fatty acid uptake and storage of neutral lipids [64], although whether adiponectin is made in intact placenta remains controversial. Additionally, higher circulating levels of insulin may serve to up-regulate placental proteins involved in lipid
transport, thereby increasing availability of fatty acids to both the placenta and the fetus. Consistent with this concept, placental trophoblast LPL activity was shown to be stimulated by hyperinsulinemic and hyperglycemic conditions, thus increasing hydrolysis of maternal lipoproteins for transport across the placenta [65].

Although fatty acids are not readily oxidized in the fetus, essential fatty acids are critical for normal fetal development, as well as the deposition of large amounts of infant body fat [66]. The human placenta is capable of transporting FFA by diffusion, and selectively increases the transport of essential fatty acids (EFA) and their long-chain polyunsaturated fatty acid derivatives (LC-PUFA) by fatty acid transporters and fatty acid binding proteins (FABPs), thereby creating a higher concentration in the fetus than in the mother [67,68]. Further, placental expression of lipoprotein receptors and receptor-related proteins allows maternal lipoproteins such as VLDL and dietary chylomicrons (CM) to be taken up by the placenta, where they must be hydrolyzed by placental LPL, or a second lesser-known placental TG hydrolase [51,66]. Additionally, the placenta expresses phospholipase A2 (PLA2), and other intracellular lipase activities to hydrolyze mono-, di-, and triacylglycerols to FFAs that can be utilized by the placenta or enter into fetal circulation. The activity of placental lipases, especially placental LPL, increases from the first to the third trimester [69], again supporting an enhanced fetal need for maternal FFA in late gestation.

Interestingly, maternal obesity appears to affect the placenta much as it does adipose tissue, in that placentas from obese mothers demonstrate increased expression of pro-inflammatory cytokines and a marked accumulation of a heterogeneous
macrophage population [70]. Additionally, placentas from obese (body fat >16%) compared to lean (body fat <8%) neonates showed a significant increase in expression of the PLA2 genes PLA2G2A and PLA2G5 (the main placenta phospholipases) as well as leptin and TNFα [71]. How this might impact placental function is not yet clear, however activation of phospholipase activity suggests inflammation may be one mechanism by which excess fat accumulates in obese neonates. Further, IL-6 and TNFα are thought to be involved in the regulation of fetal growth via modulation of both the expression and activity of the system A but not L amino acid transporters [72].

In conclusion, fetal lipid supply is regulated by both maternal circulating lipid concentrations and by the extent to which they are transported by the placenta. Maternal obesity likely impacts fetal lipid exposure, but the mechanisms by which this can alter fetal and later offspring development are still unknown. However, the ability of lipids to activate cell signaling pathways and serve as ligands for nuclear receptors suggests that aberrant lipid exposure in utero could potentially lead to the alternative regulation of multiple gene expression programs. Which programs become activated and/or inhibited is not yet known, but potential targets will be discussed in more detail below, and investigated in later chapters.

1.4 Mechanisms for Fetal Metabolic Programming by Maternal Lipids

Previous studies of maternal obesity and high-fat diet in multiple animal models provide evidence of metabolic abnormalities in the fetus, neonate, and adult offspring [73-77]. These include increased body weight and fat mass at birth and in adulthood,
reduced insulin sensitivity, increased blood glucose and cholesterol levels, increased blood pressure, reduced muscle mass, and increased lipid deposition in the fetal and adult liver. These results could be explained by an early metabolic or a potential epigenetic programming event, but mechanistic data such as the activation or inhibition of gene regulatory pathways is currently lacking. In utero exposure to excess maternal lipids likely impacts numerous pathways in developing organs such as the liver, which is the first tissue to receive the majority of post-placental nutrients, as well as other key metabolic organs such as the skeletal muscle, adipose tissue, brain, and pancreas.

The fetal liver develops early in pregnancy, beginning at week four of gestation with the generation of the hepatic bud from the ventral endoderm [78,79]. Gross hepatic morphogenesis is completed by the end of the first trimester, however, more refined cellular determination continues throughout gestation. The hepatic bud is populated by bi-potential hepatoblasts, which differentiate into mature hepatocytes or cholangiocytes that can be further refined to achieve unique cellular phenotypes [80]. As reviewed elsewhere, a vast array of genes and their transcriptional regulators are involved in the development of hepatocyte metabolic processes, including gluconeogenesis, glycogenolysis, lipid oxidation, and de novo lipogenesis [80,81], however the majority of genes underlying these processes are not highly expressed until after birth. Adding to the complexity, the liver is the primary location of hematopoietic development from week six to twenty-one of gestation, and hematopoietic stem cells account for 60 percent of total liver mass during peak hematopoiesis followed by regression to the fetal bone marrow by term [82]. Thus, the developing fetal liver is
constantly in flux over most of gestation, with large changes in cell determination and population, as well as more refined changes in cellular metabolic phenotypes.

Lipids and their pro- or anti-inflammatory derivatives can serve as transcriptional activators of multiple nuclear receptors, including the Liver X Receptor (LXR) and Peroxisome Proliferator-Acivated Receptor (PPAR) families. Interestingly, the main genetic regulators of lipid metabolism are themselves regulated by lipid exposure, as well as by inflammatory cues [83]. In the case of LXR, maternal intake of an LXR agonist led to fetal hepatic LXR activation, resulting in increased fetal liver lipogenesis [75,84,85]. In utero exposure to excess maternal lipids could impact a number of gene pathways of metabolic importance, including those for energy storage, oxidation, growth, death, differentiation, and inflammation. A number of these pathways will be reviewed below.

In rodents, maternal high-fat diet results in persistent lipid accumulation in adult offspring livers, even in the absence of post-weaning high-fat diet exposure [86,87]. This could suggest a more permanent programming effect on the liver by maternal diet, although the development of postnatal obesity may be a confounding factor.

Development of NAFLD in humans is associated with increased expression of genes associated with de novo lipogenesis such as SREBP1c, ACC, FAS, SCD1, and LXRα [88,89], as well as a decrease in expression of genes associated with hepatic fatty acid oxidation such as PPARα, CPT-1, and mitochondrial matrix proteins [89]. In a rodent model, adult offspring from high-fat fed dams demonstrated both impaired hepatic
mitochondrial metabolism and enhanced lipogenic gene expression concomitant with the development of NAFLD [90].

In addition to increasing TG storage and impairing cellular lipid oxidation, increased fetal lipids may favor formation of adipocytes over myocytes or other cell types during early organogenesis. In a sheep model of maternal over-nutrition, fetal skeletal muscle at day 60 (out of 142 days of gestation) showed small but significantly reduced fiber numbers and increased intramuscular adipocyte numbers [91]. These changes were associated with increased NF-κB activation, decreased AMPK signaling – an activator of lipid oxidation, and increased PPARγ expression – a key adipogenic transcription factor [92].

Maternal over-nutrition has also been shown to increase fetal adipose tissue expression of PPARγ, leptin and adiponectin, suggesting enhanced adipogenesis [93]. In adults, the targeted activation of PPARγ through the use of thiazolidines (TZDs, PPARγ agonists) can greatly improve insulin sensitivity by promoting adipocyte lipid storage and reducing levels of circulating free fatty acids [94], however, early activation of PPARγ or its downstream targets could promote the storage of excess lipids at the expense of oxidative pathways. In a recent mouse study, maternal exposure to PPARγ-agonists led to induction of fetal mesenchymal stem cells along the adipocyte lineage, and a reduction in the osteogenic potential in these cells, resulting in greater fat mass in adult offspring [95]. Thus, the adaptation of stem cell precursors to maternal nutrient supply likely plays an important role in cell fate and the programming of metabolic disease pathways.
It should be noted that results from programming studies in both human and animal models often show gender specificity in both the degree and type of metabolic alteration observed across tissues and species [73,96-101]. For example, the expression of diabetes in a number of animal models is sexually dimorphic and has been associated with altered hepatic metabolism. In a rat model of maternal protein restriction and intrauterine growth restriction (IUGR), only male offspring demonstrated increased incidence of type 2 diabetes with altered hepatic enzyme profiles including increased PEPCK activity [102]. Similarly, perturbations induced by bilateral uterine
artery ligation in the rat can induce IUGR, and these animals undergo a period of normalcy, followed by a male-specific alteration in hepatic fatty acid metabolism and gene expression that contributes to adult dyslipidemia [103]. Recent studies also suggest gender differences in the placenta in response to nutrients [104]. Because the placenta is a fetal organ, this suggests that gender differences in placental adaptation may impart sexually dimorphic programming outcomes even prior to birth. Thus, our studies will take both fetal and adult offspring sex into account when evaluating programming outcomes.

In addition to a gender-specific effect, an additional caveat is that metabolic programming has global and measured effects across multiple organs. Thus, it becomes difficult to assign the early origins of these disease pathways to a single maternal nutrient and a single organ when examining animal models, particularly during post-natal life. This argues strongly for studies in both genetically defined mice, as presented here, and in large animal models, such as our lab’s obese pregnancy primate model, with an emphasis on maternal and fetal analyses, when looking for early origins of disease.

1.5 Clinical Interventions to Reduce Fetal Lipid Exposure

Given the multiple metabolic gene pathways that may be targeted by excess fetal lipid exposure, the inevitable next question is how do we reverse the program? Much initial research has focused on fetus-specific therapies, including maternal antioxidant supplementation and the use of methyl donors with the intention of altering fetal
signaling pathways and the epigenetic programming of gene expression. Of course, such therapies also impact the mother, a fact that is too often ignored. Further, while epigenetic marks are more plastic during early developmental windows, and are traditionally maintained with differentiation, they are still inherently dynamic. Nonetheless, treatments designed to alter the activity of DNA and histone modifiers are currently being investigated for their utility in correcting epigenetic dysregulation [105]. For example, studies using mice expressing the Agouti allele, which is known to have variable expression due to differential methylation [106], have shown altered offspring gene expression when the maternal diet is supplemented with methyl-donors, leading to differential offspring phenotypes [107,108].

However, given that none of these therapies are specifically targeted, perhaps the more attractive approach to prevent the adverse fetal metabolic programming associated with maternal obesity is at the source; that is, prevention of initial maternal inflammation, insulin resistance, and hyperlipidemia. As maternal BMI remains the most often cited clinical correlate for newborn adiposity, there are many potential interventions, in addition to weight loss, that might impact fetal growth and development by addressing other metabolic characteristics commonly associated with obesity.

Interventions to decrease maternal lipid availability may be specifically targeted through pharmacologic interventions (niacin, fibrates, insulin) depending on if the excess fatty acids are in the form of Chylomicron-TG, VLDL-TG or FFAs. Niacin has been demonstrated to potently decrease TG in multiple studies involving non-pregnant
individuals by increasing the activity of endothelial LPL and removing chylomicron–TG from plasma, as well as decreasing hepatic TG synthesis and VLDL production. Additionally, fibrates decrease synthesis of VLDL and increase VLDL clearance, and are currently recommended for use in pregnant women with severe hypertriglyceridemia due to the risk of TG-induced pancreatitis in pregnancy [109]. Finally, insulin is known to suppress lipolysis, and the suppression of FFA production by administration of exogenous insulin to pregnant women with well-controlled GDM whose fetuses still exhibited excessive growth is speculated to be the reason why such strategies are effective in decreasing macrosomia [15].

In addition, insulin sensitizers such as Thiazolidines (TZDs) and Metformin may help to increase maternal insulin sensitivity and reduce maternal lipid mobilization. However, both agents are capable of crossing the placenta [110-112], which may have inadvertent consequences on fetal metabolism that are still unclear. Because of this, the best maternal insulin sensitizer may in fact be the simple combination of weight loss and exercise. Unfortunately, this is often complicated by difficulty with patient compliance. Thus additional lifestyle changes, such as diet modification become attractive approaches in lieu of pharmacologic intervention.

1.6 Manipulating Maternal Dietary Fat

Omega-3 (n-3) fatty acids are well recognized for their ability to promote a healthy blood lipid profile and reduce serum markers of chronic inflammation in the non-pregnant population [113-115]. N-3 fatty acids and their counterpart omega-6
(n-6) fatty acids are both polyunsaturated acids (PUFA) that are considered essential in the diet, as they cannot be synthesized de novo. Multiple mechanisms for the anti-inflammatory nature of n-3 PUFA have been suggested, including inhibition of macrophage activation by the binding of G protein-coupled receptor 120 (GPR120) [9], and the potent inflammation-resolving effects of n-3 PUFA derived lipid mediators [116]. Still, one of the most straightforward mechanisms for the effects of n-3 fatty acids is their inherent competition with n-6 fatty acids for incorporation into phospholipid membranes where their long chain versions, specifically eicosapentaenoic acid and docosahexaenoic acid (EPA and DHA, n-3 LC-PUFA) compete with arachidonic acid (AA, n-6 LC-PUFA) as substrates for eicosanoid synthesis. While AA is a precursor to many pro-inflammatory eicosanoids, EPA and DHA-derived eicosanoids do not promote such a response, and often have counter-actions to their AA-derived cousins [117,118].

Importantly, PUFA and other fatty acids can differentially impact lipid metabolism pathways. For example, saturated fatty acids appear to activate de novo lipogenesis pathways, while n-3 PUFA repress such pathways and instead turn on genes associated with fatty acid oxidation mainly via alternative regulation of transcriptional activators [119]. Maternal omega-3 supplementation in the form of cod oil decreased maternal circulating TG by about 10% compared to corn oil [120], and such supplements are known to decrease TG in the non-pregnant population.

Aside from transcriptional activation, n-3 fatty acids may also impact lipid metabolism by reducing local inflammation, which, as described earlier, has large-scale
effects on whole body lipid metabolism [119,121]. Because of their complementary actions, and based on the hunter-gatherer dietary patterns of early humans, it is thought that dietary, and hence tissue concentrations of n-3 and n-6 fatty acids should ideally be present at a 1:1 ratio in tissues [122]. However, current Western dietary trends involving increased animal and saturated fat intake are often shifted towards excess n-6 consumption at the expense of n-3. This dietary imbalance may be a partial cause of many current health problems associated with a chronic inflammatory state, including metabolic syndrome and Type 2 Diabetes, and may also alter the intrauterine environment and contribute the programming of childhood obesity.

The impact that a balanced n-3/n-6 tissue fatty acid ratio has on controlling low-grade inflammation is clearly demonstrated in the Fat-1 transgenic mouse model, which is capable of endogenously converting n-6 PUFA to n-3 PUFA via ubiquitous expression of a C. elegans-derived n-3 desaturase under a β-actin promoter [123-125]. The Fat-1 mouse has increased tissue levels of both long and short-chain n-3 PUFA [124] and increased levels of recently identified n-3 PUFA-derived pro-resolving lipid mediators, named resolvins and protectins [126]. This results in reduced inflammation and lower insulin resistance in the context of high-fat feeding [126-128], and protection from tissue damage and disease with other pro-inflammatory challenges [129-134], all without any change in dietary n-3 or n-6 PUFA intake.

Many studies have examined the relationship between increased maternal n-3 PUFA supplementation or dietary intake and maternal and infant outcomes in otherwise healthy women [135-140]; however, none of these studies examined the effect of
re-balancing n-3/n-6 PUFA ratios as an interventional approach to counter the negative effects of obese pregnancy and maternal inflammation on the development of fetal metabolic systems. Further, while studies examining interventions such as maternal antioxidant supplementation [138], maternal metformin treatment [135], or neonatal leptin administration [139] are promising, increasing the maternal n-3/n-6 ratio is particularly attractive given that maternal PUFA, especially long chain n-3 PUFA such as docosahexanoic acid (DHA), are already essential nutrients for the developing fetus [136].

Undoubtedly, there is still much to be learned about the role of maternal fuels, inflammation, and metabolic and oxidative stress on fetal development and gene regulation in obese pregnancy. A better understanding of the mechanisms behind specific nutrient-gene interactions in the context of fetal development will clearly aid in the identification of more targeted and effective means of intervention.

1.7 Hypotheses

Given our current understanding of maternal obesity and fetal metabolic programming, we hypothesize the following:

1) Maternal obesity is associated with increased chronic low-grade inflammation and disordered lipid metabolism, characterized by increased maternal insulin resistance. This results in an excess flux of lipids and pro-inflammatory cytokines to the placenta.
2) Increases in placental lipid uptake and transport lead to excess fetal lipid exposure, and altered programming of fetal metabolic pathways, particularly in the fetal liver.

3) Rebalancing the maternal n-3/n-6 ratio will reduce maternal inflammation and insulin resistance, and correct excess lipid mobilization, thus preventing maternal obesity-associated programming outcomes in the fetus and adult offspring.

In the coming chapters we will seek to address these points, first, by presenting our findings on maternal obesity and placental lipid metabolism, and how they relate to infant adiposity at birth, in an on going clinical study. Second, we will focus specifically on maternal dietary fat using a high-fat fed mouse model of obese pregnancy, identifying changes in maternal and placental inflammation and lipid metabolism in response to high fat diet. Third, we will use this same model to determine how maternal and placental inflammation can drive fetal liver lipid accumulation, and what this means for the differential activation of fetal hepatic gene pathways. Finally, we will utilize the Fat-1 transgenic mouse as a model to demonstrate how re-balancing the maternal n-3/n-6 ratio can improve maternal inflammation and insulin resistance in otherwise obese mothers, and thus mitigate fetal lipid exposure and adverse programming outcomes in the postnatal animal.
CHAPTER II

MATERNAL OBESITY, PLACENTAL LIPID TRANSPORT AND INFANT ADIPOSITY

2.1 Introduction

The incidence of obesity has reached epidemic levels in the United States and in other developed countries. Even more alarming is its increased prevalence among children and young adults. Epidemiological evidence in humans and experimental evidence in animal models demonstrates that infants born to obese and/or diabetic mothers are often large for gestational age, and at much greater risk of developing obesity and other symptoms of metabolic syndrome later in life [141,142]. The maternal intrauterine environment might be central to this disease progression, however the mechanism by which the intrauterine environment induces such developmental patterns remains elusive.

While gestational diabetes is a known risk factor for fetal macrosomia, more than half of all macrosomic births are to mothers without recognized glucose intolerance [44], suggesting maternal glucose is not the only contributor to fetal overgrowth. Further, recent studies in human pregnancy suggest that infant body composition, specifically increased adiposity and intrahepatic fat accumulation [143,144], may be more relevant than birth weight to overall infant health.

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Preliminary findings from our group suggest that elevated maternal triglycerides are a strong predictor of neonatal adiposity. Maternal triglyceride levels are often elevated in cases of pre-gravid maternal obesity, and maternal lipid metabolism differs greatly between lean and obese pregnant women. In lean women, prospective longitudinal studies using hyperinsulinemic-euglycemic clamps and indirect calorimetry demonstrated a net lipogenesis pre-gravid and in early pregnancy (12-14 weeks), but net lipolysis in late gestation (34-36 weeks). However, in obese women under similar experimental conditions, lipogenesis is reduced in early pregnancy compared to lean women, with an earlier shift from the anabolic to catabolic state and a predominance of lipolysis earlier in gestation [145].

As discussed in Chapter I, sub-clinical inflammation and hyperlipidemia may be present prior to pregnancy in obese individuals. Thus, the effects of hormones that promote the insulin resistance of pregnancy, such as placental growth hormone, human placental lactogen, leptin and TNF-\(\alpha\) [146-149], may combine with the low-grade inflammation and insulin resistance of obesity [150] to promote this observed early mobilization of maternal lipid stores.

The role of maternal lipids as a major contributor to fetal fat accretion is becoming increasingly recognized, but little is known about mechanisms regulating lipid transport across the placenta. Placental LPL is thought to be the major lipase involved in the uptake and delivery of maternal TG to the fetus, however its activity in relation to infant adiposity is unstudied. In this chapter, we will investigate the relationship between human placental LPL activity and infant adiposity at birth, and how this relationship
might be affected my maternal obesity and its downstream metabolic complications, including inflammation, insulin resistance, and hyperlipidemia.

2.2: Methods

I was awarded a TL1 fellowship from the Colorado Clinical Translational Sciences Institute (CCTSI) in the fall of 2008. As a part of this fellowship, all awardees are involved in some form of clinical project that relates to their basic science research. Under the mentorship of Dr. Linda Barbour and her many collaborators, I am involved in a clinical study examining the relationship between maternal obesity and infant adiposity. This study involves extensive metabolic characterization of the mother and infant, much of which is beyond the scope of this chapter. My focus here will be our findings as they relate to my specific role in the project: Testing the hypothesis that placental lipoprotein lipase activity is increased in maternal obesity, and contributes to increased infant adiposity at birth.

Patient Recruitment and Sample Collection Timeline

Written informed consent for all study subjects was obtained at the time of recruitment according to protocol approval by the Institutional Review Board at University of Colorado Anschutz Medical Campus (COMIRB). Healthy pregnant women were recruited to the study at 12 - 16 wks gestation. Exclusion criteria included: Age < 18 or > 35 yrs, failure of 3 hr oral glucose tolerance test (OGTT) before 24 weeks
gestation, pre-existing diabetes, multiple gestation, concurrent chronic medical problems including hypertension, HIV, hepatitis B or C, known thrombophilias, history of thromboembolism, rheumatologic disorders, or cardiac dysfunction. Other exclusions included women with a history of intrauterine growth restriction, stillbirth, or placental abruption and patients taking anticoagulants, antihypertensives, β-blockers, or glucocorticoids. All women were studied from the end of the first trimester until 2 days after delivery, and received their care at University of Colorado Hospital (UCH) and UCDAMC outpatient OB clinics, and delivered at UCH.

Figure 2.1: Study design: Patient sample collection.

This timeline describes all patient data collected, with the measures presented in this chapter highlighted in red.
Lean subjects were defined as having a pre-pregnancy BMI < 26 and obese subjects were defined as having a pre-pregnancy BMI > 28. For this chapter, I will discuss data from 18 normal glucose tolerant women (n = 9 lean, 9 obese). This includes 3 separate fasting blood draws at early (12-16 wks), mid (28-30 wks), and late (37 wks) gestation, a maternal adipose tissue biopsy at this mid gestation time point, and data collected from the placenta at time of delivery. Additionally, we use adiposity measurements obtained from newborn infants within 24 hours of delivery.

