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LONG TERM MATERNAL DIET TRANSITION RECOVERS INFLAMMATORY PHENOTYPE AND OFFSPRING OBESITY

A Thesis

by

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ABSTRACT

As the obesity epidemic continues to affect the chronic disease status of individuals worldwide, research that aims to elucidate the contribution of maternal health status to the perpetuation of the obesity cycle has become increasingly prevalent. We sought to answer how different time point interventions in maternal diet affect characteristics of obesity and presence of inflammation in the visceral adipose tissue of offspring male mice. Addressing this question, female mice were continued on either a high-fat diet or transitioned from a high-fat diet to a low-fat diet at 1, 5 or 9 weeks prior to pregnancy. Offspring male mice were continued on a HFD for 12 weeks post-weaning. Compared to NF group, the H1N group had significantly increased adipocyte diameter indicative of hypertrophy, while the H5N and H9N offspring had rescued adipocyte hypertrophy although the H5N adipocyte was significantly bigger than the HF offspring. Accordingly, the H1N and H5N offspring increased expression of key adipogenesis and fat trafficking genes compared to the NF group, which was totally reversed in H9N offspring, respectively. F4/80 positive cells indicated decreased number of adipose tissue macrophage in H9N offspring than NF offspring. However, there was increased ATM infiltration in H1N and H5N, compared to the HF offspring. Consistently, the H9N offspring also had the least amount of crown like structures among all groups. Additionally, the H1N offspring had the highest expression of *Tnf-α* and *Il-6* among all groups, while the H9N offspring expressed the similar level as the NF offspring, indicating enhanced adipose tissue inflammation in H1N offspring, which was completely reversed in H9N offspring. This result was echoed by significant upregulation of Jnk and Nf-κB in H1N group, but not H5N or H9N. In

conclusion, our study showed that a long-term, but not a short-term transition from HFD to NFD before pregnancy is efficient to block the adipocyte hypertrophy and adipose tissue inflammation induced by maternal HFD.

DEDICATION

For Matt,

I couldn't have done this without you.

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Contributors

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

As of 2016, approximately 41 million children globally were affected by childhood obesity, an increase of roughly 9 million children since the year 1990 (1). These rates are expected to continue their increase at an alarming rate, and diagnosis of chronic diseases, which have previously been identified only in adults, such as type 2 diabetes mellitus (adult-onset) and nonalcoholic fatty liver disease, are now being reported in pediatric clinical settings (2). This has resulted in further strain on healthcare systems, costing approximately $147 billion for disease management and treatment related to obesity in 2008 (3). Thus, shifting focus from treating obesity to *preventing* obesity is critical to ensure health of future generations. Strategies which offer means to address this issue are diverse, however, current research suggests that mitigating rates of pre-pregnancy obesity and gestational weight gain may be major influential factors to reduce aberrant metabolic programming and risk of developing chronic disease in offspring (4).

The Intergenerational Cycle of Obesity theory focuses on how maternal obesity propagates risk of development of chronic disease through exacerbation of inflammation and diminished function of metabolic pathways in offspring, priming another generation for the same fate as the mother (4-6). Pregnancy is a time of both subtle and pronounced changes in hormone status, inflammation, weight, and diet. Excessive weight gain and pre-existing maternal obesity can disrupt these natural changes, triggering increased risk

of maternal gestational diabetes, preeclampsia, and uncontrolled fetal exposure to inflammatory factors and circulating energy in utero (7). Research has indicated that a myriad of modifiable environmental and epigenetic factors such as micronutrient intake and methylation status may impact offspring outcomes (8-10). Targeting the critical window immediately prior to gestation by providing feasible, adherable, lifestyle modifications may assist in delaying or reversing the intergenerational cycle of obesity and aberrant metabolic outcomes