**Maternal Serum Measurements**

A fasting maternal blood sample was collected at 12-16wks, 28-30 wks, and 37 wks gestation. Serum was processed by the Adult GCRC Core and laboratories associated with the Center for human Nutrition Research, according to standard lab protocols. Maternal serum cytokines were quantitated by the Friedman lab using the Milliplex Human Serum Adipokine Panel (Millipore Corporation, Billerica MA).

**Adipose Tissue Collection**

Gluteal fat biopsies were obtained at 28-30 wks gestation in the fasted state. All subjects were instructed to follow the same recommended diet for 3 days prior to the biopsy. Biopsies were obtained from the lateral flank region to render 2 - 3 gms of adipose tissue. Following local anesthesia with 1% lidocaine, stab incisions were made through which adipose tissue was biopsied by suctioning using Coleman's manual vacuum technique [151]. Biopsied tissue was immediately placed in sterile 1 X
phosphate-buffered saline (PBS), on ice, or flash frozen in liquid nitrogen, depending on subsequent analyses.

**Flow Cytometry for Adipose Tissue Immune Cells**

A subset of the adipose tissue biopsy (2 g or less) was fractioned into adipocytes and stromal cells. Briefly, freshly biopsied adipose tissue was cleaned of visible blood clots, minced to 1 mm cubes, and digested in 20 ml Type I collagenase (Sigma-Aldrich, St. Louis, MO) solution (1 mg/ml collagenase in HBSS + 1 % BSA + 500 nM Adenosine) for 1 hr at 37°C in shaking water bath with 100 rpm agitation. The suspension was then filtered through chiffon mesh and the stromal-vascular fraction was isolated by centrifugation at 800 g for 10 min.

The pelleted stromal fraction was resuspended in red blood cell lysis buffer (155 mM NH₄Cl, 12 mM NaHCO₃, 0.1 mM EDTA) and incubated for 10 min at 4°C, then washed with 1 X PBS containing 5% BSA. For flow analysis, samples and appropriate controls were stained in 100 µl of 1 X PBS/BSA buffer containing 10% human serum. Fluorescently-conjugated primary antibodies (phycoerythrin-CD45, and allophycocyanin-CD14, eBioscience, San Diego, CA) or the respective isotype control IgGs (eBioscience) were added after 10 min of blocking, and incubated with cells for 30 min at 4°C. After staining, cells were washed and fixed for 15 min is 1% paraformaldehyde. Cell data was collected using the FACSCalibur flow cytometer and CellQuest Pro Software (BD Biosciences, San Jose, CA), and analyzed using FlowJo
software (Tree Star Inc., Ashland, OR). Positive cell populations were expressed as percent positive of total cells counted, and adjusted by isotype controls.

Placental Tissue Collection

The lab of Dr. Virginia Winn performed all placental dissection and sample collection. Immediately after term (>37 wks) delivery, placentas were weighed and placed on ice. Placenta villous samples were obtained from the central, medial, and distal regions of the placenta. For gene expression analyses, central villous samples were rinsed in sterile 1 X PBS before freezing in liquid nitrogen. For LPL activity analysis, a villous sample from each region was collected in 1 X PBS and placed on ice for immediate processing, as described below.

Placental RNA Extraction and qPCR

Placental villous RNA extraction and cDNA synthesis was performed by graduate student Diane Gumina. Briefly, frozen central villous samples were placed in RLT Buffer (Qiagen) containing 1 % β-mercaptoethanol and homogenized at 4°C using a Polytron homogenizer. Homogenate was then processed using a QIAshredder (Qiagen), and subsequently extracted using the Qiagen RNeasy Mini kit per manufacturer’s instructions. RNA samples were tested for quality using the Experion (BioRad), and cDNA synthesis was then performed using the iScript cDNA synthesis kit (BioRad).
Real-time quantitative PCR was performed by Rachel Janssen in the Friedman laboratory. Relative gene expression was determined using iQ SYBR Supermix (Bio-Rad, Hercules, CA) with primers provided by IDT PrimeTime qPCR Primers (Integrated DNA Technologies). Primers were designed to be specific for exon regions of human inflammation and lipid transport-related genes: LPL, CD36, FABP-4, IL-1β, IL-6, TNFα, F4/80, iNOS, Arg-1, and Arg-2 (primer sequences - Appendix A). Reactions were run in duplicate on an iQ5 Real-Time PCR Detection System (Bio-Rad) along with a no-template control per gene, and normalized to ribosome protein L13A and ubiquitin C expression using the comparative threshold cycling method.

**Placental Lipoprotein Lipase Activity**

Placenta LPL activity was determined using the methods described by Eckel *et al.* [152]. Briefly, freshly harvested villous samples were minced in ice-cold Krebs-Ringer-phosphate (KRP) buffer (pH 7.4) to 1 – 2 mm³ pieces. Tissue-bound LPL was heparin-released by incubating 100 mg of minced tissue in 0.5 ml of KRP buffer containing 15 µg/ml heparin sulfate for 45 min at 37°C and 100 rpm. Subsequent to brief centrifugation at 4°C, a 100 µl aliquot of supernatant containing the heparin-released enzyme was then removed and incubated with 100 µl of a ¹⁴C-triolein phosphatidylcholine-stabilized substrate for an additional 45 min at 37°C and 100 rpm. The reaction was solubilized and extracted by addition of 3.4 ml of Belfrage solution containing chloroform, methanol and heptane, followed by addition of 0.96 ml of a bicarbonate buffer and agitation. The ¹⁴C-labeled fatty acids were partitioned by
centrifugation for 20 min at 4°C. A 500µl aliquot of the resulting aqueous supernatant containing LPL-released $^{14}$C-FFAs was counted by β-scintillation (LS6000TA; Beckman Coulter, Brea, CA).

$^{14}$C-oleic acid was used to control for extraction efficiency, and individual results were normalized to a heparinized rat plasma internal standard. For each villous region, 2 minced samples were heparin released, and measurement of LPL activity for each heparin-released sample was performed in duplicate. Final villous activity was calculated as the average activity of the central, medial, and distal villous samples. Whole placental LPL activity was estimated by multiplying villous activity by placental weight.

**Anthropometry**

Dr. Melanie Reece performed all infant anthropometric measurements. Infant adiposity at birth was determined by skin fold thickness within 24 hrs of delivery. Briefly, skinfold measurements were performed in triplicate using Harpenden calipers (British Indicators, St Albans, U.K., resolution 0.2mm) at four different sites: triceps, subscapular, suprailiac, and midthigh. Lengths (body and limb), weight, head circumference (HC), abdominal circumference (AC) and limb circumference were also measured. Occipitofrontal (OFC) head, chest, abdomen, mid-thigh and mid-arm circumferences were measured, in triplicate, and measurements plotted on the Lubchenco Growth Chart. Body composition was estimated using the sum of skin folds according to the Dauncey method [153].
**Data Analysis**

Differences between lean and obese women were determined by 2-tailed Student’s t test using Graph Pad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA). When variances were not equal, a 2-tailed t test with Welch’s correction was performed. Correlations were performed for two separate variables using a 2-tailed P-value. All data are expressed as the mean ± standard error of mean (SEM), and differences were determined to be significant at P<0.05. During analysis, gestational age was identified as an independent explanatory variable for villous LPL activity. Further analyses were thus conducted after adjusting villous LPL activity for gestational age using the Fit Model from JMP Pro 9 software (SAS Institute Inc., Cary, NC).

**2.3 Results**

**Characterization of Lean and Obese Mothers**

Lean and obese maternal groups did not differ in maternal age or parity. Additionally, there was no significant increase in incidence of caesarean section, propensity towards male or female infants, or difference in gestational age, all of which could confound data (Table 2.1). While previous groups have demonstrated increased birth weight and placental weight associated with male sex, this was not observed within this cohort. There was, however, an unexpected trend for an increase in the
number of male infants born to obese mothers, but this did not reach significance with such a small sample number.

Table 2.1: Maternal demographics.

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>Parity</th>
<th>Labor/CS</th>
<th>M/F</th>
<th>GA at Del’y</th>
<th>BMI</th>
<th>Weight Gain (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>30.5 ± 0.9</td>
<td>0(6), 1(2), 2(1)</td>
<td>8L / 1CS</td>
<td>5M / 4F</td>
<td>39.8 ± 0.5</td>
<td>22.0 ± 0.4</td>
<td>13.9 ± 0.9</td>
</tr>
<tr>
<td>Obese</td>
<td>29.0 ± 0.7</td>
<td>0(6), 1(2), 2(1)</td>
<td>7L / 2CS</td>
<td>7M / 2F</td>
<td>39.8 ± 0.4</td>
<td>*31.7 ± 1.1</td>
<td>14.4 ± 2.1</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM of n = 9 mothers per maternal group; *P<0.05 vs. Lean.

Despite a significant difference in BMI between lean and obese mothers (P<0.001; Table 2.1), we observed no difference in maternal weight gain (Table 2.1) or serum fasting blood glucose (FBG), insulin, or TG across gestation, with the exception of a slight elevation in FBG in obese mothers at 37wks (P<0.002; Table 2.2). For both groups, fasting insulin and TG levels did significantly increase with advancing gestation, as expected, but the degree of increase was not significantly exacerbated by maternal obesity (Table 2.2).

Table 2.2: Maternal fasting serum.

<table>
<thead>
<tr>
<th></th>
<th>Early Gestation</th>
<th>Mid Gestation</th>
<th>Late Gestation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Insulin</td>
<td>Glucose</td>
<td>TG</td>
</tr>
<tr>
<td>Lean</td>
<td>8.1 ± 0.9</td>
<td>75.7 ± 1.0</td>
<td>86.1 ± 5.7</td>
</tr>
<tr>
<td>Obese</td>
<td>9.9 ± 1.4</td>
<td>80.3 ± 1.3</td>
<td>104.3 ± 8.9</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM of n = 9 mothers per maternal group; *P<0.05 vs. Lean.
In line with this lack of metabolic phenotype differences between lean and obese mothers, there was also no difference in infant birth weight, placental weight, or adiposity between maternal groups (Figure 2.2).

**Figure 2.2: Placenta and Infant characteristics between lean and obese mothers.**

Placentas and infants were weighed at birth. Infant body composition was determined by skin fold measurements collected within 24hrs of delivery. Data are expressed as maternal group mean ± SEM for n = 9 mothers per maternal group.

**Markers of Maternal Obesity-Associated Inflammation**

Maternal adipose tissue was sampled from the upper hip area, anatomically proximal to the lateral anterior side of the abdomen. Although not abdominal or visceral fat per se, it is thought to have similar metabolic characteristics to abdominal fat given it is taken from a similar region. Thus, we would expect to find increased levels of adipose tissue macrophages in such depots of obese individual if metabolic
inflammatory pathways were being activated. However, despite increased fat mass in obese mothers, we did not observe a significant increase in percent of stromal adipose tissue cells that were CD45+CD14+, primarily a signature for macrophages, but also neutrophils and dendritic cells to a lesser extent (Figure 2.3).

Figure 2.3: Maternal adipose tissue macrophages.
Maternal gluteal adipose tissue samples were separated into adipocyte and stromal cells, and the percent of stromal cells that were CD45+CD14+ were determined by flow cytometry. Data are expressed as maternal group mean ± SEM for n = 9 mothers per maternal group.

Additionally, we found no significant difference between measured serum cytokine and adipokine levels in lean versus obese mothers, however maternal Leptin and MCP-1 trended higher in obese mothers, as would be expected with increased fat mass, but this did not reach significance given a large degree of variability and small sample size (Figure 2.4). Adiponectin, a well-reported insulin sensitizer, also trended higher in
obese mothers, which was unexpected given that obesisty is typically associated with reduced circulating adiponectin levels, concomitant with reduced insulin sensitivity. While unexpected, this data is consistent with the lack of metabolic abnormalities in this obese patient cohort.

![Bar charts showing cytokine and adipokine levels for lean and obese mothers.](image)

**Figure 2.4:** Maternal fasting serum cytokine and adipokine levels. Cytokine and adipokines were titered for mid gestation maternal serum samples. Data are expressed as maternal group mean ± SEM for n = 7 lean and n = 8 obese mothers.
Impact of Placenta on Infant Birth Weight and Adiposity

Among all groups, placental weight significantly correlated with infant birth weight ($r = 0.60, P = 0.009$; Figure 2.5), and, to a slightly lesser extent, infant adiposity at birth ($r = 0.50, P = 0.04$; Figure 2.5).

Figure 2.5: Correlations between placental weight and infant outcomes.

Increasing placenta weight positively correlated with increasing infant birth weight and adiposity. Data are from $n = 18$ mothers across all BMIs.

Placental villous LPL activity was measured for all placentas at the time of delivery.

Upon initial examination, placental villous activity appeared unrelated to infant adiposity at birth ($r = 0.19, P = 0.45$; Figure 2.6).

However, given that villous LPL activity was measured per gram of tissue, but total placental sizes varied within our maternal cohort, we also estimated whole placental LPL activity by multiplying villous LPL activity by total placental weight. Using this
estimation, placental LPL activity approached, but did not reach significance to correlate with infant adiposity ($r = 0.42, P = 0.08$; Figure 2.6).

**Figure 2.6: Correlation of villous and placental LPL activity and infant adiposity.** Placental LPL activity was estimated by multiplying villous LPL activity by placental weight. Data are from $n = 18$ mothers across all BMIs.

While placental LPL activity is thought to increase with advancing gestation, we observed a significant negative correlation between villous LPL activity and maternal gestational age within the window of term delivery ($>37$ wks gestation; $r = 0.53, P = 0.02$; Figure 2.7). Importantly, these small differences in maternal gestational age at term did not correlate with any other maternal measurements or infant outcomes.

To determine whether this influence of gestational age could be interfering with our initial findings, we adjusted our original villous LPL activity measurements for this variable. Consequently, we identified an improved relationship between villous LPL activity and infant adiposity at birth ($r = 0.43, P = 0.08$; Figure 2.7). This relationship became quite strong with the inclusion of placental weight, and thus an estimate of
overall placental LPL activity ($r = 0.65, P = 0.003$; Figure 2.7). Importantly, the relationship between placental LPL activity and infant percent body fat was stronger than with placental weight alone.

![Graphs showing correlations between placental LPL activity and infant adiposity](image)

**Figure 2.7:** Impact of gestational age on villous LPL activity.

Gestational age at term negatively correlated with villous LPL activity. Activity was adjusted for this effect and correlations between villous and placental LPL activity and infant adiposity re-analyzed. Data are from $n = 18$ mothers across all BMIs.

The relationship between adjusted placental LPL activity and infant adiposity held at 2 weeks of age (by repeated skin fold measurements), however the correlation was weaker ($r = 0.52, P = 0.03$; Figure 2.8), further underscoring the impact of postnatal factors on infant body composition after birth. Placental weight alone was no longer associated with infant adiposity at 2 wks (Figure 2.8).
Determinants of Placental LPL Activity

Not surprisingly, given the lack of difference in placental weight, infant birth weight, or infant adiposity between lean and obese mothers, neither villous nor placental LPL activity differed between maternal groups (Figure 2.9).

Importantly, villous LPL gene expression also did not correlate with villous LPL activity (Figure 2.10), nor did villous expression of other proteins involved in fatty acid transport. Additionally, villous expression of genes associated with a more pro-inflammatory placental microenvironment, including macrophage markers and pro-inflammatory cytokines, did not appear to correlate with villous LPL activity.

Figure 2.8: Relationship between placental LPL activity and placental weight with infant adiposity at 2 weeks of age.

Infant body fat percent was determined by repeated infant skin fold measurements at 2 wks post-delivery. Data are from $n = 18$ mothers across all BMIs.
Figure 2.9: Villous and placental LPL activity between lean and obese mothers.

Data are expressed as maternal group mean ± SEM for $n = 9$ lean and $n = 9$ obese mothers. Both raw and gestational age (GA)-adjusted LPL activities are shown.
Interestingly, while there were no observed differences in placental pro-inflammatory genes in obese versus lean placentas (Figure 2.11), there was a trend for reduced villous expression of genes associated with fatty acid transport in obese subjects, but with only FABP-4 reaching statistical significance ($P<0.05$; Figure 2.11). This change in gene expression may be compensatory, but the mechanisms for this are not yet clear.
Figure 2.11: Placental gene expression between lean and obese mothers.

Placental villous gene expression for $n = 9$ lean and $n = 7$ obese mothers. Data are expressed as maternal group mean $\pm$ SEM; *P<0.05 vs. lean.
In looking for a possible explanatory variable for placental villous LPL activity, we ran correlations with all previously discussed maternal measurements, but no significant relationships were identified. However, total adjusted placental LPL activity was positively associated with the change in maternal fasting insulin between 12-16 wks and 26-28 wks gestation ($r = 0.48$, $P = 0.05$; Figure 2.12). This was different than placental weight alone, which instead correlated with the change in maternal fasting insulin between 12-16 wks and 37 wks gestation ($r = 0.53$, $P = 0.03$; Figure 2.12). Both interactions suggest a possible relationship between maternal insulin levels and placental growth and capacity for nutrient transport.

![Figure 2.12](image.png)

**Figure 2.12:** Correlations between placental measurements and maternal insulin.

Gestational age-adjusted placental LPL activity was used in measurements. All maternal mothers were fasted prior to serum collection. Data are from $n = 18$ mothers across all BMIs.
2.4 Discussion

Humans are born with the highest percent fat of any species, and roughly 90% of this fat deposition occurs in the last 10 weeks of pregnancy, increasing exponentially to 7 grams per day near term [51,154-156]. Recent evidence suggests that maternal obesity may exacerbate this process, leading to excessive neonatal fat accumulation [157-159], and potentially placing the infant on a trajectory towards early metabolic disease. In this study, we examine the potential maternal correlates of infant adiposity, with a focus on maternal metabolism and placental lipid transport.

Our primary findings suggest a strong relationship between placental weight, placental villous LPL activity and infant adiposity at birth, as estimated by anthropometric skin fold measurements. The strong relationship between placental weight and infant birth weight is a well-established paradigm [160-162], but the relationship between placental weight and infant adiposity has only recently gained attention [163]. To our knowledge, this is the first time that placental LPL activity, in addition to placental weight alone has been associated with such outcomes. Importantly, we found that both placental weight and villous LPL activity (once adjusted for gestational age) were independently associated with infant adiposity at birth. However, it was the combination of the two measurements that yielded the strongest relationship, suggesting that the inter-relation between placental size and lipid transport have combinatorial effects on overall lipid flux to the fetus.

Recent data suggests that fetal-placental glucose and amino acid utilization rates are highest at 22-26 weeks and decrease near term, in contrast to lipid transport which
is maximal in the third trimester, coincident with rapid fetal fat accretion [155,164].

Thus, our observation of reduced villous LPL activity with advancing gestation within term pregnancies was initially surprising. However, the placenta is known to age over the course of gestation [165], with late gestation placentas showing evidence of villous thinning, syncytial knots, and apoptosis [166]. These very late gestation changes may explain the decrease in LPL activity that we observed, particularly in placentas over 40 weeks of age.

While the use of skin fold measurements to determine relative body composition are by no means the gold standard, it was important for us to be able to quickly measure infant adiposity within 24 hours of delivery, before extra-uterine influences (especially feeding practices) significantly influenced the infant’s body composition and insulin sensitivity. It has previously been shown that measured and derived anthropometry from weight, length, head circumference, mid-arm circumference, and triceps skinfolds offer maximum predictive value for body composition in neonates when compared to dual energy x-ray absorbitomity (DXA) scan [167].

Importantly, we did not identify any clear maternal factors, specifically those associated with maternal obesity, which might be driving this paradigm for increased villous LPL activity and fetal fat accretion. This may be partially due to the fact that, within our cohort of mothers, common measures associated with maternal obesity were not increased – including maternal inflammation, insulin resistance, or hyperlipidemia. With no clear delineation between maternal lean and obese metabolic phenotypes, it is perhaps not surprising that we observed no difference in infant birth weight or
adiposity, or placental weight and villous LPL activity between these groups.

Additionally, this may also underscore the poor predictive value of maternal BMI as a classifier for metabolically healthy versus unhealthy mothers.

Independent of maternal BMI, maternal fasting insulin levels did appear to play a role in placental development, specifically with regard to placental weight – an overlapping factor in the measure of total placental LPL capacity. Given the strong association between placental weight and both infant weight and adiposity, as well as the relative contribution of placental size to overall nutrient flux, this relationship may be an important player in pregnancies characterized by maternal insulin resistance and consequent hyperinsulinemia.

While a multitude of studies have reported differential expression of placental lipid transport genes in the context of maternal obesity and GDM, we observed no relationship between villous gene expression of LPL and measures of villous LPL activity. This underscores the need for measurements beyond gene expression analysis, such as protein level, localization, and activity, before conclusions can be drawn, particularly for enzymes such as LPL that are known to be regulated post-transcriptionally [168].

It is possible that, despite a lack of delineation between obese and lean mothers, maternal factors beyond those presented here may be important for the regulation of villous LPL activity. For instance, given that postprandial hyperglycemia is more predictive of macrosomia than fasting hyperglycemia, it may be that postprandial TGs and NEFAs may be more closely tied with fetal fat accretion than fasting hypertriglyceridemia. Further, a number of placental and maternal hormones
associated with pregnancy, such as placental growth hormone, human placental lactogen, and estrogen, may play important roles in regulating placental growth and lipid transport. However, their measurement was out of the scope of this current study.

In conclusion, villous LPL activity and placental weight appear to have combinatorial effects on infant adiposity at birth. While the hormonal control mechanisms for the regulation of villous LPL activity are still unclear, the relationship between maternal fasting insulin levels and placental growth may be important, however whether this is a direct effect of insulin on the placenta requires further testing. Aside from fasting insulin levels, we were not able to identify any maternal obesity-specific factors that may be contributing to this mechanism of infant fat accretion in this small sub-set of relatively metabolically healthy mothers, which underscores the need for corroboration of these findings in a larger group of women, including those with more pronounced metabolic pathology. Further, studies in animal models of obese pregnancy, with more standardized maternal metabolic phenotypes, may help to clarify how maternal obesity might impact placental lipid transport, and the ramifications this may have on the developing fetus.
CHAPTER III

IMPACT OF MATERNAL OBESITY AND HIGH-FAT DIET ON MATERNAL INFLAMMATION, INSULIN RESISTANCE, AND LIPID METABOLISM IN A MOUSE MODEL

3.1 Introduction

The perinatal period is emerging as a critical window for developmental programming of future metabolic disease. Substantial animal data and human epidemiological evidence suggests that maternal obesity during pregnancy increases the risk for obesity and metabolic syndrome in the offspring. However, the mechanism by which this occurs is still unclear. While many studies in animal models support altered programming of lipid metabolism pathways in adult offspring of obese mothers, these studies are inherently weakened by their lack of mechanistic data from the mother, placenta, and fetus. In fact, pregnancy itself is often treated as a black box, and very little is known about which maternal and placental factors associated with obese pregnancy are responsible for inducing the reported metabolic changes in offspring.

As discussed in Chapters I and II, placental lipid flux is minimal during the first and second trimester and exponentially increases in later gestation with enhanced maternal lipolysis and placental lipid transport, which coincides with fetal adipose tissue

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3 Portions of this chapter are from 169. Heerwagen MJ, Stewart MS, de la Houssaye BA, Janssen RC, Friedman JE (2013) Transgenic increase in n-3/n-6 fatty acid ratio reduces maternal obesity-associated inflammation and limits adverse developmental programming in mice. PLoS One 8: e67791.
development [170,171]. A driving force in fetal lipid accretion relates to physiologic insulin resistance in the mother during the second half of gestation, when it is common to see a two to three fold increase in maternal serum triglycerides [172]. This increased substrate load, ultimately, is believed to create a concentration gradient driving lipid flux to the fetus.

As discussed in Chapter I, pregnancies complicated by maternal obesity are associated with low grade inflammation and pre gravid insulin resistance, which, when combined with the metabolic stress of pregnancy, may result in exaggerated serum lipid levels and increased placental lipid transport [173,174]. Additionally, as discussed in Chapter II, it appears that, independent of obesity, the regulation of maternal lipid transport to the fetus by placental lipoprotein lipase may be another mechanism by which the fetus is exposed to excess lipids in utero leading to increased fat accretion by birth.

Increased lipid transfer across the placenta to the developing fetus may be associated with additional pathological changes in inflammation and metabolic gene programs, leading to early dysregulation of lipid metabolism after birth. Previously, in a non-human primate model of maternal obesity, we reported that maternal HFD during pregnancy results in increased fetal hepatic lipid accumulation, oxidative stress, and apoptosis [75], accompanied by a dramatic reduction in fetal plasma omega-3 (n-3) polyunsaturated fatty acids (PUFA) and a more pro-inflammatory cytokine profile [175]. These findings suggest not only enhanced lipid flux to the fetus, but also a pro-inflammatory exposure, possibly originating from the mother and/or placenta.
Given the ethical considerations when dealing with pregnant subjects, animal models are necessary to obtain more detailed data on the potential mechanistic changes that occur in pregnancies complicated by maternal obesity. In this chapter, we will utilize a high-fat fed mouse model of obese pregnancy to test the hypothesis that maternal obesity and high-fat diet increase maternal inflammation and insulin resistance, resulting in increased placental lipid and cytokine exposure.

3.2 Methods

Animal Breeding and Diet

All animal studies were approved by the University of Colorado Institutional Animal Care and Use Committee and carried out in strict accordance with the guidelines set forth by the Guide for the Care and Use of Laboratory Animal by the National Institutes of Health.

Eight-week-old C57BL6/J female mice were placed on a 45% kcal HFD (Research Diets D12451, Appendix B) or a 10% kcal control diet (CD, Research Diets D12450B, Appendix B) and fed ad libitum for 8 wks prior to mating with a chow-fed male. Successful mating was determined by post-copulatory plug, and defined as E0.5. Males were removed post-coitously, and mothers were maintained on experimental diet throughout pregnancy. Mothers were weighed weekly from the start of experimental diet, then at day 0.5 and 18.5 of gestation. Pups were analyzed on embryonic day 18.5.
(n = 12-14 mothers per group). Fetal sex was determined by tail-DNA PCR for presence or absence of Y-chromosome SRY gene (Primers – Appendix A). Mothers were euthanized by isoflurane anesthesia followed by cervical dislocation and exsanguination. Fetal pups were euthanized by decapitation and exsanguination (Figure 3.1).

Figure 3.1: Study design: Maternal diet and breeding scheme
Mothers were fed either control or high-fat diets (CD, HFD respectively), for 8 weeks prior to mating. Diet was maintained through pregnancy, and mothers, fetuses, and placenta were harvested at embryonic day 18.5.

Tissue Collection
At day 18.5 of gestation, mothers were fasted 4 hrs in a clean cage. Maternal fasting blood was obtained from the tail vein, and glucose levels measured using the Accu-Check Aviva monitoring system (Roche Diagnostics, Indianapolis IN). Mothers were then euthanized by isoflurane anesthesia followed by exsanguination by cardiac
puncture. Maternal blood and tissues were quickly collected, rinsed in PBS and processed according to respective analyses listed below. Fetal-placental units were then quickly dissected from the uterine horns, fetal membranes were dissected away, and the placenta and fetus were weighed and measured prior to fetal decapitation. Blood and tissues were collected and processed as described below.

**Serum Analyses**

Blood was collected from mothers by cardiac puncture post-anesthesia and allowed to clot for 10 min at room temperature. Serum was isolated by centrifugation at 5000 g for 10 min, aliquoted and snap-frozen and stored at -80°C. Serum insulin and adiponectin levels were analyzed by Enzyme Immuno Assay (EIA, ALPCO Diagnostics) per manufacturer’s instructions. Serum non-esterified fatty acids (NEFA/FFA), triglycerides (TG) and free glycerol levels were quantified by an enzymatic, colorometric assay (Wako Diagnostic, Richmond VA and Sigma-Aldrich, St. Louis MO, respectively) per manufacturer’s instructions. All measurements were quantified by microplate reader (Molecular Devices, Sunnyvale CA) using standards provided by the manufacturer. Maternal serum pro-inflammatory cytokine levels were determined using the Mouse Inflammation Antibody Array I (RayBiotech Inc., Norcross, GA) per manufacturer’s instructions. For each membrane, 125 µl of serum from four separate mothers was pooled and diluted 1:2 with blocking buffer, three membranes (n = 12 animals) were used per experimental condition, and results were normalized to the relative expression of IgG positive control internal standards.
Flow Cytometry for Adipose Tissue and Placental Macrophages

Subsequent to euthanasia, the left perigonadal adipose tissue depot was dissected and rinsed in DMEM containing 10% FBS. Both adipose tissue and whole placentas were minced to 1 mm cubes and digested in the same media containing 500 U/ml Type I Collagenase (Sigma-Aldrich) for 1 hr at 37°C with agitation. The suspension was then filtered through chiffon mesh and the stromal-vascular fraction was isolated by centrifugation at 500 g for 5 min. The pelleted fraction was resuspended in 1 X PBS containing 5% BSA.

For flow analysis, samples and appropriate controls were stained in 100 µl of 1 X PBS/BSA buffer containing 2 µl Mouse SeroBlock FcR (AbD Serotec, Raleigh, NC). Fluorescently-conjugated primary antibodies (phycoerythrin-F4/80, and allophycocyanin-CD11c, eBioscience, San Diego, CA) or the respective isotype control IgGs (eBioscience) were added after 10 min of blocking, and incubated with cells for 30 min at 4°C. After staining, cells were washed and fixed for 15 min in 1% paraformaldehyde. Cell data was collected using the FACSCalibur flow cytometer and CellQuest Pro Software (BD Biosciences, San Jose, CA), and analyzed using FlowJo software (Tree Star Inc., Ashland, OR). Positive cell populations were expressed as percent positive of total cells, and adjusted by isotype controls.

Placental RNA Extraction and qPCR

Freshly harvested placentas were equilibrated in RNAlater (Qiagen, Valencia, CA) for 24 hrs prior to storage at -20°C. Thawed samples were homogenized in TRIzol
Reagent (Life Technologies, Grand Island, NY) and total RNA was isolated according to manufacturer’s instructions. Real-time quantitative PCR was performed using iQ SYBR Supermix (Bio-Rad, Hercules, CA) or TaqMan Primer-probe sets (Life Technologies) with primers provided by IDT PrimeTime qPCR Primers (Integrated DNA Technologies). Primers were designed to be specific for exon regions of inflammation and lipid transport-related genes: LPL, CD36, FABP-4, IL-1β, IL-6, TNFα, F4/80, iNOS, and Arg-1 (Primers – Appendix A). Reactions were run in duplicate on an iQ5 Real-Time PCR Detection System (Bio-Rad) along with a no-template control per gene, and normalized to GAPDH and ubiquitin C expression using the comparative threshold cycling method.

**Maternal Tissue Triglyceride Quantitation**

Flash-frozen maternal liver and skeletal muscle samples were homogenized in 1 ml of ice-cold methanol, and lipids were extracted using 1:2 methanol:chloroform followed by 0.6 volumes of distilled water, and solubilization for 1 hr end-over-end at 4°C. The polar and non-polar phases were then separated by centrifugation, and the lower non-polar phase was isolated and dried under nitrogen. The dried samples were then resuspended in isopropanol + 2% Triton X-100 for TG quantitation by glycerol equivalents using Infinity Triglycerides Reagent (Thermo Scientific, Waltham, MA) and a standard curve using glycerol standard solution (Sigma-Aldrich) per manufacturer’s instructions. Resultant TG concentrations were normalized to starting tissue weight.
**Tissue Histology**

To visualize lipid droplet deposition, maternal livers and placentas (in cross-section) were cryoembedded in Tissue-Tek OCT Compound (Sakura Finetek U.S.A., Inc., Torrence, CA). 5 μM cryosections were stained using a 1% Oil Red O in propylene glycol solution. Slides were fixed in ice-cold formalin, equilibrated in 100% propylene glycol, and stained for 10 min in pre-warmed 1% Oil Red O solution. Stained sections were then differentiated in 80% propylene glycol, rinsed in distilled water, and counterstained with Mayer’s Hematoxylin (Sigma-Aldrich).

**Placental Lipoprotein Lipase Activity Measurements**

Assay was performed as described in Chapter II utilizing whole, freshly harvested, placentas ($n = 4$ placentas/mother).

**Placenta Lipid Analysis by GC-MS**

Protocols used for lipid extraction and GC-MS methodology were originally optimized by Dr. Bryan Bergman, who assisted with analysis. Frozen placenta samples were lyophilized overnight prior to lipid extraction, and dry weights recorded. Lyophilized samples were then homogenized in 1 ml of ice-cold methanol, and lipids were extracted using 1:2 methanol:chloroform followed by 0.6 volumes of distilled water, and solubilization for 1 hr end-over-end at 4°C. The polar and non-polar phases were then separated by centrifugation, and the lower non-polar phase was isolated and dried under nitrogen.
Resuspended samples were separated by solid phase extraction (SPE) using a Supelco aminopropyl phase column (Sigma-Aldrich). Phospholipid and TG fractions were methylated using 0.5 M sodium methoxide in methanol, and fatty acid methyl esters (FAMEs) were analyzed by gas chromatography-mass spectrometry (GC-MS) using a DB-23 column and helium as a carrier gas. Temperature was programmed from 140-240°C at 15°/min. Results were normalized to internal extraction standards and resulting concentrations were normalized to dry tissue weight.

Data Analysis

To correct for a common maternal environment, placental results are expressed as a single average for each mother. Male and female placentas were initially analyzed separately, and subsequently combined after no between-group sexual dimorphism was identified.

Differences between maternal CD and HFD groups were determined by 2-tailed Student’s t test using the Graph Pad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA). When variances were not equal, a 2-tailed t test with Welch’s correction was performed. All data are expressed as the mean ± standard error of mean (SEM), and differences were determined to be significant at $P<0.05$. 
3.3 Results

Maternal HFD Induces Obese Phenotype in Late Gestation Mothers

Maternal pre-pregnancy weight was significantly increased in HFD mothers relative to CD mothers ($P<0.05$; Figure 3.2). This difference in weight was maintained throughout gestation, with both groups demonstrating roughly equal rates of weight gain during pregnancy, such that at the time of harvest, HFD mothers were still significantly heavier than CD mothers ($P<0.05$; Figure 3.2).

![Figure 3.2: Maternal pre-pregnancy and gestational weight gain.](image)

Maternal weight 8 wks prior to mating and at E18.5 (late gestation). Late gestation weight was estimated by subtracting the complete fetal-placental litter weight from the weight of the mother. Results are the average of $n = 12 - 14$ mothers per group. Data represented as mean ± SEM; *$P<0.05$ vs. WT-CD.
To determine the effects of HFD on classic markers of obesity-associated inflammation, but this time in the context of pregnancy, we assessed both adipose tissue macrophage content and serum pro-inflammatory cytokine levels in late gestation (E18.5) mothers.

HFD mothers demonstrated increased adipose tissue macrophage content, as measured by percent F4/80+ cell population in stromal fractions by flow cytometry ($P<0.02$; Figure 3.3). However, no difference in M1 polarization of macrophages was observed between groups based on CD11c co-positivity, a previously established M1 phenotypic marker [30].

Despite increases in adipose tissue macrophages in HFD mothers, we were not able to identify any increases in placental macrophage content by flow cytometry for F4/80+ cells (Figure 3.4), or by F4/80 gene expression (Figure 3.4). However, we did observe HFD-associated increases in placental gene expression of the pro-inflammatory cytokine IL-1β, as well as the expression ratio of inducible nitric oxide synthase (iNos) to arginase-1 (Arg-1), which is a rough estimate of the ratio of M1 to M2 macrophage polarization ($P<0.05$; Figure 3.4). Both these measurements suggest that while there may not be more macrophages present, they could be more M1 polarized, thus creating a more pro-inflammatory microenvironment.
Figure 3.3: Maternal adipose tissue macrophages.

Adipose tissue (AT) macrophage quantitation by flow cytometry. Antibodies against mouse macrophage marker F4/80 and M1 macrophage marker CD11c were used to determine the percent of macrophages present and their relative M1 polarization in maternal AT stroma at E18.5. Results are the average of \( n = 12 - 14 \) mothers per experimental group. Data represented as mean ± SEM; \(* P<0.05\) vs. WT-CD.
Maternal HFD Increases Systemic Inflammation and Insulin Resistance

In order to measure a number of circulating pro-inflammatory cytokines with a small volume of serum, we utilized the Mouse Inflammation Antibody Array. HFD mothers showed elevated levels of 12 circulating pro-inflammatory cytokines known to be involved in immune cell activation, proliferation and chemotaxis ($P<0.05$, Figure 3.5). Maternal leptin levels, while trending higher in HFD mothers, were not significantly elevated.

Figure 3.4: Markers of placental inflammation.

(Top) Flow cytometry for F4/80+ and CD11c+ macrophages in collagenase-digested whole placentas ($n=6$ mothers per group, average of 2 placentas per mother). (Bottom) Placental gene expression of select pro-inflammatory cytokines and macrophage markers by quantitative PCR ($n=9$ mothers per group, 3 RNA pools of 3 mothers each). Data represented as mean ± SEM; *$P<0.05$ vs. WT-CD.
This increase in maternal inflammation in HFD mothers also corresponded with an elevation in fasting insulin and glucose levels, and thus a consequent increase in their homeostatic model assessment of insulin resistance (HOMA-IR) score, a rough measure of insulin resistance ($P<0.05$ and $P<0.01$, respectively; Table 3.1). Importantly, while maternal fasting glucose levels in HFD mothers were significantly elevated relative to CD mothers ($P<0.05$; Table 3.1), HFD mothers did not demonstrate overt fasting hyperglycemia. Maternal fasting TG levels did not differ between groups, but maternal
Fasting free fatty acid (FFA) levels were lower in HFD mothers ($P<0.02$; Table 3.1), which was unexpected and the opposite of what is typically observed in non-pregnant HFD animals. No differences in serum high molecular weight (HMW) adiponectin were observed.

**Table 3.1: Maternal E18.5 fasting serum measurements**

<table>
<thead>
<tr>
<th>Mother</th>
<th>Glucose (mg/dL)</th>
<th>Insulin (ng/mL)</th>
<th>HOMA-IR</th>
<th>FFA (mq/L)</th>
<th>TG (mg/dL)</th>
<th>Glycerol (mg/dL)</th>
<th>Adiponectin ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-CD</td>
<td>95 ± 6</td>
<td>0.41 ± 0.06</td>
<td>2.12 ± 0.26</td>
<td>0.98 ± 0.05</td>
<td>22.98 ± 3.97</td>
<td>15.51 ± 0.61</td>
<td>1.17 ± 0.27</td>
</tr>
<tr>
<td>WT-HFD</td>
<td>*117 ± 7</td>
<td>*0.83 ± 0.13</td>
<td>*6.21 ± 1.34</td>
<td>*0.75 ± 0.04</td>
<td>*18.23 ± 1.31</td>
<td>*15.46 ± 0.87</td>
<td>*1.40 ± 0.17</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM of $n = 12 - 14$ mothers per maternal group; *$P<0.05$ vs. CD.

**Maternal Tissue Lipid Accumulation Increased With Pregnancy**

Given that maternal inflammation and insulin resistance is often associated with increased ectopic lipid deposition due to poor suppression of adipose tissue lipolysis, lipids were extracted from maternal liver and skeletal muscle samples and TG levels relative to tissue weight were quantified. Surprisingly, we detected no differences in either maternal liver or skeletal muscle TG levels between CD and HFD mothers (Figure 3.6). It should be noted, however, that the metabolic stress of pregnancy may be sufficient to mask such changes, as can be seen by the large increase in maternal hepatic lipids by Oil Red O staining, even in CD mothers (Figure 3.6).
Figure 3.6: Maternal late gestation tissue triglycerides.

(Left) Maternal skeletal muscle and liver TG were extracted and quantified in \( n = 7 \) CD mothers and \( n = 10 \) HFD mothers. (Right) Representative Oil-Red-O staining of maternal liver lipid droplets in cryosectioned E18.5 livers (10X magnification). Data represented as mean ± SEM.
Maternal HFD Alters Fetal-Placental Growth

Fetuses from HFD mothers had significantly larger placentas versus CD mothers at E18.5, as demonstrated by both an increase in placental weight and surface area ($P<0.02$; Figure 3.7). Fetal weights in HFD mothers trended lower, leading to a reduced fetal-placental ratio overall ($P<0.01$; Figure 3.7).

Figure 3.7: Placental and fetal gross measurements.

Average fetal and placental weights for each litter. Placentas were weighed subsequent to removal of umbilical cord and fetal membranes. Placental surface area was calculated from measured diameter on maternal side. (Right) Representative images of fetal-placental unit size for each maternal group. All data are expressed as mean ± SEM of the litter average for each mother (n=12-14 mothers per experimental group); *$P<0.05$ vs. WT-CD.
This pattern suggests reduced placental efficiency and restricted fetal growth relative to placental size. While fetal sex significantly affected placental and fetal weights in both maternal groups, no sexual dimorphism was apparent with respect to maternal HFD.

There was no significant difference in litter male/female sex ratio between groups; however, litter size was reduced from an average of 10 to 8 pups in HFD mothers, with a concomitant increase in rate of resorptions ($P<0.01$; Table 3.2).

### Table 3.2: Litter characteristics between CD and HFD mothers.

<table>
<thead>
<tr>
<th>Mother</th>
<th>Pups/Litter</th>
<th>Resorptions/Litter</th>
<th>Fetal Deaths/Litter</th>
<th>M/F Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-CD</td>
<td>10.1±0.4</td>
<td>0.67±0.25</td>
<td>0.08±0.08</td>
<td>0.89±0.18</td>
</tr>
<tr>
<td>WT-HFD</td>
<td>*8.5±0.5</td>
<td>*1.50±0.27</td>
<td>0.36±0.13</td>
<td>1.22±0.26</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM of $n = 12 - 14$ mothers per maternal group; *$P<0.05$ vs. CD.

**Increased Placental Lipid Accumulation With Maternal HFD**

To better identify cumulative differences in fetal-placental lipid exposure over gestation, GC-MS was performed on placental lipid extracts. Placentas from HFD mothers showed increased TG levels relative to CD mothers ($P<0.01$ Figure 3.8), and the lipid composition of placental TG and phospholipid extracts had more saturated fat relative to monounsaturated and polyunsaturated fat (Figure 3.8). This change in lipid character is not unexpected, as it is reflective of the maternal HFD, which is rich in saturated fat.
This increase in placental TG was further confirmed by Oil Red O staining of HFD and CD placenta tissue sections, viewed in cross-section. This technique also allowed us to further localize the previously identified lipid accumulation to the decidual zone of HFD placentas (Figure 3.9).
**Figure 3.9: Placenta lipid droplet staining.**

Oil Red O staining of representative placenta cryosections to localize increased TG deposition quantitated in Figure 3.8 (lipid droplet = red, cell nuclei = blue). Placentas are seen in cross section, with 10X and 40X magnification of decidual (left) and labyrinth (right) sides.
Placental LPL Activity Increased With Maternal HFD

Given that we did not observe an increase in fasting serum TG with maternal HFD, we sought to clarify whether this difference in placental lipid accumulation could be due in part to differential lipid uptake we examined the expression of a limited set of genes related to lipid transport. However, no differences in placental LPL, CD36, or FABP-4 expression were observed between HFD and CD mothers (Figure 3.10). Nevertheless, given that the regulation of placental transport is often post-transcriptional and affected by environmental changes, placental LPL activity was assayed on freshly dissected E18.5 placentas from a subset of mothers. In line with the increased placental TG deposition observed in HFD mothers, placental LPL activity was significantly elevated ($P<0.02$; Figure 3.10).

![Figure 3.10: Placental lipid uptake.](image)

(Left) Placental gene expression of select genes involved in fatty acid transport by quantitative PCR ($n = 9$ mothers per group, 3 RNA pools of 3 mothers each). (Right) Placental LPL hydrolase activity. Activity was measured in 3 - 4 placentas from each mother and averaged, with $n=6$ mothers per experimental group. All data are expressed as mean ± SEM; *$P<0.05$ vs. WT-CD.
3.4 Discussion

Maternal pre gravid obesity increases the risk for numerous obstetric complications such as preeclampsia and gestational diabetes mellitus [176,177], but also increases sub-clinical risk factors, such as maternal and placental inflammation [178-183]. While inflammation and insulin resistance are well-recognized characteristics of both pregnancy and obesity, little is known about the mechanisms linking these maternal changes with changes in fetal-placental development or, perhaps more importantly, the pathogenesis of obesity in offspring. Our previous research in non-human primates shows that both maternal obesity and high-fat feeding increase fetal exposure to lipids and pro-inflammatory cytokines [75,175], which we hypothesize may originate from maternal and placental inflammation and inflammation-associated insulin resistance.

In this chapter, we utilized a mouse model of obese pregnancy to better identify metabolic changes in the mother and placenta that may impact fetal developmental programming. Mothers placed on HFD prior to and during pregnancy demonstrated significant increases in weight gain as well as classic hallmarks of an obese phenotype, including increases in F4/80+ macrophage accumulation in adipose tissue depots, and systemic increases in pro-inflammatory cytokines associated with immune cell activation, proliferation and chemotaxis. Maternal inflammation was also associated with increased maternal insulin resistance, as indicated by increased maternal fasting insulin levels and, to a lesser degree fasting glucose levels, leading to an increase in their homeostatic model of assessment of insulin resistance (HOMA-IR) score. These
findings of increased low-grade inflammation and reduced insulin sensitivity without overt hyperglycemia are consistent with the progression of obesity-related metabolic syndrome in non-pregnant mouse models [183-185].

Surprisingly, we did not detect any increase in F4/80+ macrophage accumulation in the placenta of HFD mothers, as has been previously reported in human pregnancies complicated by obesity [178]. However, our methods involved utilization of the whole placenta, and therefore may simply have lacked the sensitivity to detect regional changes in cell populations. Histological studies to better characterize regional changes in placental cell populations would help to clarify these findings. However, increases in IL-1β and reduced Arg-1 relative to iNos gene expression in HFD mothers still suggested a more inflamed placental phenotype overall, and were consistent with previous findings in primates and humans [178,181,183].

Interestingly, we did not observe any evidence of maternal hyperlipidemia with HFD, as is often reported in human pregnancy complicated by pre-gravid obesity. This could be due to differences in mouse versus human metabolism during pregnancy, including both maternal and placental response to fasting, and rate of tissue and placental lipid clearance. Further studies utilizing real-time measurements and labeled lipid substrates would be helpful in identifying overall maternal lipid flux throughout gestation, as our measurements provide only a single snapshot from late gestation. It should also be noted that we did see a small, but significant increase in maternal fasting blood glucose. These fasting blood glucose levels were still below what we observe for a lean non-pregnant female, and so do not suggest overt gestational
diabetes, however even slight increases may possibly contribute to changes in placental and fetal growth patterns.

In the hopes of clarifying maternal lipid trafficking, beyond serum measurements, we measured the level of TG deposition in maternal liver and skeletal muscle. Here too, we found no differences between CD and HFD mothers. However, the level of TG, particularly in maternal liver, was very high, and thus may have masked more subtle differences due to diet. As the maternal liver is known to up-regulate *de novo* lipogenesis in late gestation to supply lipid for lactation, as well as utilize lipids as an energy source to salvage glucose for the fetus, the excess TG in the maternal liver is not unexpected.

In accordance with previous groups, our mouse model of obese pregnancy produced fetuses with mild evidence of growth restriction [75,186,187]. While these findings are somewhat contrary to the large for gestational age (LGA) infants common to human pregnancies complicated by obesity, this may simply be due to species-specific differences in developmental age and fat mass at birth. More concerning than the trend in reduced fetal growth was the evidence of reduced placental efficiency, with WT-HFD mothers producing slightly smaller fetuses with significantly larger placentas [188]. This reduced fetal-placental ratio was recently reported by another group [189], and may be indicative of previously described impairments in placental perfusion and vascular defects related to maternal obesity and HFD [181,183,190,191]. Contrary to our findings, some groups have observed reduced placental size with maternal HFD [186], which may be partially explained by differences
in mouse strains, diet composition, duration of feeding, gestational age at time of harvest, as well as maternal age at time of conception [192]. Clearly, the maternal environment plays a key role in the proper establishment, growth, and function of the placenta [193], and the mechanisms by which maternal obesity and HFD can impair these processes requires further examination. For instance, histological analysis of placental pathology, particularly markers of altered vascularization would be informative.

Corresponding with previous groups’ findings in the human and rodent placenta [146,194], maternal HFD led to a significant increase in lipid deposition in the decidua, and to a lesser extent, the labyrinth regions of the placenta. This was observed despite no evidence of late gestation maternal hyperlipidemia. It is well established that decidual cells have the capacity to store lipid, as this is one of the characteristic features of the decidual reaction in early gestation, while cells within the murine placental labyrinth are primarily involved in nutrient exchange rather than storage, which may explain the differential TG deposition between placental regions.

Previous groups have reported an association between maternal obesity and increased placental expression of LPL and various other fatty acid transporters and binding proteins [146,194-198]. However, we detected no differences in LPL, nor CD36 or FABP-4, at the level of expression. Instead, HFD mothers demonstrated increased LPL enzymatic activity, suggesting, as in Chapter II, that placental lipid uptake may be regulated by more than simply gradient dynamics.
Importantly, what increased placental size, inflammation and lipid deposition may mean for the developing fetus is still unclear. Given the trend for reduced fetal growth in HFD mothers, increased placental size could either be a primary response to pro-growth signals from the mother, or a compensatory response due to inefficient placental perfusion and feedback from the fetus. Future studies to better understand overall placental perfusion, vascularization, and endothelial function would be helpful in clarifying the reason for disproportionate placental growth, and the potential role for placental lipids and inflammation in such pathology. Ultimately, however, what may be most important is the identification of what the fetus sees from this placenta, including potential excess lipid and pro-inflammatory cytokine exposure. How the fetus responds to such a stimulus likely has important consequences for the adaptive programming of fetal metabolic pathways, and may help us to identify which variables are necessary to recapitulate the adverse metabolic programming observed in adult offspring.
CHAPTER IV

FETAL RESPONSE TO MATERNAL OBESITY AND HIGH-FAT DIET IN A MOUSE MODEL

4.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is now the most common cause of adult chronic liver disease in the United States [199]. The occurrence of NAFLD in children has also increased, from the first case report in 1983 to a current prevalence of 8 to 11 percent in adolescents [200-203]. The tripling of childhood obesity in the past thirty years is likely a significant contributor to this recent epidemic [204,205], with rates of NAFLD in obese adolescents reported to be as high as 38 to 83 percent [206,207]. NAFLD describes a spectrum of pathology from isolated hepatic steatosis to more severe necro-inflammatory non-alcoholic steatohepatitis (NASH). The natural history of NASH in pediatrics is unknown, but in adults approximately one third of patients with NASH will have progressive inflammation and fibrosis, ending in cirrhosis within 5 to 10 years [208].

Pediatric NAFLD is a histologic diagnosis with greater than 5 percent steatosis in the absence of other specific cause, and NASH is the more severe form with steatosis, inflammation, and variable degrees of fibrosis [209]. For unclear reasons, pediatric NASH is characteristically different from the adult form, with geographically unique portal inflammation and fibrosis [210,211]. The pathogenesis of NAFLD and ensuing

4 Portions of this chapter are from 169. Heerwagen MJ, Stewart MS, de la Houssaye BA, Janssen RC, Friedman JE (2013) Transgenic increase in n-3/n-6 fatty acid ratio reduces maternal obesity-associated inflammation and limits adverse developmental programming in mice. PLoS One 8: e67791.
NASH is likely multifactorial and has been described by a “two-hit” or more recently a “multiple-hit” hypothesis [212,213]. Hepatic lipid accumulation, commonly regarded as the first hit, results in the characteristic lipid droplets seen in hepatic steatosis, which is thought to be a protective mechanism to minimize lipotoxicity [214,215]. Secondary events from lipid overload can lead to overt inflammation and steatohepatitis, and likely involve a combination of factors including mitochondrial dysfunction, oxidative stress, and pro-inflammatory cytokine production [213,216,217].

While obesity is the most common risk factor for NAFLD, gene polymorphisms associated with lipid metabolism, inflammatory cytokines, fibrotic mediators and oxidative stress also appear to play a role in both susceptibility and severity of pathology [218]. Additionally, there is now growing evidence that exposures prior to birth, such as maternal obesity, may contribute to an individual’s risk of developing metabolic diseases such as NAFLD.

Because a definitive diagnosis for NAFLD requires a liver biopsy, neonatal studies for NAFLD are limited in humans due to its invasive nature. In animal models, maternal obesity clearly shows an association with early onset NAFLD, even prior to birth [219-221]. We previously demonstrated in non-human primates that maternal obesity and a high fat diet (HFD) during gestation promote fetal hepatic steatosis and lipotoxicity in the early third trimester [222]. Further, this steatotic phenotype persisted into the juvenile period when HFD was absent, suggesting persistent hepatic programming. Recently, two innovative human studies utilized magnetic resonance technology as a non-invasive means to screen for steatosis in newborn infants;
Brumbaugh et al. found a 68 percent increase in intrahepatocellular lipid content in newborns born to pregnancies complicated by maternal obesity and insulin resistance [223], and Modi et al. reported an 8.6 percent increase in intrahepatocellular lipid content for each one point increase in maternal BMI [224]. Both groups found a direct correlation with maternal BMI and neonatal fatty liver. Importantly, neonatal fatty liver did not correlate with newborn adiposity itself, suggesting that factors associated with maternal obesity, such as excess serum lipids, are independently and specifically associated with newborn fatty liver.

As discussed in Chapter I, the developmental programming of fatty liver is well characterized in animal models. However, these studies focus on juvenile and adult offspring, and knowledge of a fetal mechanism for the observed NAFLD pathology is sorely lacking. While the increase in fetal hepatic lipid content we observed in our primate model can be attributed to the increased availability and transfer of maternal plasma lipids, persistent lipid accumulation post-partum suggests a more permanent metabolic change. Within the liver, two potential mechanisms for aberrant lipid accumulation are increased hepatic de novo lipogenesis and/or reduced fatty acid oxidation. Defects in either pathway could impact not only liver function, but also whole-body metabolism, greatly increasing the risk of metabolic disorders in adult offspring. In this chapter, we utilize a mouse model of obese pregnancy to better characterize in utero lipid exposure, and its down stream impact on fetal hepatic gene expression and lipid metabolism pathways.
4.2 Methods

Animal Breeding and Diet

All animal studies were approved by the University of Colorado Institutional Animal Care and Use Committee and carried out in strict accordance with the guidelines set forth by the Guide for the Care and Use of Laboratory Animal by the National Institutes of Health. This chapter discusses the fetal results from the maternal-fetal pairs first described in Chapter III.

Tissue Collection

Fetal-placental units were quickly removed from the uterine horns of the pregnant mothers described in Chapter III. Fetal membranes were dissected away, and the placenta and fetus were weighed and measured prior to fetal decapitation and drainage of trunk blood. Fetal livers were then dissected and either embedded in OCT, snap frozen and stored at 80°C, or equilibrated in RNALater (Qiagen, Valencia, CA) for 24 hr at 4°C prior storage at -20°C.

Serum Analyses

Fetal blood was collected by decapitation and trunk drainage, and then allowed to clot for 10 min at room temperature. Serum was isolated by centrifugation at 5000 g for 10 min, aliquoted and snap-frozen and stored at -80°C. Due to low blood volumes in fetal samples, pup serum was pooled prior to further analysis. Serum
insulin and levels were analyzed by Enzyme Immuno Assay (EIA, ALPCO Diagnostics) per manufacturer’s instructions. Serum non-esterified fatty acids (NEFA/FFA), triglycerides (TG) and free glycerol levels were quantified by an enzymatic, colorimetric assay (Wako Diagnostic, Richmond VA and Sigma-Aldrich, St. Louis MO, respectively) per manufacturer’s instructions. All measurements were quantified by microplate reader (Molecular Devices, Sunnyvale CA) using standards provided by the manufacturer.

**Fetal Liver Lipid Analysis by GC-MS**

Fetal liver lipids were extracted, quantitated and characterized by GC-MS as described for placentas in Chapter III.

**Tissue Histology**

Fetal livers were sectioned and stained as described for placentas and maternal livers in Chapter III.

**RNA Samples For Microarray and Quantitative PCR**

RNA was extracted from fetal livers as described for placentas in Chapter III. RNA was resuspended in RNase-Free water, quantified, and then pooled. Each pool contained equal amounts of RNA from 3 maternally-unique male fetal livers, with 3 separate pools created for each maternal condition (CD, HFD). These 6 pools (3 pools per group) were then used for all cDNA synthesis and gene expression analyses (microarray, qPCR).
**Fetal Liver Gene Expression by Microarray**

Pooled fetal liver RNA was sent to Dr. Kartik Shankar at the University of Arkansas for Medical Sciences for gene expression analysis by microarray utilizing the Affymetrix Mouse Genome 430 2.0 array chip (Affymetrix, Santa Clara CA). Initially, only 2 pools (of 3 maternally-unique fetal livers) per maternal group were analyzed, with a third pool of fetal liver RNA added into the analysis at a later date after more mothers were harvested. Due to significant batch differences between these two analysis time points, the results were refined using ComBat batch correction software (Broad Institute, Cambridge MA) to normalize data prior to further analyses. The final results represent an n of 9 maternally-unique fetal livers per maternal group, condensed into 3 pools of 3 fetal livers.

**Microarray Analysis**

Microarray gene expression results were analyzed using GenePattern software (Broad Institute, Cambridge MA), including Differential Expression Analysis, Comparative Marker Expression and Heat Map Imager programs for baseline gene expression differences between maternal CD and HFD groups. Given the small number of groups (n = 3 per maternal condition), and relatively small differences in gene expression, a permissive false detection rate (FDR) of less than 0.3 and fold change greater than 1.2 was used for preliminary identification of potential differentially expressed genes. In addition, Gene Set Enrichment Analysis (GSEA) was performed to better identify
cumulative pattern differences based on multiple, smaller gene expression changes, yielding an overall tissue gene expression phenotype.

**Realtime PCR for Confirmation of Microarray Results**

Subsequent to microarray analysis, real-time quantitative PCR was performed on cDNA from the same fetal liver RNA pools ($n = 3$ pools per maternal condition) using iQ SYBR Supermix (Bio-Rad, Hercules, CA) with primers provided by IDT PrimeTime qPCR Primers (Integrated DNA Technologies). Primers were designed to be specific for exon regions of select genes suggested to be up or downregulated by microarray: Scd2, Fads1, Fads2, Acss1, Acss2, Rdh11, Hmgcs1, Hmgcr, Atp5g1, Ndufs7, Cox5b, Uqcr11, Sdhb, Cycs, Ech1, Hmox1, RetSat, Txn2, Ghr, Plin5 (Primers – Appendix A). Reactions were run in duplicate on an iQ5 Real-Time PCR Detection System (Bio-Rad) along with a no-template control per gene, and normalized to GAPDH and ubiquitin C expression using the comparative threshold cycling method.

**Fetal Liver Fatty Acid Oxidation**

Fetal liver oxidation of palmitate was carried out in collaboration with Dr. Kristen Boyle in our lab, as previously described by Berggren et. al. [225]. Individual fetal livers were freshly harvested ($n = 4$/mother, 5 mothers per experimental group) and placed in 200 μL of a modified sucrose-EDTA medium containing 250 mM sucrose, 1 mM EDTA, and 10 mM Tris - HCl, pH 7.4. Samples were minced thoroughly with scissors and then
diluted 20-fold with additional sucrose-EDTA buffer. Tissue was placed on ice and homogenized with a glass pestle on glass for 30 s.

For the reaction, 40 μL of homogenate was then added to incubation wells on a sealed, modified, 48-well plate with a channel cut between the adjacent trap wells. The trap wells contained 200 μL of 1 N sodium hydroxide for the collection of liberated 

$^{14}$CO$_2$. A $^{14}$C-labeled lipid substrate solution containing 0.2 mM palmitate, 100 mM sucrose, 10 mM Tris - HCl, 5 mM potassium phosphate, 80 mM potassium chloride, 1 mM magnesium chloride, 0.1 mM malate, 2 mM ATP, 1 mM dithiothreitol, 0.2 mM EDTA, 1mM L-carnitine, 0.05 mM coenzyme A, and 0.5% fatty acid-free bovine serum albumin was then added to initiate reaction.

Following 30 min of incubation at 37°C, 100 ul of 70% perchloric acid was added to terminate the reaction. The trap wells were sampled for label incorporation into $^{14}$CO$_2$, which was determined by scintillation counting. In addition to complete oxidative products ($^{14}$CO$_2$), incomplete oxidative products (acid-soluble metabolites, ASM) were also measured using the supernatant from the original sample wells. The ratio of complete ($^{14}$CO$_2$) to incomplete (ASM) radiolabeled products was used to provide an index of fatty acid oxidation efficiency.

**Mitochondrial Enzyme Activity**

Measurements of mitochondrial enzyme activities were performed by Dr. Kristen Boyle. Mitochondrial supernatants (post 600 g) were isolated from fetal liver samples in Zheng buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EGTA, pH 7.4).
The supernatants were used to assay activity of respiratory chain enzyme complex I, citrate synthase (CS), aconitase, and β-hydroxyacyl CoA dehydrogenase (β-HAD) on a Synergy H1 microplate reader (Biotek, Winooski, VT). Enzyme assays for respiratory chain complexes and CS were performed as described by Kendrick et al. [226], with minor modifications for microplate reading. For complex I and CS, enzyme activities were calculated as initial rates (nmol/min). For aconitase assay, mitochondrial samples were incubated with appropriate reaction buffers for 2 min at 30°C, after which specific activators were added and reactions were followed for 5 min at specified wavelengths. Zheng buffer was used as a blank and run in duplicate with each assay run. Aconitase activity was measured as described by Xu et al. [227] with modifications for microplate reading. All assays were performed in duplicate. The protein content of each sample was determined using a bicinchoninic acid assay (BCA assay). All activities were normalized to the total protein content of each sample.

Additional Data Analysis

To correct for a common maternal environment, fetal results are expressed as a single average for each mother. Male and female data were analyzed separately, and subsequently combined after no between-group sexual dimorphism was identified. Differences between maternal CD and HFD groups were determined by 2-tailed Student’s t test using the Graph Pad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA). When variances were not equal, a 2-tailed t test with Welch’s correction
was performed. All data are expressed as the mean ± standard error of mean (SEM), and differences were determined to be significant at \( P < 0.05 \).

### 4.3 Results

As described in Chapter III, fetuses from HFD mothers demonstrated a significant reduction in growth relative to placental size. Their placentas were heavier, with increased surface area, and showed evidence of increased lipid deposition and a pro-inflammatory microenvironment. In this chapter, we will discuss what corresponding changes occurred in the fetus, with a specific focus on the fetal liver.

**No Observed Changes in Fetal Serum With Maternal HFD**

Despite a trend for reduced growth in HFD fetuses, there were no significant changes in pooled fetal serum levels of glucose, insulin, TG, or FFA between maternal groups (Table 4.1). Given the low volume of fetal serum, we attempted to use a multiplex ELISA to detect fetal levels of the pro-inflammatory cytokines IL-1β, IL-6, and TNFα, but levels in all experimental groups at E18.5 were below the detectable range of the assay in the majority of samples and therefore are not reported here.

<table>
<thead>
<tr>
<th>Table 4.1: Fetal E18.5 fasting serum.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mother</strong></td>
</tr>
<tr>
<td>WT-CD</td>
</tr>
<tr>
<td>WT-HFD</td>
</tr>
</tbody>
</table>

Measurements were conducted on pooled litter serum for each mother. Data are expressed as mean ± SEM for the fetal serum of \( n = 12 - 14 \) mothers per maternal group.
Maternal HFD Promotes Fetal Hepatic Lipid Accumulation

To determine whether the pattern of placental lipid accumulation seen in HFD mothers was also apparent in the fetal liver, GC-MS was performed on fetal liver lipid fractions. As with the placenta, fetal livers from HFD mothers showed increased TG levels relative to CD mothers (P<0.02, respectively; Figure 4.1). Oil Red O staining of cryosectioned tissue confirmed these results, showing diffuse accumulation of lipid droplets throughout the fetal liver of HFD mothers (Figure 4.1).

![Figure 4.1: Fetal liver triglycerides.](image)

(Left) Quantification of fetal liver TG by GC-MS on extracted tissue TG lipid fractions (n = 6 maternally unique fetal livers per experimental group). (Right) Oil Red O staining of representative fetal liver cryosections to localize TG (lipid droplet = red, cell nuclei = blue, 10X magnification). All data are expressed as mean ± SEM for each maternal group; *P<0.05 vs. WT-CD.
Maternal diet also significantly impacted the composition of the triglyceride lipid fractions from the fetal liver. Specifically, HFD fetal liver TG were composed of more polyunsaturated fatty acids (PUFA) relative to CD fetal livers, while CD fetal livers were instead composed of relatively more saturated and mono-unsaturated lipid (Figure 4.2). These differences are primarily explained by a disproportionate increase in the amount of PUFA the fetal hepatic TG fractions from HFD mothers, with smaller increases in saturated and monounsaturated fatty acids (Figure 4.2).

![Figure 4.2: Fetal liver triglyceride composition.](image)

**Figure 4.2:**  Fetal liver triglyceride composition.

Quantification of saturated fatty acids (Sat FA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) within TG lipid fractions. All data are expressed as mean ± SEM for each maternal group; *P<0.05 vs. WT-CD.

Importantly, these differences are not reflective of differences in the composition of fat within the mothers’ respective diets. The composition of TG PUFA did not differ between CD and HFD fetal livers, however both were primarily composed of linoleic
(18:2, n-6), arachidonic acid (10:4, n-6) and n-3 docosahexanoic acid (22:6, n-3), with an average overall n3:n6 ratio of about 1:4 in both HFD and CD (Figure 4.2), while maternal diets had a starting n3:n6 ratio of about 1:8. The composition of fetal liver phospholipid fractions did not differ significantly between groups.

**Changes in Fetal Liver Gene Expression With Maternal High-Fat Diet**

Pooled RNA samples (n = 3 per maternal group) containing the RNA from 3 maternally-unique fetal livers were analyzed by microarray to determine whether increased hepatic lipid deposition was associated with *in utero* gene expression changes. Interestingly, results for fetal livers from HFD mothers suggested an increase in mitochondrial genes associated with oxidative metabolism, including genes for subunits of ATP synthase, cytochrome-c oxidase, and NADH dehydrogenase. Additionally, an important regulator of lipid droplet formation, perilipin 5 was significantly upregulated. Further, HFD fetal livers showed a decrease in genes associated with *de novo* lipogenesis and cholesterol synthesis, including steroyl-CoA desaturase 2 and HMG-CoA synthase and reductase. Also, a down-regulation in both the fads1 and fads2 genes, which encode the delta-5 and delta-6 desaturases (respectively) required for the conversion of short chain PUFA to their long chain biologically active counterparts were down-regulated in HFD fetal livers (Figure 4.3).
Figure 4.3: Heat map of fetal liver gene changes.

Up (red) and down (blue) -regulated genes between CD and HFD fetal liver pooled samples (n = 3 maternally-unique fetal livers per pool, 3 pools per experimental group). Cut off for analysis were fold change >1.2, false detection rate (FDR)<0.3, P<0.05.
Confirmation of these gene changes by qPCR demonstrated the same patterns as observed by microarray, but with only fatty acid desaturase 2 (Fads2), Acetyl-CoA synthetase 2 (Acss2), retinol saturase (RetSat), and perilipin 5 (Plin5) reaching statistical significance, likely due to low sample number and reduced sensitivity with pooling samples (Figure 4.4). All these changes support an overall increase in the influx of fatty acids, particularly long chain PUFA into the developing fetal liver.

**Figure 4.4:** Quantitative PCR for validation of fetal liver microarray.

Results expressed as mean ± SEM for n = 3 maternally-unique fetal livers per pool, 3 pools per experimental group; *P<0.05 vs. WT-CD.
In order to better identify cumulative changes in gene expression pathways, we performed Gene Set Enrichment Analysis (GSEA) using the microarray gene expression data. Results for biological process pathways suggest a significant enrichment for substrate metabolism, particularly fatty acid metabolism, in HFD fetal livers relative to CD (Table 4.2).

Table 4.2: Fetal liver GSEA - Biological processes enriched with HFD.

<table>
<thead>
<tr>
<th>Biological Process Name</th>
<th># Genes</th>
<th>Enrichment Score</th>
<th>p-value</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CELLULAR_RESPIRATION</td>
<td>19</td>
<td>0.7011</td>
<td>0.0000</td>
<td>0.0147</td>
</tr>
<tr>
<td>FATTY_ACID_OXIDATION</td>
<td>17</td>
<td>0.6991</td>
<td>0.0000</td>
<td>0.0230</td>
</tr>
<tr>
<td>AEROBIC_RESPIRATION</td>
<td>15</td>
<td>0.7048</td>
<td>0.0000</td>
<td>0.0432</td>
</tr>
<tr>
<td>AROMATIC_COMPOUND_METABOLIC_PROCESS</td>
<td>25</td>
<td>0.6002</td>
<td>0.0000</td>
<td>0.0379</td>
</tr>
<tr>
<td>AMINO_ACID_METABOLICPROCESS</td>
<td>69</td>
<td>0.4719</td>
<td>0.0000</td>
<td>0.0413</td>
</tr>
<tr>
<td>AMINO_ACID_CATABOLIC_PROCESS</td>
<td>21</td>
<td>0.6073</td>
<td>0.0078</td>
<td>0.0543</td>
</tr>
<tr>
<td>NITROGEN_COMPOUND_CATABOLICPROCESS</td>
<td>25</td>
<td>0.5809</td>
<td>0.0020</td>
<td>0.0495</td>
</tr>
<tr>
<td>ORGANIC_ACID_METABOLIC_PROCESS</td>
<td>151</td>
<td>0.3966</td>
<td>0.0000</td>
<td>0.0688</td>
</tr>
<tr>
<td>AMINE_CATABOLIC_PROCESS</td>
<td>23</td>
<td>0.5799</td>
<td>0.0064</td>
<td>0.0819</td>
</tr>
<tr>
<td>CARBOXYLIC_ACID_METABOLIC_PROCESS</td>
<td>149</td>
<td>0.3948</td>
<td>0.0000</td>
<td>0.0754</td>
</tr>
<tr>
<td>ELECTRON_TRANSPORT_GO_0006118</td>
<td>41</td>
<td>0.4888</td>
<td>0.0060</td>
<td>0.0929</td>
</tr>
<tr>
<td>CELLULAR_LIPID_CATABOLIC_PROCESS</td>
<td>28</td>
<td>0.5360</td>
<td>0.0040</td>
<td>0.0958</td>
</tr>
<tr>
<td>GENERATION_OF_PRECURSOR_METABOLITES_AND_ENERGY</td>
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<td>0.4015</td>
<td>0.0000</td>
<td>0.0950</td>
</tr>
<tr>
<td>MITOCHONDRION_ORGANIZATION_AND_BIOGENESIS</td>
<td>44</td>
<td>0.4671</td>
<td>0.0080</td>
<td>0.0937</td>
</tr>
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<td>ENERGY_DERIVATION_BY_OXIDATION_OF_ORGANIC_COMPOUNDS</td>
<td>35</td>
<td>0.4933</td>
<td>0.0058</td>
<td>0.1281</td>
</tr>
<tr>
<td>AMINO_ACID_AND_DERIVATIVE_METABOLIC_PROCESS</td>
<td>90</td>
<td>0.4051</td>
<td>0.0000</td>
<td>0.1255</td>
</tr>
<tr>
<td>INDUCTION_OF_APOPTOSIS_BY_EXTRACELLULAR_SIGNALS</td>
<td>26</td>
<td>0.4976</td>
<td>0.0201</td>
<td>0.1598</td>
</tr>
<tr>
<td>COENZYME_METABOLIC_PROCESS</td>
<td>35</td>
<td>0.4662</td>
<td>0.0193</td>
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</tr>
<tr>
<td>MONOCARBOXYLIC_ACID_METABOLIC_PROCESS</td>
<td>70</td>
<td>0.4068</td>
<td>0.0039</td>
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</tr>
<tr>
<td>MITOCHONDRIAL_TRANSPORT</td>
<td>20</td>
<td>0.5276</td>
<td>0.0195</td>
<td>0.1984</td>
</tr>
<tr>
<td>REGULATION_OF_HEART_CONTRACTION</td>
<td>19</td>
<td>0.5278</td>
<td>0.0254</td>
<td>0.2077</td>
</tr>
</tbody>
</table>

Results follow moderately permissive cut-off values of \( P<0.01 \), FDR<0.20.
Interestingly, as opposed to metabolic pathways, the gene enrichment analysis for CD fetal livers instead showed an increase in pathways necessary for cell cycle progression and cell division. This suggests that cells in HFD fetal livers may not only be more metabolically active, but may be comparatively more senescent when compared to CD fetal livers (Table 4.3). However, the false detection rate (FDR) for these enriched groups was relatively high when compared to HFD fetal livers, suggesting a certain degree of variability.

Table 4.3: Fetal liver GSEA – Biological processes enriched with CD.

<table>
<thead>
<tr>
<th>Biological Process Name</th>
<th># Genes in Process</th>
<th>Enrichment Score</th>
<th>p-Value</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHROMOSOMESEGREGATION</td>
<td>26</td>
<td>-0.5811</td>
<td>0.0039</td>
<td>0.3037</td>
</tr>
<tr>
<td>G1_S_TRANSITION_OF/MITOTIC_CELL_CYCLE</td>
<td>27</td>
<td>-0.5384</td>
<td>0.0078</td>
<td>0.5707</td>
</tr>
<tr>
<td>CYTOKINESIS</td>
<td>17</td>
<td>-0.6118</td>
<td>0.0103</td>
<td>0.3907</td>
</tr>
<tr>
<td>CELL_DIVISION</td>
<td>19</td>
<td>-0.5922</td>
<td>0.0100</td>
<td>0.3426</td>
</tr>
<tr>
<td>M_PHASE</td>
<td>95</td>
<td>-0.4004</td>
<td>0.0000</td>
<td>0.3642</td>
</tr>
<tr>
<td>MITOTIC_CELL_CYCLE</td>
<td>132</td>
<td>-0.3795</td>
<td>0.0000</td>
<td>0.3664</td>
</tr>
<tr>
<td>M_PHASE_OF/MITOTIC_CELL_CYCLE</td>
<td>74</td>
<td>-0.4213</td>
<td>0.0000</td>
<td>0.3365</td>
</tr>
<tr>
<td>MITOSIS</td>
<td>72</td>
<td>-0.4144</td>
<td>0.0101</td>
<td>0.3801</td>
</tr>
<tr>
<td>CELL_CYCLE_PROCESS</td>
<td>165</td>
<td>-0.3502</td>
<td>0.0000</td>
<td>0.4361</td>
</tr>
<tr>
<td>CELL_CYCLE_PHASE</td>
<td>146</td>
<td>-0.3516</td>
<td>0.0021</td>
<td>0.4751</td>
</tr>
</tbody>
</table>

Results follow permissive cut-off of P<0.05, with no FDR cutoff set.

**No Functional Changes Observed in Fetal Liver Mitochondrial Metabolism**

Given the gene changes in HFD fetal livers associated with increased mitochondrial metabolism, we assayed the ability of freshly harvested fetal liver homogenates to oxidize lipid substrate *in vitro* (*n* = 4 livers per mother, 4 - 6 mothers per group). However, we observed no significant difference in the complete oxidation of 14C-labeled palmitate to 14CO2 between groups (Figure 4.5). Furthermore, there was
no difference in the level of partially oxidized $^{14}$C-labeled acid-soluble metabolites (ASMs), and thus no difference in mitochondrial oxidative efficiency, as measured by the ratio of complete ($^{14}$CO$_2$) to incomplete (ASM) oxidation (Figure 4.5). Importantly, the rate of fatty acid oxidation in fetal livers was roughly one third of what we traditionally observe in healthy adult animals, suggesting that this metabolic pathway has not completely matured by E18.5.

In addition to overall fatty acid oxidation, we measured the activity of individual mitochondrial enzymes associated with fatty acid β-oxidation, the TCA cycle, and the mitochondrial respiratory chain. First, we assayed citrate synthase activity, the initial enzyme of the TCA cycle whose constitutive activity is used as a measure of overall mitochondrial number in a cell, and thus a normalizing factor for all other mitochondrial enzyme activity. We observed no significant difference in citrate synthase activity between fetal livers exposed to maternal HFD versus CD, indicating no increase in mitochondrial number (Figure 4.5).

In keeping with the above data, there was also no change in β-hydroxyacyl-CoA-dehydrogenase (fatty acid β-oxidation, β-HAD), aconitase (TCA cycle), or complex I (respiratory chain) activity between groups, whether alone or normalized to citrate synthase activity (Figure 4.6).
Figure 4.5: Fetal liver fatty acid oxidation.

Quantitation of *in vitro* oxidation of $^{14}$C-labeled palmitate to $^{14}$CO$_2$ (FAO), incomplete oxidation (ASM), and estimation of oxidative efficiency (FAO/ASM) in freshly harvested fetal liver homogenates. Results are the average of $n = 4$ fetal livers/mother, $n = 5$ mothers per experimental group, and are displayed as maternal mean ± SEM.
Figure 4.6: Fetal liver mitochondrial enzyme activity.

Fetal liver citrate synthase activity, an accepted measure of mitochondrial number, was used to normalize all other measurements to determine overall mitochondrial enzyme activity. Results are the average of $n = 4$ fetal livers/mother, $n = 5$ mothers per experimental group, and are displayed as maternal mean ± SEM.
4.4 Discussion

There is now growing evidence that maternal obesity, independent of diabetes, contributes to adverse metabolic outcomes in children, including insulin resistance, obesity, and metabolic syndrome [141,228,229]. Therefore, it is not surprising that maternal obesity may also be an important risk factor for pediatric NAFLD. In the current study we utilized a HFD mouse model of obese pregnancy to examine the impact of both obesity and HFD on fetal hepatic lipid exposure, and how such an exposure alters hepatic gene expression and fatty acid metabolism.

Despite a trend for reduced growth efficiency in HFD relative to CD fetuses, we observed no significant changes in fetal serum levels of glucose, insulin, TG, or FFA. Importantly, blood glucose levels from HFD fetuses did not reflect the elevation observed in HFD mothers. However, further experiments such as maternal and fetal blood sampling across gestational time points, or tracking of a maternally-administered labeled substrate, would aid in any definitive conclusions regarding maternal nutrient transport and fetal exposure to these maternal nutrients in the context of maternal obesity and HFD.

Along the same lines of measuring exposure-over-time versus acute serum levels, our primary observation in HFD fetuses was an increase in TG deposition in the fetal livers. Specifically, there was an increase in the amount of PUFA within these TG, with smaller increases in saturated and monounsaturated fatty acids. Lipid accretion is critical for normal fetal development. Particularly important is maternal supply of essential PUFA, which are required for proper cell membrane structure and function,
and cannot be synthesized by the fetus [164]. However, excess lipids may be cytotoxic and induce metabolic dysfunction. In adults, lipotoxicity is caused by increases in intracellular lipids, resulting in toxic levels of ceramides, diacylglycerols and reactive oxygen species (ROS). These lipid species can activate cellular stress and inflammation pathways, and consequently lead to cell death. Excess fetal lipid exposure prior to the development of fetal adipose tissue may, therefore, be an important mechanism not only for the observed ectopic lipid deposition in the fetal liver, but for potential activation of cellular stress pathways.

Increases in hepatic lipid influx and synthesis relative to utilization and export can lead to cellular lipotoxicity, and is fundamental in the pathophysiology of adult NAFLD [230]. The liver is a main regulator of lipid homeostasis, and as such it carries out multiple metabolic processes, including de novo lipogenesis, fatty acid esterification, lipoprotein processing and export, and β-oxidation for energy. In adults, this imbalance occurs with the development of hepatic insulin resistance, but whether this is the case in the developing fetus is still unknown. Thus, we sought to elucidate whether the increased lipid deposition we observed in HFD fetal livers might alter the expression of key genes associated with these metabolic processes, and possibly identify genetic culprits of later offspring NAFLD development. Interestingly, our completely unbiased approach of gene expression analysis by microarray yielded gene changes almost exclusively associated with hepatic metabolic processes, particularly those involved in fatty acid oxidation and synthesis.
The specific gene expression changes we observed in HFD fetal livers, as well as the pathways indicated to be enriched by GSEA, were associated with both fatty acid catabolism and storage, while genes associated with de novo lipogenesis, specifically fatty acid desaturation and elongation as well as cholesterol synthesis, were down regulated relative to CD fetal livers. Importantly, the most striking take away from the changes in fetal liver gene expression was how closely HFD fetal livers resembled the gene expression profile of a fasted liver; specifically, the genes regulated by the activation of the nuclear receptor PPARα, and its antagonism of the LXR-SREBP1c transcriptional network [231,232].

PUFA and their eicosanoid derivatives are the main ligands for PPARα, which is classically activated in the liver by influxes of lipids from adipose tissue lipolysis, which occurs with the low insulin levels of fasting. PPARα in turn transcribes genes necessary for utilizing those fatty acids for energy (fatty acid oxidation), as well as genes necessary for storage of un-utilized fatty acids, for instance lipid droplet coat proteins. Opposing this is the fed liver state, where elevated insulin levels suppress lipolysis and lead to the activation of sterol regulatory element-binding protein 1c (SREBP-1c), which is responsible for the expression of many genes necessary for the de novo lipogenesis of lipids from any excess glucose supplied during feeding. LXR, an activator of SREBP1c [233], often competes with PPARα for their shared requisite heterodimer retinoic receptor X (RXR). Additionally PUFA themselves have been shown to antagonize SREBP1c targets [233]. Both of these mechanisms rely on lipids, and specifically PUFA, to set the delicate balance between the fed and fasted states of the liver.
Thus, our data suggest that an excess flux of maternal lipids, especially PUFA, to the developing fetal liver may result in the activation of PPARα gene targets, as well as the storage of excess lipid in cytoplasmic lipid droplets. Whether these transcriptional changes actually resulted in increased mitochondrial fatty acid oxidation is less clear. Further studies to confirm the activation of PPARα, such as confirmation of nuclear enrichment as well as target promoter binding, would aid in supporting this concept.

It should be noted that despite these gene changes, de novo lipogenesis is limited in the fetus [234], and while mature hepatocytes routinely process fatty acids for ATP synthesis via β-oxidation, lipid oxidation in utero is limited, with glucose instead being the primary metabolic fuel [235]. This is supported by our findings of reduced overall fatty acid oxidation in the fetal liver relative to adult animals, which may also explain why the up-regulation in fatty acid metabolism genes that we observed in HFD fetal livers did not correspond with an increase in the ability of the HFD fetal liver to oxidize palmitate relative to CD fetal livers. However, this assay only yields data for the overall fatty acid oxidative capacity of the tissue, rather than the actual in vivo rate, so there may still be differential regulation within the fetus despite the immaturity of the system.

The changes we observed in fetal liver gene expression in response to maternal HFD are somewhat counter to those reported for juvenile and adult mouse offspring from HFD mothers. These offspring are reported to have reduced hepatic mitochondrial oxidative capacity, including decreased mitochondrial electron transport complex (ETC) I,II/III, and IV activity, and reduced carnitine palmitoyltransferase 1 (CPT-1) expression, resulting in reduced basal hepatic fatty acid oxidation and a blunting
in oxidative response to post-weaning HFD challenge [236,237]. While these changes fit with a paradigm of maladaptive programming, and a hepatic gene expression profile common to NAFLD patients, our results suggest a proper fetal adaptive response to hepatic lipid influx, with gene sets necessary to either store or metabolize such an influx increased. Interestingly, in a mouse model of nutrient restriction, hepatic genes involved in lipid metabolism were also found to be increased in newborn offspring [238], suggesting that early regulatory events in utero are particularly sensitive to nutrient availability. Also, it is important to note that this may further support an obese pregnancy model that has yielded a more growth-restricted phenotype, which could potentially be confounding the interpretation of our data.

Alternatively, the contrast between our fetal results and the results from others in adult offspring from HFD mothers may be an example of an acute versus chronic response to stimulus, and may be separate from later postnatal pathology. For instance, exposure to maternal HFD during the critical lactation period may exacerbate the development of fatty liver in juvenile offspring. Alternatively, a potential increase in fatty acid oxidation could have direct consequences on liver health if it is improperly carried out, for instance by damaged mitochondria, leading to decreased fatty acid oxidation in adulthood.

Impairments in mitochondrial metabolism can often become a chicken-or-egg scenario, with reduced mitochondrial function leading to metabolic dysregulation, and dysregulation further impairing mitochondrial function. Such a paradigm is frequently cited in the pathology of adult NAFLD and NASH. Mitochondria are the major endpoint
in fatty acid metabolism; however, in excess, fatty acids can promote their dysfunction. *In utero* exposure to excess lipids may potentiate both mitochondrial dysfunction and resulting oxidative damage. Our findings in the non-human primate clearly demonstrated hepatic oxidative stress as early as the third trimester concomitant with the previously discussed steatosis [222]. This is reinforced by data in rodent models, showing maternal over-nutrition resulted in activation of hepatic oxidative stress pathways in neonatal pups, which persisted into adolescence, even after weaning on to a control diet [239].

Ultimately, this mitochondrial damage can create a vicious cycle of reduced oxidative capacity and increased oxidative stress, which is thought to be one of the tenets underlying the development of NASH. Importantly, free radical damage is also known to induce cellular senescence and apoptosis pathways. Given our findings for the relative enrichment in gene expression pathways associated with cell growth and division in CD fetal livers relative to HFD, it may be worthwhile to determine if ROS-induced DNA damage is contributing to fetal hepatocellular senescence. This potential pathway is supported by data in adolescent mice offspring from HFD mothers demonstrating a programmed senescent phenotype in the liver, concomitant with the reduced expression of antioxidant genes [240].

It is not clear, at this point, whether the gene expression changes we observed play a causal role in the developmental programming of pediatric NAFLD, or are in fact evidence of proper hepatic function. However, the presence of increased lipid droplets in the fetal liver could itself be indicative of an early shift in metabolic
patterning towards a more pro-storage phenotype. This is similar to the development of NAFLD in adults, which is known to occur in stages, the first being steatosis. These animals could thus be more susceptible to later liver challenges, for instance continued exposure to HFD during lactation and early adolescent stages, leading to further disease progression.

As with all gene expression data, it is difficult to delineate between causal and compensatory gene expression. For instance, should mitochondrial fatty acid oxidation capacity be impaired, these genes may be up-regulated to maintain normal mitochondrial function. Thus, whether these gene changes are in fact a causal contribution to potential programming pathology is not yet clear. However, our findings do clearly demonstrate a unique fetal environment imparted by maternal obesity and HFD, where excess placental lipid uptake and transport of PUFA, as well as monounsaturated and saturated fatty acids, to the developing fetus results in fetal hepatic lipid accumulation and acute changes in gene expression that correspond with an increase in PUFA influx. Normalizing these changes with a targeted intervention at the level of maternal and placental lipid metabolism is an important next step in identifying the causal role of fetal lipid exposure on the downstream programming of metabolic pathways. Further, whether these changes have postnatal consequences warrants further examination.
5.1 Introduction

The prevalence of pediatric obesity has increased dramatically over the past two decades and is presenting at a progressively younger age, implicating the importance of early life events in children’s long-term metabolic health. As discussed throughout these chapters, both human and animal studies strongly support the idea that early fetal and neonatal exposure to an obese maternal environment can have long-term negative consequences. However, our knowledge of which specific maternal factors are responsible for these consequences, and therefore potential pathways for intervention, remains limited.

Chronic low-grade inflammation is often implicated in the pathogenesis of the metabolic syndrome and insulin resistance in the non-pregnant obese population [184,185]. In fact, in non-pregnant animal models, disassociating obesity from its characteristic adipose tissue inflammation is a particularly effective means of preventing insulin resistance and other downstream metabolic complications [241-246], suggesting that maternal metabolic inflammation may be an important therapeutic target. While

\[184,185\] Portions of this chapter are from 169. Heerwagen MJ, Stewart MS, de la Houssaye BA, Janssen RC, Friedman JE (2013) Transgenic increase in n-3/n-6 fatty acid ratio reduces maternal obesity-associated inflammation and limits adverse developmental programming in mice. PLoS One 8: e67791.
maternal systemic and placental inflammation are now recognized as prominent features of pregnancies complicated by obesity [178-183,247], little is known about the causal role this inflammation may play in altering fetal-placental developmental programming.

One comparatively simple way to reduce metabolic inflammation is by increasing tissue levels of anti-inflammatory n-3 PUFA relative to pro-inflammatory n-6 PUFA [248-251], as evidenced by the Fat-1 transgenic mouse previously described in Chapter I. In this chapter, we utilize the Fat-1 transgenic mouse on HFD to demonstrate that increasing the maternal n-3/n-6 PUFA ratio can effectively reduce maternal metabolic inflammation, thereby reversing HFD-associated fetal and placental lipid exposure in the absence of a change in maternal obesity. In addition, we show that protecting the mother from obesity-induced chronic inflammation attenuates the development of long-term metabolic consequences of maternal obesity and HFD in WT offspring. On the basis of these results, we discuss how maternal inflammation may play a direct role in the pathways involved in the metabolic programming of the infant.

5.2 Methods

Animal Breeding and Diet

All animal studies were approved by the University of Colorado Institutional Animal Care and Use Committee and carried out in strict accordance with the guidelines
set forth by the Guide for the Care and Use of Laboratory Animal by the National Institutes of Health.

Transgenic Fat-1 C57BL6/J male mice were provided courtesy of Dr. J.X. Kang [123], and bred to wild type (WT) C57BL6/J females to obtain roughly 50% transgene-positive mixed litters. As described in Chapter III, eight-week-old female littermates, either WT or hemizygous Fat-1, were then placed on a 45% kcal high-fat diet (HFD) or a 10% kcal control diet (CD) and fed ad libitum for 8 wks prior to mating with a WT chow-fed male.

Fetal pups were analyzed at embryonic day 18.5 (n = 12-14 mothers per group), using the approaches discussed Chapters III and IV. Additionally, a second group of offspring from a second group of mothers was allowed to deliver (n = 10 mothers per group). These offspring were weaned onto either CD or HFD ad libitum, and analyzed at 20 wks of age (Figure 5.1).

As discussed in Chapters III and IV, fetal sex was determined by tail-DNA PCR for SRY gene. Additionally, for all fetal and adult offspring from Fat-1 mothers, genotyping for presence or absence of the fat-1 transgene was carried out (Primers – Appendix A), and only WT offspring from Fat-1 mothers were included in analysis. For adult offspring studies, litters were standardized to 8 pups on postnatal day 1 to control for nutrient bias during lactation. At time of analysis, mothers and adult offspring were euthanized by isoflurane anesthesia followed by cervical dislocation and exsanguination, and fetal pups were euthanized by decapitation and exsanguination (Figure 5.1).
Figure 5.1: Study design: Inclusion of transgenic mother and adult offspring
Mothers were fed either control or high-fat diets (CD, HFD respectively), for 8 weeks prior to mating as first described in chapter 3. For this chapter, an interventional third of a Fat-1 transgenic mother placed on HFD was added. Diet was maintained through pregnancy, and mothers, fetuses, and placentas were harvested at embryonic day 18.5, or allowed to give birth and adult offspring analyzed at 20 wks of age. Only the WT fetuses, placentas, and offspring from Fat-1 mothers were analyzed.

Flow Cytometry for Adipose Macrophages

Adipose tissue macrophages were isolated, quantitated, and characterized by flow cytometry as previously described in Chapter III.

Serum Analyses

Maternal, fetal, and adult offspring serum glucose, insulin, and lipids were measured as previously described in Chapters III and IV.
Placental and Fetal Liver Lipid Analysis by GC-MS

Placental and fetal liver lipids were quantititated and characterized as previously described in Chapters III and IV.

Adult Offspring Metabolic Analyses

Offspring weight was recorded at weaning and every-other week thereafter. At 20 weeks of age, animals were fasted 5 hrs before blood glucose was measured from the tail vein and recorded by glucometer. An insulin tolerance test (ITT) was then performed. Briefly, a bolus of recombinant insulin was injected IP at 0.0075 U/kg body weight, and blood glucose readings were taken from tail blood by glucometer at 0, 10, 20, 30, 45, 60, 75, and 90 mins post-injection. Body composition (fat and lean mass) was determined 3 days post-ITT by EchoMRI™ Body Composition Analyzer (EchoMRI, Houston, TX).

Adult Offspring Liver Triglyceride Quantitation

Adult offspring liver TG was quantitated biochemically as previously described for maternal livers in Chapter III.

Adult Offspring Liver Histology

Adult offspring liver was cryosectioned and stained as described for the placenta and fetal livers in Chapters III and IV, respectively.
**Adult Offspring Liver Fatty Acid Oxidation**

Adult offspring hepatic fatty acid oxidation was performed as previously described for fetal livers in Chapter IV.

**Data Analysis**

To correct for a common maternal environment, results for both fetal and adult offspring were expressed as an average for each mother, with siblings treated as replicates. Only WT placentas, fetuses, and adult offspring from Fat-1 mothers were included in these averages. For fetal phenotyping, male and female data were analyzed separately, and pooled after no between-group sexual dimorphism was identified, while analyses in adult offspring were separated by sex. Differences between maternal groups (and their offspring) were determined by 1-way ANOVA with Bartlett’s test for equal variance using the Graph Pad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA). When variances were not equal, a non-parametric Kruskall-Wallis test and Gaussian approximation were used. Post-hoc Student’s t tests were performed when necessary to identify between group differences. For experiments involving a time course (weight gain, ITT), a 2-way ANOVA with time as a repeated measure variable was used, with Bonferroni post-tests to compare replicate means. All data are expressed as the mean ± standard error of mean (SEM), and differences were determined to be significant at $P<0.05$. 

5.3 Results

This chapter will focus on the efficacy of maternal expression of the fat-1 transgene in the context of HFD, given our previous findings. Thus, the data previously generated from WT-CD and WT-HFD maternal groups in Chapters III and IV is presented again here for comparison purposes.

Maternal obesity-associated inflammation is suppressed by Fat-1 transgene

Fat-1 and WT mice gained similar weight on the HFD prior to mating and during pregnancy relative to CD mothers ($P<0.05$; Figure 5.2).

![Figure 5.2: Maternal pre-pregnancy and gestational weight gain.](image)

Maternal weight for 8 wks prior to mating, and at E18.5 (late gestation). Late gestation weight was estimated by subtracting the complete fetal-placental litter weight from the weight of the pregnant mother. Results are the average of $n = 12 - 14$ mothers per experimental group. Data represented as mean ± SEM; *$P<0.05$ vs. WT-CD.
To determine whether fat-1 expression had any effect on the obesity-associated inflammation we observed in Chapter III, we again assessed both adipose tissue macrophage content and serum pro-inflammatory cytokine levels in late gestation (E18.5) Fat1-HFD mothers. Despite similar weight gain on HFD, Fat1-HFD mothers did not demonstrate the same increase in adipose tissue macrophage content as WT-HFD mothers, as measured by percent F4/80+ cell population in stromal fractions by flow cytometry (P<0.02; Figure 5.3).

Figure 5.3: Maternal adipose tissue macrophages.

Adipose tissue (AT) macrophage quantitation by flow cytometry. Macrophage marker F4/80 and M1 macrophage marker CD11c were used to determine the percent of macrophages present and their relative M1 polarization in maternal AT stroma at E18.5. Results are the average of n = 12 - 14 mothers per experimental group. Data represented as mean ± SEM; *P<0.05 vs. WT-CD.
Additionally, serum levels of circulating pro-inflammatory cytokines in Fat1-HFD mothers were reduced to the level of WT-CD mothers ($P<0.05$, Figure 5.4), further supporting a degree of protection from a HFD-related pro-inflammatory response offered by the fat-1 transgene.

Figure 5.4: Maternal serum cytokine levels.

Relative maternal serum pro-inflammatory cytokine levels at E18.5 by membrane-bound antibody array. Results are the average of three membranes per maternal group, with each membrane incubated with the pooled serum from four separate mothers. Data represented as mean ± SEM; *$P<0.05$ vs. WT-CD and Fat1-HFD.

Importantly, this protection carried over to the placenta as well, where IL-1β expression and the ratio of arginase-1 (Arg-1) to iNos expression, previously observed to be significantly increased in WT-HFD placenta ($P<0.05$; Figure 5.5), were again reduced in the WT placenta from Fat1-HFD mothers.
This reduction in overall inflammatory load in Fat1-HFD mothers also corresponded with a reduction in fasting insulin levels, and a consequent reduction in HOMA-IR score relative to WT-HFD mothers ($P<0.05$ and $P<0.01$, respectively; Table 5.1). In fact, there were no significant differences in maternal late gestation fasting serum between Fat1-HFD and WT-CD mothers for any parameters measured (Table 5.1).

Table 5.1: Maternal E18.5 fasting serum.

<table>
<thead>
<tr>
<th>Mother</th>
<th>Glucose (mg/dL)</th>
<th>Insulin (ng/mL)</th>
<th>HOMA-IR</th>
<th>FFA (mg/L)</th>
<th>TG (mg/dL)</th>
<th>Glycerol (mg/dL)</th>
<th>Adiponectin (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-CD</td>
<td>95 ± 6</td>
<td>0.41 ± 0.06</td>
<td>2.12 ± 0.26</td>
<td>0.98 ± 0.05</td>
<td>22.98 ± 3.97</td>
<td>15.51 ± 0.61</td>
<td>1.17 ± 0.27</td>
</tr>
<tr>
<td>WT-HFD</td>
<td>*117 ± 7</td>
<td>*0.83 ± 0.13</td>
<td>*6.21 ± 1.34</td>
<td>*0.75 ± 0.04</td>
<td>18.23 ± 1.31</td>
<td>15.46 ± 0.87</td>
<td>1.40 ± 0.17</td>
</tr>
<tr>
<td>Fat1-HFD</td>
<td>104 ± 6</td>
<td>0.53 ± 0.08</td>
<td>2.86 ± 0.61</td>
<td>0.86 ± 0.06</td>
<td>18.62 ± 2.85</td>
<td>16.46 ± 0.91</td>
<td>1.20 ± 0.14</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM of $n = 12 - 14$ mothers per maternal group; *$P<0.05$ vs. WT-CD and Fat1-HFD.

Figure 5.5: Placenta inflammatory gene expression.

Placental gene expression of select pro-inflammatory cytokines and macrophage markers by quantitative PCR ($n = 9$ mothers per group, 3 RNA pools of 3 mothers each). Data represented as mean ± SEM; *$P<0.05$ vs. WT-CD and Fat1-HFD.
**Reduced Fetal/Placental Ratio is Ameliorated in Fat1-HFD Mothers**

As described in Chapter III, Fetuses from WT-HFD mothers had significantly larger placentas versus WT-CD mothers at E18.5, and fetal weights in WT-HFD mothers trended lower than in WT-CD mothers, leading to a reduced fetal-placental ratio overall. This pattern was prevented in WT fetuses from Fat1-HFD mothers, who demonstrated similar placental and fetal growth to WT-CD mothers (Figure 5.6A-C).

![Figure 5.6: Fetal and placental gross measurements.](image)

(A) Representative images of fetal-placental unit size for each maternal group. (B) Average fetal and placental weights for each litter. Placentas were weighed subsequent to removal of umbilical cord and fetal membranes. (C) Average calculated placental surface area (SA) and fetal/placental (F/P) weight ratio for each litter. Placental surface area was calculated from measured diameter on maternal side. All data are expressed as mean ± SEM of the litter average for each mother (n=12-14 mothers per experimental group), with only WT fetal-placental units included in maternal averages for Fat1-HFD group; *P<0.05 vs. WT-CD and Fat1-HFD.
Additionally, as with the WT maternal groups, there were no significant changes in pooled fetal serum levels of glucose, insulin, TG, or FFA from Fat1-HFD mothers (Table 5.2).

Table 5.2: Fetal E18.5 fasting serum.

<table>
<thead>
<tr>
<th>Mother</th>
<th>Glucose (mg/dL)</th>
<th>Insulin (ng/mL)</th>
<th>FFA (mq/L)</th>
<th>TG (mg/dL)</th>
<th>Glycerol (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-CD</td>
<td>116 ± 16</td>
<td>1.78 ± 0.47</td>
<td>0.11 ± 0.02</td>
<td>26.53 ± 1.78</td>
<td>6.58 ± 0.72</td>
</tr>
<tr>
<td>WT-HFD</td>
<td>95 ± 9</td>
<td>1.35 ± 0.17</td>
<td>0.09 ± 0.01</td>
<td>26.90 ± 1.00</td>
<td>5.45 ± 0.52</td>
</tr>
<tr>
<td>Fat1-HFD</td>
<td>102 ± 12</td>
<td>1.88 ± 0.44</td>
<td>0.11 ± 0.01</td>
<td>24.89 ± 2.85</td>
<td>4.56 ± 0.64</td>
</tr>
</tbody>
</table>

Measurements were conducted on pooled litter serum for each mother. Data are expressed as mean ± SEM for the fetal serum of n = 12 - 14 mothers per maternal group.

**Maternal fat-1 Expression Protects Against Placental and Fetal Lipid Accumulation**

As previously described in chapters 3 and 4, placental and fetal liver lipid extracts were analyzed by GC-MS to determine if Fat1-HFD mothers demonstrated the same pattern of lipid deposition observed in WT-HFD mothers. Interestingly, despite high-fat feeding and weight gain, WT placentas and fetal livers from Fat1-HFD mothers had similar TG deposition to WT-CD mothers, which was visually supported by reduced size and number of lipid droplets by Oil Red O staining (Figure 5.7).

Additionally, given the expected impact of the fat-1 transgene on PUFA species, tissue differences in n-3/n-6 PUFA ratios were examined in placenta and fetal liver phospholipid fractions, where the majority of PUFA localize. While maternal HFD did not impair n-3 PUFA transfer, as seen by an equal stepwise increase in n-3/n-6 ratios from the placenta to the fetus in WT-CD and HFD mothers, maternal fat-1 transgene expression increased both placental and fetal liver n-3/n-6 ratios, primarily due to greater levels of the long chain n-3 PUFA DHA (figure 5.8).
Figure 5.7: Placental and fetal liver TG.

(Left) Quantification of placental and fetal liver TG by GC-MS on extracted tissue TG lipid fractions \( n = 6 \) maternally unique placenta-fetal liver pairs per experimental group. (Right) Oil Red O staining of representative placenta and fetal liver cryosections to localize increased TG deposition quantitated (lipid droplet = red, cell nuclei = blue, 10X magnification). All data are expressed as mean ± SEM; *\( P<0.05 \) vs. WT-CD and Fat1-HFD.
**Figure 5.8:** Placental and fetal liver n-3 and n-6 PUFA ratio.

GC-MS characterization of tissue phospholipid fractions to measure relative levels of n-3 and n-6 PUFA. All data are expressed as mean ± SEM; #P<0.05 vs. WT-CD and WT-HFD.
Corresponding with decreased lipid deposition relative to WT-HFD mothers, placental LPL activity in WT placentas from Fat1-HFD mothers was reduced to level of WT-CD mothers (Figure 5.9). Importantly, across all maternal groups, average placental LPL activity for each mother significantly correlated with degree of maternal insulin resistance, as measured by HOMA-IR ($r=0.59$, $P<0.02$; Figure 5.9).

![Graph showing placental LPL activity and maternal insulin resistance](image)

**Figure 5.9:** Placental LPL hydrolase activity.

Activity was measured in 3 - 4 placentas from each mother and averaged, with $n=6$ mothers per experimental group. (Right) Correlation of average maternal placental LPL activity and maternal insulin resistance, as measured by HOMA-IR. All data are expressed as mean ± SEM; *$P<0.05$ vs. WT-CD and Fat1-HFD.
WT Offspring from Fat-1 Mothers Selectively Protected from Adverse Programming

**Weight gain, body composition and insulin sensitivity.** To determine whether the protective properties of maternal fat-1 expression extended into adult offspring, WT pups from all mothers were weaned onto either CD or HFD for a total of 6 experimental groups, which were further divided by male/female sex, and analyzed at 20 wks of age. Groups are labeled by “Maternal Genotype – Maternal Diet”/”Offspring Genotype – Offspring Diet,” with a total of 6 groups.

In both sexes, weight did not differ between maternal groups at weaning; however, by 16wks post-weaning male and female WT-HFD/WT-CD offspring were significantly heavier relative to WT-CD/WT-CD offspring ($P<0.05$; Figure 5.10). While Fat1-HFD/WT-CD offspring demonstrated a pattern of increased weight gain relative WT-CD/WT-CD offspring, this difference was not significant. As expected, high-fat feeding led to increased weight gain in all groups. However, while that weight gain was significantly increased in male WT-HFD/WT-HFD offspring relative to both WT-CD/WT-HFD and Fat1-HFD/WT-HFD groups at 6 - 10 wks post-weaning, by 16 wks on diet this difference was no longer present (Figure 5.10). All female offspring groups weaned onto HFD gained significantly more weight than those weaned onto CD, but no programmed exacerbation in weight gain was apparent (Figure 5.11)
Figure 5.10: Male offspring weight gain.

WT Offspring from each maternal group (n = 10 mothers per group) were weaned on to CD, and weighed every other week for 16 weeks. All offspring data is averaged per mother, and only data for male offspring is presented, with siblings treated as replicates. For Fat-1 mothers, only WT offspring were included in analyses. All data are expressed as mean ± SEM and labeled according to maternal group; *P<0.05 vs. WT-CD.
Figure 5.11: Female offspring weight gain.

WT Offspring from each maternal group (n = 10 mothers per group) were weaned on to CD, and weighed every other week for 16 weeks. All offspring data is averaged per mother, and only data for male offspring is presented, with siblings treated as replicates. For Fat-1 mothers, only WT offspring were included in analyses. All data are expressed as mean ± SEM and labeled according to maternal group; *P<0.05 vs. WT-CD.
Male WT-HFD/WT-CD offspring had increased fat mass relative to male WT-CD/WT-CD and Fat1-HFD/WT-CD offspring \((P<0.05; \text{ Figure 5.12})\), who had similar body compositions. Female offspring on CD showed this same pattern between maternal groups, but the difference did not reach statistical significance (Figure 5.13). As with overall weight gain, both male and female offspring weaned onto HFD had significantly more fat mass, but the difference between maternal groups was no longer significant after 16 wks on diet. Lean mass did not differ between maternal groups, however HFD increased lean mass as well in all animals (Figures 5.12 and 5.13).

![Graphs showing body composition of male and female offspring](image)

**Figure 5.12: Male offspring body composition.**

Adult offspring body composition (fat and lean mass) at 16 wks post-wean, as determined by Echo MRI. All offspring data is averaged per mother, and only data for male offspring is presented, with siblings treated as replicates. For Fat-1 mothers, only WT offspring were included in analyses. All data are expressed as mean ± SEM and labeled according to maternal group; \(*P<0.05\) vs. WT-CD.
Differences in insulin sensitivity were determined by insulin tolerance test (ITT).

Male WT-HFD/WT-CD offspring demonstrated a reduced insulin response relative to male WT-CD/WT-CD and Fat1-HFD/WT-CD offspring ($P<0.05$; Figure 5.14), who displayed markedly similar serum glucose curves. On HFD, all male offspring had reduced insulin response relative to CD males, however the difference between WT-HFD/WT-HFD males and the two other groups was no longer statistically significant. While female offspring on CD were more insulin sensitive than those weaned onto HFD, there was no difference observed between maternal groups in either condition.
Figure 5.14: Adult offspring insulin sensitivity by insulin tolerance test (ITT).

Glucose curves in response to insulin tolerance test on fasted adult offspring 16 wks post-weaning. All offspring data is averaged per mother ($n = 10$ mothers), with siblings treated as replicates. For Fat-1 mothers, only WT offspring were included in analyses. All data are expressed as mean ± SEM and labeled according to maternal group; *$P<0.05$ vs. WT-CD and Fat1-HFD.
**Programming of downstream metabolic complications.** While both male and female offspring demonstrated similar programming patterns in terms of weight gain and fat mass, the difference in weight gain between female WT-CD/WT-CD and WT-HFD/WT-CD offspring was not as robust in comparison to males, with a non-significant trend for increased fat mass, and relative maintenance of insulin sensitivity in all groups. Additionally, for both sexes, high-fat feeding appeared to mask potential programming of body weight and fat mass by the time of harvest. Because of this, only male offspring weaned onto CD were examined further for additional metabolism-associated pathology.

First, as the programming of fatty liver by maternal HFD has been widely reported, we examined the livers of male CD offspring for TG content. In accordance with other groups, WT-HFD/WT-CD males had significantly higher levels of liver TG (Figure 5.15), which was confirmed by Oil Red O staining demonstrating the development of very fatty livers (Figure 5.15). Importantly, male Fat1-HFD/WT-CD offspring showed a significant reduction in liver TG relative to WT-HFD/WT-CD offspring, but still maintained an increased level relative to WT-CD/WT-CD offspring.

Increased hepatic TG deposition is often associated with a reduced rate of hepatic lipid oxidation, among other factors. To determine whether this may be true for offspring from HFD mothers, we tested the *in vitro* fatty acid oxidative capacity of freshly harvested offspring liver homogenates, as previously described in Chapter 4. We observed no significant difference in the complete oxidation of \(^{14}\)C-labeled palmitate between groups, however there was a significant reduction in partially-oxidized
acid-soluble metabolites (ASMs) in WT-HFD/WT-CD offspring relative to WT-CD/WT-CD offspring (Figure 5.16). This produced a trend for increased oxidative efficiency in WT-HFD/WT-CD offspring, as observed by the ratio of complete oxidation/incomplete oxidation, however this did not reach significance (Figure 5.16).

Figure 5.15: Adult male CD offspring liver triglycerides.

(Left) Liver TG quantitated in by colorimetric assay on tissue lipid extracts. (Right) Lipid droplets visualized in by Oil Red O staining of representative liver cryosections (lipid droplet = red, cell nuclei = blue, 20X magnification). All offspring data is averaged per mother, with siblings treated as replicates. For Fat-1 mothers, only WT offspring were included in analyses. All data are expressed as mean ± SEM and labeled according to maternal group; *P<0.05 vs. WT-CD; †P<0.05 vs. WT-HFD.
Figure 5.16: Adult male CD offspring liver fatty acid oxidation.

Quantitation of *In vitro* oxidation of $^{14}$C-labeled palmitate to $^{14}$CO$_2$ (FAO), incomplete oxidation (ASM), and estimation of oxidative efficiency (FAO/ASM) in freshly harvested liver homogenates. Results are the average of $n = 5$ maternally-unique male CD offspring per experimental group, and are displayed as maternal mean ± SEM; $^*P<0.05$ vs. WT-CD/WT-CD and Fat1-HFD/WT-CD.
Given the greater weight gain, adiposity and liver TG deposition in male WT-HFD/WT-CD offspring, obesity-associated inflammation was also assessed.

Adipose tissue depots of WT-HFD/WT-CD male offspring showed greater macrophage content, as well as increased M1 polarization ($P<0.001$ and $P<0.01$, respectively; Figure 5.17) relative to WT-CD/WT-CD offspring. This pattern of increased adipose tissue inflammation was not observed in Fat1-HFD/WT-CD offspring.

Figure 5.17: Adult male CD offspring adipose tissue macrophages.

Adipose tissue (AT) macrophage quantitation by flow cytometry. Macrophage marker F4/80 and M1 macrophage marker CD11c were used to determine the percent of macrophages present and their relative M1 polarization in adult offspring AT at 16 wks post-weaning. Results are the average of $n = 8$ maternally-unique male CD offspring per experimental group, and are displayed as maternal mean ± SEM; *$P<0.05$ vs. WT-CD/WT-CD.
Lastly, serum insulin levels were elevated in WT-HFD/WT-CD offspring only (\(P<0.02\); Table 5.3), further supporting a programmed increase in insulin resistance in WT-HFD/WT-CD offspring, with Fat1-HFD/WT-CD offspring showing levels more similar to WT-CD/WT-CD offspring. There were no additional differences in fasting serum Glucose, TG, or FFA levels between groups.

Table 5.3. Adult WT offspring fasting serum measurements.

<table>
<thead>
<tr>
<th>Offspring</th>
<th>Glucose (mg/dL)</th>
<th>Insulin (ng/mL)</th>
<th>FFA (mq/L)</th>
<th>TG (mg/dL)</th>
<th>Glycerol (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-CD/WT-CD</td>
<td>151 ± 10</td>
<td>2.04 ± 0.52</td>
<td>0.76 ± 0.13</td>
<td>92.4 ± 9.7</td>
<td>49.1 ± 3.6</td>
</tr>
<tr>
<td>WT-HFD/WT-CD</td>
<td>164 ± 8</td>
<td>*6.44 ± 1.71</td>
<td>0.59 ± 0.06</td>
<td>116.3 ± 6.5</td>
<td>47.1 ± 3.9</td>
</tr>
<tr>
<td>Fat1-HFD/WT-CD</td>
<td>154 ± 9</td>
<td>3.53 ± 0.98</td>
<td>0.51 ± 0.06</td>
<td>111.1 ± 9.0</td>
<td>41.0 ± 2.3</td>
</tr>
</tbody>
</table>

Measurements were performed on the 20 wk old fasting serum collected from male adult offspring sampled from \(n = 8\) mothers per experimental group. All offspring were weaned onto CD. Data are expressed as mean ± SEM; *\(p<0.05\) vs. WT-CD and Fat1-HFD.

5.4 Discussion

In the current study, we used the Fat-1 mouse model to demonstrate that increasing the maternal n-3/n-6 tissue fatty acid ratio can reduce maternal obesity-associated inflammation without any manipulation of maternal weight, thereby preventing fetal exposure to maternal metabolic impairments, and mitigating the adverse metabolic programming of adult offspring.

Our findings demonstrate that maternal expression of the \(fat-1\) transgene protects mothers from increases in obesity-associated F4/80+ macrophage accumulation in adipose tissue depots, and systemic increases in pro-inflammatory cytokines associated with immune cell activation, proliferation and chemotaxis. Importantly, this protection occurred despite equal weight gain between WT and Fat-1
HFD-fed mothers. This reduction in inflammation also corresponded with a reduction in maternal insulin resistance, as measured by HOMA-IR, with Fat1-HFD mothers not differing significantly from WT-CD mothers. These findings are consistent with the previously reported protection by the fat-1 transgene on HFD-associated metabolic inflammation and insulin resistance [126-128]. Importantly, this also carried over to the placentas from Fat1-HFD mothers, which did not show the same pro-inflammatory pattern as those from WT-HFD mothers.

Additionally, the increase in lipid deposition in both the placenta and fetal liver with maternal HFD was mitigated in Fat1-HFD mothers. Again, this was seen despite no detectable changes in maternal serum lipids between Fat-1 and WT mothers. Rather, a potential difference in placental lipid uptake, as measured by changes in placental LPL activity, was observed. Given that LPL is both an established gatekeeper in the tissue uptake of lipoprotein particles, and known to be regulated at a post-translational and tissue-dependent level [168,252], it follows that alterations in the maternal endocrine environment, such as increased inflammation and insulin levels, could impact its hydrolase activity [168,193].

Importantly, we did not observe any evidence of reduced n-3 PUFA in fetuses from HFD mothers, as our earlier findings in fetal plasma from non-human primates suggested [175]. However, we were only able to assess fetal tissue levels, due to the very low volume of fetal mouse plasma. Fetuses from all maternal groups had relatively high n-3 PUFA phospholipid levels compared to maternal diet, consistent with the theory that the placenta has a preferential uptake mechanism for n-3 PUFA
specifically, and maternal PUFA in general, relative to other lipid species [51,253,254].

It is important to note that we detected a significant increase in placental and fetal liver phospholipid DHA levels, and thus an increased n-3/n-6 PUFA ratio in WT offspring from Fat-1 mothers, which cannot be ruled out as a second potential mechanism of protection against adverse programming.

Critical to the above findings was the question of whether the protective effects observed in Fat1-HFD mothers had a long-term impact on offspring metabolic health, so we assessed the obesity phenotype in the adult offspring from a second cohort of mothers. Adult offspring were weaned onto either CD or HFD, however by the time of harvest at 16 wks post-diet, it was determined that the obesogenic effects of HFD were enough to mask the more subtle changes due to maternal HFD. Thus the majority of our analyses focused on offspring weaned onto CD. Male WT offspring from Fat1-HFD mothers weaned onto CD demonstrated reduced programming of weight gain, adiposity, fatty liver, adipose tissue macrophages, hyperinsulinemia, and insulin resistance relative WT-HFD offspring. In the majority of measures, Fat1-HFD/WT-CD offspring did not significantly differ from controls; however some trends in programming were not completely reversed, but rather mitigated. This underscores the complexity of the maternal-fetal obesity paradigm; where it is likely that many factors related to maternal obesity and HFD, in addition to the metabolic inflammation reported here, also impact the development of fetal metabolic systems.

It should be noted that, despite similar trends, we did not observe the same degree of severity in programming in female offspring as we did in males. Whether
this is due to actual differences in programming, or instead baseline sex differences in tendency towards metabolic disease, still remains to be clarified. As discussed in Chapter I, many animal models do show sexually dimorphic programming outcomes, particularly a susceptibility of male offspring to metabolic disorders resulting from maternal dietary fat exposure [255-259].

Our findings illustrate the potential to reduce adverse fetal programming events by indirectly limiting maternal inflammation to improve the metabolic environment in which the placenta and fetus develops. Transgenic increase of maternal n-3/n-6 PUFA reduces obesity-related inflammation, which is associated with improved maternal insulin sensitivity and reduced placental and fetal hepatic lipid accumulation. The mechanism by which excess cytokines and lipids can program fetal metabolic systems is still being elucidated, and there are likely many tissue targets and mechanisms. As discussed in Chapter IV, increased placenta and fetal hepatic lipid droplets are likely a method of cellular lipid storage and are not inherently damaging. However, our previous observations of increased fetal hepatic lipotoxicity and oxidative stress in primate fetal livers [75], as well as findings on the programming of fatty liver in adult offspring [10,147,260-262], suggest that an aberrant influx of lipids into a still-developing tissue may have both short- and long-term consequences on tissue function [1].

Given no demonstrable impairment of hepatic lipid oxidation in WT-HFD/WT-CD male offspring, there are likely other factors contributing to their fatty liver pathology. For instance, the significant reduction in acid-soluble metabolites (ASM) levels in
WT-HFD/WT-CD adult offspring livers after in vitro exposure to labeled palmitate may be indicative of reduced mitochondrial lipid uptake upon substrate exposure, and potentially a propensity to store whether than oxidize that substrate.

In adult humans, the development of NAFLD is often concomitant with the progression of hepatic insulin resistance and consequent un-checked de novo lipogenesis. While our results in the fetal liver suggest no changes in the de novo pathway at the fetal stage, the development of hepatic insulin resistance itself may have in utero origins. This is not to say, however, that the mechanisms of maternal HFD programming are localized to the fetal liver. On the contrary, the effects are likely global, and impact the regulation of many inter-connecting systems, including nutrient metabolism, insulin signaling, and appetite/satiety pathways.

To our knowledge, this is the first time that maternal n-3/n-6 PUFA status and HFD have been studied in combination, with a focus on preventing the adverse metabolic programming attributed to maternal obesity. Utilization of the Fat-1 mouse allows for the isolation of the n-3/n-6 PUFA variable in otherwise genetically identical sibling females, and without any other change in maternal obesity. However, use of a transgene prevents us from ruling out protective effects that may be established during early development and puberty, for instance improved oocyte quality. While we sought to reduce the relative impact of this by exposing mothers to HFD only after they had reached adulthood, early benefits of the fat-1 transgene cannot be eliminated.

Additionally, we cannot delineate whether it is the increase in n-3 PUFA or the reduction in n-6 PUFA that is most important. Both of these essential fatty acids play
an important role in fetal development, as indicated by the preferential uptake of long chain PUFA by the placenta [51,253,263]. Studies have associated maternal n-6 PUFA status with increased adiposity in childhood [264], and n-3 PUFA status with beneficial effects on fetal growth and placental function [137] and reduced adiposity [265]. However, the overall impact of balancing maternal n-3/n-6 PUFA status is still unclear [266], particularly as none of these studies include obese pregnancy.

It is likely that both the excess of n-6 PUFA and paucity of n-3 PUFA, characteristic of more Western-style high-fat diets, play a functional role in adverse obese pregnancy outcomes. Given that n-3 and n-6 PUFA are both broken down to their most biologically active eicosanoid products by the same enzymatic pathways, it follows that a 1:1 ratio of the two is ideal [116,267]. Thus, potential therapies should focus on not only increasing maternal n-3 PUFA intake, but correspondingly reducing dietary n-6 PUFA and overall saturated fat intake as well.

In summary, our findings demonstrate a novel mechanism to reduce maternal inflammation and insulin resistance in a mouse model of obese pregnancy. Targeting early maternal metabolic inflammation by increasing maternal n-3/n-6 PUFA status reduces fetal-placental lipid exposure, and limits the development of adverse metabolic outcomes in adult offspring. Our results suggest that early intervention at the level of maternal inflammation and dietary fat balance may be a promising target for future preventative therapy in pregnancies complicated by maternal obesity and its corresponding metabolic consequences.
CHAPTER VI

CONCLUSIONS

The Developmental Origins of Disease Hypothesis posited by Dr. David Barker argues that exposure to an adverse environment during critical windows of cellular plasticity results in increased risk of later life disease [268]. During these critical periods, changes in the local environment can impact the “programming” of gene expression pathways, with consequent long-term changes in organ function and/or growth [269]. Multiple observational and experimental studies in humans and animals have demonstrated that fetal exposure to stressors such as maternal malnutrition and environmental toxins results in the programming of later life disease [270].

Importantly, the crux of the Barker Hypothesis focuses on how in utero under-nutrition programs a “thrifty” phenotype, resulting in metabolic mismatch to a postnatal obesogenic environment [271,272]. However, the opposite scenario of maternal hyper-nutrition and obesity may also have long lasting effects on offspring metabolic health. In particular, maternal inflammation, insulin resistance, and altered lipid metabolism may be a driving force in aberrant fetal lipid exposure, and its downstream programming consequences.

6.1 Maternal Obesity and Determinants of Infant Adiposity

While large for gestational age (LGA) infants are known to be at increased risk for development of obesity and cardiovascular disease [2,3,43] it is becoming apparent that
abnormal infant body composition, even in cases of normal weight infants, also plays a
significant role in this disease trajectory. Increased birth weight can be a factor of
increased lean mass or fat mass (or both), but the increased fat mass is much more
strongly associated with later life central adiposity and insulin resistance [273]. While
maternal glucose and amino acid transport are commonly considered the driving forces
in the creation of fetal lean mass, maternal lipids, specifically triglycerides (TG), may be
important determinants of infant fat mass, given the limited capacity for fetal de novo
lipogenesis. However, which maternal factors play a role in infant adiposity, and how
this process is regulated, is still being determined.

In Chapter II, we discussed an ongoing clinical study examining the relationship
between maternal obesity and infant adiposity, where we identified a significant role for
the placenta, specifically villous lipoprotein lipase (LPL) activity. Both villous LPL
activity and placenta weight significantly correlated with infant percent body fat at
birth, as estimated by skin fold measurements collected within 24 hours of delivery.
Importantly, it was the combination of placental weight and villous LPL activity, an
estimation of overall placental LPL capacity, which was most strongly associated with
infant adiposity.

While there was no clear relationship between maternal metabolism and villous
LPL activity in this small sample size, maternal fasting insulin levels did positively
correlate with placental weight and total placental LPL activity, which includes placental
weight in its measurement. The regulation of placental development, specifically size,
is an ongoing field of research, however elevated maternal fasting insulin levels may be
one mechanism, along with insulin like growth factors (IGF1, IGF2), which are more well-established players in placental growth.

Our conclusions in this small sub-set of human subjects were hindered by the lack of clear delineation between lean and obese mothers within this cohort, particularly in terms of metabolic complications commonly associated with pre gravid obesity. Further, none of the infants in the study were born with an overt excess of body fat, such that the changes we studied were particularly subtle, with much overlap between study groups. In the postnatal period, the strongest predictor of childhood obesity is when infants exceed their growth curves during the first year to 18 months of life. Thus, whether the more global programming of obesity would appear later in these infants is still unknown.

While our findings are novel in the establishment of placental lipid hydrolysis directly correlating with a logistical infant outcome, a larger cohort, including patients with more severe obesity-related pathology, may help to clarify whether this is a mechanism for increased fetal lipid exposure in the context of obese pregnancy.

6.2. Mouse Model of Maternal Obesity: Maternal and Placental Outcomes

Maternal obesity is known to be associated with increased fat mass and metabolic disease in adult offspring. Some of this fat mass may come early, as a result of increased lipid flux to the developing fetus, promoting excess infant adiposity. As discussed in chapter II, the placenta appears to play a primary role in regulating the degree of this lipid exposure. However, the connection between this placental
regulation and maternal obesity is not yet well defined. Many factors associated with maternal obesity, including inflammation, insulin resistance, and consequent hyperlipidemia may play a role in nutrient sensing and transport by the placenta. In chapter III, we sought to characterize these particular aspects of obesity using a high-fat diet (HFD) mouse model of obese pregnancy.

We observed many classic markers of obesity were present in late gestation HFD mothers, including increased maternal adipose tissue macrophages, serum pro-inflammatory cytokines, and elevated insulin and glucose levels when compared to control diet (CD) mothers. However, we did not observe any fasting hyperlipidemia, which is typically seen in non-pregnant females of this strain of mice after high-fat feeding. Despite this apparent lack of excess lipid mobilization, we found that maternal obesity and HFD corresponded with increased placental size and decidual TG deposition. The composition of these TG was consistent with a high saturated fat diet.

The placentas from HFD mothers also exhibited increased IL-1β gene expression, and increased expression of i-Nos relative Arginase 1. However, we were not able to identify any increase in the number of placental macrophages, as has been previously reported in one study of human placentas from obese mothers with GDM. Additionally, we did not identify any gene expression changes in placental lipid transporters, LPL, CD36, or FABP4, however these proteins can be regulated by localization, and therefore total placental gene expression would miss these subtle differences. Instead, we found an increase in placental LPL enzymatic activity, which
as discussed in chapter I, appears to be regulated independently of gene expression. This increased activity may partially explain the increased placental TG deposition.

While placentas from HFD mothers were significantly larger, fetal growth was comparatively restricted, as has been reported previously in other rodent and primate models. In chapter IV, we sought to clarify how these changes in the mother and placenta altered the fetal environment, with a particular focus on the exposure of the fetal liver to excess maternal lipids.

6.3. Mouse Model of Maternal Obesity: Fetal Outcomes

The link between maternal obesity and the programming of adverse metabolic outcomes in offspring is well established. However, evidence describing what occurs in the developing fetus in response to an obese maternal environment is sorely lacking. In chapter 4, we sought to determine whether the fetal liver is also exposed to the excess lipids first seen in their placentas, and how this exposure might alter gene expression programs.

Despite a trend for reduced fetal growth, and a significant reduction in the fetal/placental weight ratio in HFD mothers, we found no significant differences in fetal serum for glucose, insulin, TG, or FFA. Still, despite no evidence of increased serum lipids, we identified increased TG deposition in the fetal liver. Interestingly, this TG was composed of relatively more PUFA when compared to the TG of CD mothers, which is inconsistent with a maternal diet high in saturated fat. The TG composition is more
suggestive of preferential transport by the placenta, a mechanism that is well established.

Microarray analysis of fetal liver gene expression identified a pattern of gene regulation from HFD fetal livers consistent with a cellular influx of lipid, particularly polyunsaturated fatty acids (PUFA). Specifically, pathways involved in the metabolism and storage of fatty acids were enriched relative to CD fetal livers, while CD fetal livers were comparatively enriched for pathways associated with cell division and proliferation, potentially pointing to a more senescent state in HFD fetal livers. This could have important implications for the later ability of HFD offspring livers to cope with additional nutritional stress in the postnatal environment.

While changes in gene expression suggested increased fetal liver fatty acid metabolism in HFD mothers, we did not observe any differences in ability to perform in vitro fatty acid oxidation between CD and HFD fetal livers. This may be due to the relative immaturity of this system in utero, when oxygen tension is comparatively reduced and antioxidant defense enzyme expression is still low [233,274]. Additionally, these gene expression changes may be partially compensatory, either because of the immature oxidative metabolism described above, or perhaps early metabolic dysfunction at the mitochondrial level in HFD fetal livers. Given the excess fetal liver TG, and no evidence of increased de novo lipogenesis, these findings suggest that fatty acids may be transported from the maternal circulation and re-esterified in the fetal liver if they cannot be readily used as a fuel source.
Under normal physiologic conditions, respiratory chain complex activity is induced by increases in cellular lipid influx, however in non-alcoholic fatty liver disease (NAFLD), pathological reduction in these proteins’ function and expression has been implicated as a mechanism of impaired fatty acid oxidation [275]. Importantly, in adult NAFLD, this is also tied to an unchecked increase in de novo lipogenesis and the negative feedback of Malonyl-CoA on the mitochondrial fatty acid transporter CPT-1. However, based on our findings this pathology does not appear to be occurring in utero with maternal HFD, as has been hypothesized by multiple groups reporting such outcomes in juvenile and adult offspring [236,237]. Thus, the mechanism for the adverse programming of hepatic lipid metabolism remains unclear, although our subsequent findings in adult offspring do allude to altered lipid substrate handling, as described below. Future studies examining pathways associated with insulin resistance and cellular damage, particularly oxidative stress and increased apoptosis may help to clarify the points of lasting impact of maternal HFD on fetal liver lipid metabolism.

6.4. The Maternal N-3/N-6 Fatty Acid Ratio as a Therapeutic Target

Our findings from the previous chapters underscore how maternal obesity is associated with increases in low-grade markers of inflammation and insulin resistance. Additionally, maternal obesity and HFD increase placental and fetal gestational lipid exposure. Whether these factors are interrelated, and which parts might play a role in the fetal programming of obesity and metabolic disease is less clear. In Chapter V, we used an established transgenic mouse model, which exhibits reduced inflammation and
insulin resistance during high-fat feeding, as an interventional third group to identify
whether correcting these aspects of maternal obesity would improve offspring
outcomes.

As previously discussed, the Fat-1 transgenic mouse model converts n-6
polyunsaturated fatty acids (PUFA) to n-3 PUFA via ubiquitous expression of a C. elegans-derived n-3 desaturase under a β-actin promoter. This is a particularly
attractive means of reducing maternal inflammation as it utilizes an essential nutrient
already required by the developing fetus. Additionally, while the Fat-1 mouse is
upheld as a model for reduced inflammation, n-3 PUFA have also been shown to
improve blood lipid profiles and prevent hyperlipidemia. While we did not observe any
increase in fasting TG in late gestation HFD mothers, we cannot rule out hyperlipidemia
earlier in gestation or post-prandial delay in lipid clearance, particularly given the
evidence of increased placental TG.

Fat-1 mothers on HFD demonstrated reduced levels of late gestation adipose tissue
macrophages and serum pro-inflammatory cytokine levels relative to WT-HFD mothers.
This also corresponded with reduced insulin and HOMA-IR score. For all of these
measurements Fat1-HFD mothers did not significantly differ from WT-CD mothers,
despite weight gain comparable to WT-HFD mothers. Suggesting that many of the
downstream consequences of maternal obesity can be disassociated from weight gain
by balancing maternal n-3 and n-6 tissue fatty acid levels.

Interestingly, these improvements in maternal metabolism also led to a
normalization of placental and fetal growth back to the pattern of WT-CD mothers.
Further, we did not observe the same increase in placental and fetal liver TG in Fat1-HFD mothers as we did in WT-HFD mothers, which corresponded with reduced placental LPL activity.

Most significant, these improvements in maternal-fetal outcomes were important for limiting or preventing obesity, fatty liver, metabolic inflammation, and insulin resistance, particularly in the adult male offspring. While male offspring from WT-HFD mothers weaned onto CD demonstrated increased weight gain, body and liver fat, and whole body insulin resistance, these outcomes were partially prevented or even reversed in WT offspring from Fat1-HFD mothers. Our results suggest that reducing excess maternal inflammation may be a promising target for preventing adverse fetal metabolic outcomes in pregnancies complicated by maternal obesity.

6.5. Alternative Hypotheses

As stated in Chapter I, we began this research with the following hypotheses:

1) Maternal obesity is associated with increased chronic low-grade inflammation and disordered lipid metabolism, characterized by increased maternal insulin resistance. This results in an excess flux of lipids and pro-inflammatory cytokines to the placenta.

2) Increases in placental lipid uptake and transport lead to excess fetal lipid exposure, and altered programming of fetal metabolic pathways, particularly in the fetal liver.
3) Rebalancing the maternal n-3/n-6 ratio will reduce maternal inflammation and insulin resistance, and correct excess lipid mobilization, thus preventing maternal obesity-associated programming outcomes in the fetus and adult offspring.

Throughout these chapters we have demonstrated that maternal obesity, in a mouse model, is associated with increased maternal inflammation, insulin resistance, and placental and fetal lipid accumulation, and re-balancing the maternal n-3/n-6 ratio improves these measures and adult offspring metabolic outcomes. However, given our inability to identify an overt increase in maternal fasting lipids, coupled with our complicated findings in human obese pregnancy, there are some alternative explanations and/or considerations that should be discussed, particularly in regard to the regulation of placental lipid transport and mechanisms for the programming of fatty liver in adult offspring exposed to maternal obesity.

**Maternal and Placental Adaptations to Obese Pregnancy**

Our findings support the idea of maternal obesity and high-fat feeding promoting excess fetal lipid exposure, particularly in our mouse model of obese pregnancy. While our human data lacked a clear obese phenotype with which to draw such conclusions, broader clinical studies do support increased placental lipid transport, infant adiposity, and infant hepatic lipid deposition with increasing maternal BMI [7,48,157,159,195,198].
The mechanism for control of this excess lipid flux, as well as programming outcomes is less clear. While placental weight and LPL activity appear to contribute to overall fetal lipid exposure, how both placental growth and lipid hydrolase activity are regulated is still relatively unknown. Although data from our mouse model of obese pregnancy suggested a correlation between placental LPL activity and maternal HOMA-IR score, this was not true in our clinical studies. This lack of correlation may be due to a small sample size, as findings from other groups do support an increase in placental LPL activity under combined hyperglycemic and hyperinsulinemic conditions [69].

Additionally, there may be alternative endocrine regulation beyond the scope of our maternal serum measurements. For instance, the levels of maternal estrogen, growth hormone and glucocorticoids, known regulators of LPL in other tissues [252,276,277], may be different in lean versus obese pregnancy. Additionally, placental growth hormone and IGF 1 and 2 are known to play a role in the regulation of placental growth, and may be associated with altered maternal insulin sensitivity [146,147,149].

Further, we did not identify any maternal hyperlipidemia in either humans or mice coincident with maternal obesity. While for our clinical data this may simply be due to our small sample size, as elevated fasting TG are widely reported in pregnancies complicated by maternal obesity, our results in mice may reflect a species-specific difference. For instance, our measurement of placental LPL per gram of tissue was roughly 5-fold higher in the mouse versus the human placenta. This could aid in
explaining not only the lack of hyperlipidemia in HFD mothers, but also the relatively low fasting TG overall when compared to non-pregnant animals.

**Fetal Response to Obese Pregnancy**

It is important to acknowledge that our mouse model of obese pregnancy yielded fetuses that were mildly growth restricted, which may be a complicating variable when attempting to identify a mechanism for adverse metabolic programming due to maternal obesity. This is not to say that programming risks are only associated with fetal overgrowth, or that maternal obesity necessitates such growth in humans, but is merely an acknowledgement that there may be multiple paradigms at play in regards to maternal obesity and fetal growth trajectories.

While this trend for reduced fetal weight is commonly reported in rodent models of obese pregnancy, and largely ignored in favor of proven long-term outcomes, we cannot rule out that such outcomes could instead be attributed to the classic Barker Hypothesis of fetal programing, characterized by catch-up growth and maladaptation to the mismatch of under-nutrition *in utero* versus sufficient nutrition after birth. This is commonly due to excess visceral fat deposition, at least in humans.

It should be noted, however, that with traditional growth restriction we also see a smaller placenta as well. Thus, our observation of reduced placental efficiency, with increased placental relative to fetal size, is likely more of a placental phenotype, and not a true recapitulation of growth restriction.
In keeping with this idea of maladaptation, is the continued belief that maternal HFD programs a fatty liver phenotype due to programmed reduction in the expression of fatty acid oxidizing genes, and increased expression of de novo lipogenesis genes. However, our results suggest the opposite, with the fetus responding to a fatty acid influx in such away that, if programmed, could be beneficial for consumption of diets rich in fat after birth.

However, given the clear programming of fatty liver in adult male offspring from WT-HFD mothers, the mechanisms for these fatty liver gene expression patterns observed by other groups may not be directly programmed, as we originally hypothesized, but instead be a secondary result of other programmed pathology such as hepatic inflammation, insulin resistance, and/or mitochondrial dysfunction.

*In utero* exposure to excess blood lipids could lead to the induction of fetal pro-inflammatory pathways, and prime the offspring for an enhanced inflammatory response. Inflammation is a major component in the pathophysiology of non-alcoholic fatty liver disease (NAFLD), as it relates to development of insulin resistance and disease progression to necro-inflammatory non-alcoholic steatohepatitis (NASH). Numerous animal models of maternal obesity have demonstrated inflammatory changes in adult offspring liver, corresponding with the progression of NAFLD, including increased hepatic levels of TNFα and IL1β [278], altered hepatic insulin signaling proteins, increased hepatic fibrogenesis [219], and enhanced Kupffer cell density [262]. However, given that all these changes occurred with the concomitant development of offspring obesity, the relative impact of pro-inflammatory changes *in utero* is still
unclear. While we did not identify an overt increase in fetal liver inflammation, smaller changes or initial kupffer cell activation could explain both the programmed metabolic inflammation in adult male offspring, and the alleviation of this in Fat1-HFD mothers.

The kupffer cell is the tissue macrophage of the liver, and its activation may be critical for the development of insulin resistance and NASH [279]. Kupffer cell activation is regulated by a number of factors, including the binding of cell surface Toll-Like Receptors (TLRs). TLRs are pattern recognition receptors that sense pathogen-associated molecular patterns (PAMPs) such as LPS and bacterial DNA, but can also be activated by lipids [280]. TLRs promote inflammation by stimulating local cytokine production via NF-κB activation in the presence of ligand. They can also promote insulin resistance via activation of stress kinase pathways. Additionally, lipotoxicity within hepatocytes themselves can promote the activation of stress kinases, inflammation and consequent insulin resistance as well [281].

Evidence in the literature for early activation of kupffer cells within the fetal liver is currently lacking, however our laboratory’s recent findings in the non-human primate suggest that maternal HFD can ‘prime’ their pro-inflammatory response, such that kupffer cells derived from juvenile offspring have an exacerbated response to stimulus prior to any observed development of obesity (publication in review). In utero exposure to the increased placentally-derived pro-inflammatory cytokines and lipids observed in our non-human primate model [175], as well as local hepatic pro-inflammatory stimuli, could promote such a priming and thus place the fetal liver on
a path toward postnatal metabolic and inflammatory dysfunction, resulting in progression through NAFLD to NASH with continued metabolic challenge.

As previously stated, many animal studies have indicated increased *de novo* lipogenesis in obese adult offspring from high-fat fed mothers [138,236,282]. While the early stimulus for enhanced *de novo* lipogenesis leading to the pathologic development of NAFLD remains unclear based on our gene expression findings, programmed changes in inflammatory or stress pathways could promote hepatic insulin resistance, which plays an important role in shifting fuel utilization toward steatosis [275]. Insulin resistance results in elevated hepatic glucose uptake, which is shuttled into the fatty acid synthetic pathway as acetyl-CoA, providing substrate for *de novo* lipogenesis enzymes acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). Sterol regulatory element-binding protein 1c (SREBP-1c) regulates the expression these genes, and is traditionally activated by insulin. Importantly, SREBP-1c appears to remain insulin sensitive, even in the face of hepatic glucose insulin resistance, as seen in inflammatory NAFLD [275,283,284]. Thus, in the pediatric liver, programming of inflammation-related insulin resistance could result in continued triglyceride synthesis in the presence of hyperinsulinism, as we observed in adult male offspring from HFD mothers.

**Alternative Therapeutic Mechanisms of Maternal N-3/N-6 Fatty Acid Ratio**

Our interventional technique of re-balancing the maternal n-3/n-6 ratio is complicated by the fact that n-3 fatty acids have been reported to be metabolically
beneficial in multiple ways, making it difficult to identify which aspect of maternal obesity is the most important interventional target. While the beneficial effects of n-3 fatty acids are most often attributed to their anti-inflammatory properties, they have also been shown to impact lipid metabolism, specifically by the promotion of cellular fatty acid oxidation and enhanced blood lipid clearance [231,285-287], such that they are considered to have mild hypolipidemic effects. To what extent this is n-3 fatty acid specific, or a quality of PUFA in general is less clear, as many studies do not delineate between n-3 and n-6 PUFA in their measurements.

Thus, increasing the maternal n-3/n-6 ratio may also impact maternal lipid metabolism separate from its regulatory effects on inflammation. While our data did not suggest such an effect, at least not at the level of maternal fasting serum TG, it is difficult to say whether there was an improvement in maternal blood lipid levels when we did not detect any HFD-related hyperlipidemia at the outset, which may be related to differences in mouse versus human lipid metabolism during pregnancy.

Additionally, we observed reductions in both inflammation and insulin resistance in Fat1-HFD mothers, but as these pathologies are inter-related it is difficult to separate one from the other. Thus, the relative contribution of pro-inflammatory cytokines, insulin, glucose, and perhaps post-prandial lipids to our observed placental and fetal phenotypes is not clear. Further maternal studies supplying and/or removing each of these stimuli individually would be helpful in clarifying our observed outcomes.

Finally, while we have assumed the direct benefits offered by maternal expression of the fat-1 transgene are maternal, increased fetal tissue levels of n-3 fatty acids,
particularly DHA could also be contributing to the reduction in adverse programming. This may also not be simply fetal, but early postnatal exposure to increased n-3 fatty acids during lactation, which have been shown to be increased in Fat-1 mouse milk. As this is a particularly important phase during mouse development, given that mice are born relatively premature compared to humans, differential milk composition is likely an important variable in long term programming effects. While we did observe a correction in the HFD placental and fetal phenotype in Fat-1 mothers, supporting the importance of maternal fat-1 expression in utero, additional cross-fostering studies would help in clarifying the degree of protection offered between the in utero and lactation stages of development.

6.6. Clinical Perspectives

Further studies are needed in both humans and animal models to better characterize the role of events prior to birth in the programming of metabolic disease, as well as identify potential therapeutic interventions. However, improving maternal metabolic adaptations associated with pre-gravid obesity, including insulin resistance and low-grade inflammation, may be a desirable therapeutic target during pregnancy.

Our results suggest that adjusting maternal dietary fat consumption may be a good place to start, especially when pharmaceutical treatment is not warranted. Key to this approach is the holistic perspective of a compositional change in diet, including increasing the consumption of n-3 fatty acids relative to n-6 fatty acids, and increasing PUFA intake relative to saturated fats. All while minimizing excess dietary fat
consumption. Such an approach becomes particularly important when considering the adoption of low-glycemic, high-fat diets in patients with Gestational Diabetes Mellitus (GDM). While these diets aid in controlling maternal glycemic levels and excess fetal growth, whether they protect against excess adiposity is another matter entirely.

Ideally, these modifications in dietary fat consumption would be just that – dietary. However, fish oil and other n-3 fatty acid supplements may be an alternative means of balancing n-3/n-6 fat intake. Unfortunately, the particular composition of such supplements – including long chain versus short chain n-3 fatty acids, dosage, and methods to prevent oxidation vary greatly, and it is still unclear what is the most effective. Further, without conscious reduction in other dietary fat, particularly animal-derived sources, benefits from supplements may be relatively minimal.

Additionally, our findings of increased fetal hepatic lipid accumulation with maternal HFD, and our hypothesis that this may place the infant on a trajectory for early development of fatty liver, underscores the importance of monitoring early childhood weight gain and body composition, as well as improving pediatric screening methods for intrahepatic fat accumulation. Such methods would be helpful to better identify at-risk populations, and at an earlier age, so that immediate action can be taken to minimize disease progression.

6.7. Future Directions

The primary focus of our continued research within this mouse model of obese pregnancy will be to better characterize both the fetus and adult offspring by identifying
metabolic changes that are established in utero and maintained in adult offspring. The global nature of the maternal obesity insult suggests that multiple small changes in gene function might be important for altering the trajectory of liver metabolism. Therefore, identifying these maintained metabolic changes would help provide a more mechanistic reason to explain what, at this point, are relatively observational findings regarding maternal HFD and adult offspring programming outcomes.

One of our main focuses will be to determine whether the observed insulin resistance in adult offspring from HFD mothers was initiated by events in utero, as insulin resistance could contribute to both fatty liver and systemic metabolic defects. There are a number of potential pathways that dampen the insulin-signaling cascade, attenuating insulin action in liver, skeletal muscle, and adipose tissue. In the canonical pathway, insulin binding stimulates auto-phosphorylation of the insulin receptor, allowing insulin receptor substrates (IRS-1, IRS-2) to dock. Increased IRS-1 serine phosphorylation is one of the primary mechanisms associated with inhibition of insulin signaling, and is known to be phosphorylated by lipid, ROS, and inflammation-activated stress kinases. Investigation into whether these kinase pathways are activated in either the fetus or adult liver, and whether they are involved in inhibitory signaling of the insulin pathway, will help us clarify the timing of hepatic insulin resistance – and thus determine whether it is more a cause or consequence of early programming events.

In addition to insulin resistance, increased lipid deposition in muscle and liver can lead to mitochondrial dysfunction and an impaired ability to oxidize fatty acids due to an
increase in reactive oxygen species (ROS) production and consequent oxidative stress [288,289]. Currently, very little is known about the control of fatty acid oxidation in fetal mitochondria. However, there is some evidence that mitochondrial dysfunction precedes insulin resistance and hepatic steatosis and may contribute to the natural history of NAFLD in an obese rodent model [290]. Moreover, recent studies in mice have demonstrated that maternal diet-induced obesity increases mitochondrial ROS and oxidative stress in both mouse zygotes, and even pre-conception oocytes [291], and that periconceptual exposure to high-energy substrates such as fatty acids and proteins results in perturbed oocyte and embryo mitochondrial metabolism [292,293]. Studies in vitro support this idea that low-level acquired mitochondrial injuries may persist into embryonic life [294,295].

While our data do not support reduced hepatic oxidative capacity, it is less clear whether or not any in utero fatty acid oxidation might not also come with consequent oxidative injury. Fatty acid oxidation contributes to normal free radical leak from mitochondria, which can lead to cell damage if not balanced by antioxidant enzymes such as glutathione peroxidase (GPX) and superoxide dismutase (SOD). Interestingly, hepatic expression of these enzymes is quite low until after birth [274,296]. The reduced oxidative capacity of the fetal liver, coupled with an immaturity in antioxidant defense systems, may promote the accumulation of excess ROS and partially oxidized lipid intermediates under conditions of excess lipid influx. Supporting this are the findings in a mouse model of maternal HFD combined with an antioxidant supplementation cocktail during gestation and lactation, which showed improved redox
homeostasis and oxidative stress in the fetus, in addition to normalizing metabolic
parameters and total body adiposity at 2 months of age [138]. Further, there is
evidence that maternal HFD may induce mitochondrial damage and ROS production
even earlier in mouse embryonic development, including in the oocyte prior to
fertilization [186]. We hope to clarify this by measuring the degree of oxidative stress
within fetal liver samples. We will do this by determining the levels of oxidized lipid,
protein, and DNA products, as ROS themselves are far too labile to accurately quantify.

Early hepatic damage by ROS could explain later pathology. However, given the
regenerative capacity of the liver, a programmed change in a regulator of cellular
metabolism may be of more importance. The sirtuins are a family of NAD+ dependent
deaetylases that act as nutrient sensors, and have a role in post-translational protein
activity specifically regarding energy mobilization and homeostasis [297,298]. Sirtuin-3
(SIRT3), specifically, is a key regulator of mitochondrial function and antioxidant
capacity, and SIRT3 knockout results in reduced respiratory chain function and increased
production of free radicals from lipid excess [297]. In a HFD mouse model of NAFLD,
we showed decreased hepatic SIRT3 activity and increased oxidative damage of
mitochondrial proteins [299]. Further, rat weanlings from HFD mothers were shown to
have decreased hepatic SIRT3 expression along with impaired SIRT3 activation [237],
suggesting an early role for SIRT3 in mitochondrial dysfunction.

SIRT1, similar to SIRT3, is a key cellular deactelyase important in post-translation
regulation of various proteins and genes involved in glucose and lipid homeostasis.
Importantly, SIRT1 positively regulates peroxisome proliferator-activated receptor alpha
(PPARα) transcription of target proteins involved in fatty acid oxidation. Further, reduced SIRT1 expression and activity decreases PPARα transactivation, and increases HFD-susceptibility, as seen in SIRT1 knockout mice [300]. Interestingly, fetal livers of non-human primates exposed to a maternal high fat diet had decreased SIRT1 expression, protein, and activity in the presence of heightened oxidative stress [222,301].

The mechanisms of sirtuin regulation remain to be fully elucidated, however increased sirtuin expression is known to be closely linked to periods of caloric restriction [297,302]. Further, sirtuins have been shown to be upregulated by peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α), the co-activator of PPARα, and a master regulator of mitochondrial biogenesis [303]. In newborn cord blood, methylation of the PGC1α promoter correlated with pre-gravid maternal BMI [304]. This gene silencing was supported in a mouse model of maternal HFD, where they observed reduced PGC1α expression in the liver of adult male offspring [305].

Thus, we would like to investigate whether the fetal livers in our mouse model may have targeted changes in SIRT1 and SIRT3 protein expression and/or function, given their regulation of many of the pathways identified in our microarray analysis.

In summary, our current data supports an in utero adverse metabolic programming event that presents as obesity, fatty liver, and insulin resistance in adult male offspring, which is mitigated by balancing the maternal n-3/n-6 fatty acid ratio. Now that we have established the efficacy of both the HFD and the intervention, our next steps will be to utilize the samples collected from the fetal and adult livers to better
identify mechanistic changes in insulin signaling and cellular and oxidative stress pathways that might explain our observed outcomes.

6.8 Concluding Remarks

The prevalence of obesity in the developed world has increased markedly over the last 20 years in every country, in each race/ethnic group studied, and in both men and women. Considering the prevalence of obese and overweight adult women, and the fact that pregnancy itself induces a state of insulin resistance and inflammation, maternal obesity may be the most common health risk for the developing fetus. The notion that an abnormal maternal metabolic environment may lead to permanent changes in key organs and the fetal programming of adult disease, is increasingly gaining acceptance. However, the mechanisms involved in generating such responses are only now being elucidated.

One of the most challenging goals in this field is to uncover how maternal metabolism can permanently alter gene expression programs in the fetus. While many pathways have been proposed based on adult pathologies, including programmed reductions in fatty acid oxidation and activation of pro-adipogenic and lipogenic pathways, in utero mechanisms for such changes are still lacking.

Given these challenges, what may be most important is better characterization of early pathology, beyond the scope of up/down gene changes. For instance, excess infant fat mass, ectopic lipid deposition, and early indicators of insulin resistance. Perhaps, if we can identify the initiating pathology, we can then begin to identify the
insult and its intervention. Ultimately, in addition to current practices, an effective means to limit the obesity epidemic may begin in the womb.
REFERENCES


APPENDIX A

PCR PRIMER SEQUENCES

Chapter II – Human Placenta Primers

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Chapters III and IV – Mouse Placenta Primers

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APPENDIX B

MOUSE EXPERIMENTAL DIETS

CD Manufacturer Information:

Description
Rodent Diet with 10% kcal% fat.

Used in Research
Obesity
Diabetes

Packaging
Product is packed in 12.5 kg box. Each box is identified with the product name, description, lot number and expiration date.

Lead Time
IN-STOCK. Ready for next day shipment.

Gamma-Irradiation
Yes. Add 10 days to delivery time.

Form
Pellet, Powder, Liquid

Shelf Life
Most diets require storage in a cool dry environment. Stored correctly they should last 6 months.

Control Diets
Used as a control diet for D12451 and D12492

Sucrose Content
35% Sucrose
See D12450J, K, H for other options.

Formula

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Total 1055.05 4057


*Typical analysis of cholesterol in lard = 0.72 mg/gram.
Cholesterol (mg)/4057 kcal = 54.4
Cholesterol (mg)/kg = 51.6
**HFD Manufacturer Information:**

**Product Data - D12451**

**Description**
Rodent Diet with 45% kcal% fat.

**Used in Research**
- Fatty Liver
- Inflammation
- Obesity
- Diabetes

**Packaging**
Product is packed in 12.5 kg box. Each box is identified with the product name, description, lot number and expiration date.

**Lead Time**
IN-STOCK. Ready for next day shipment.

**Gamma-Irradiation**
Yes. Add 10 days to delivery time.

**Form**
- Pellet, Powder, Liquid

**Shelf Life**
Most diets require storage in a cool dry environment. Stored correctly they should last 6 months.

**Control Diets**
D12450B, D12450H, D12450K

**Formula**

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</tr>
</tbody>
</table>

**Total**

|            | 858.15 | 4057  |


*Typical analysis of cholesterol in lard = 0.72 mg/gram. Cholesterol (mg)/4057 kcal = 167.8 Cholesterol (mg)/kg = 195.5*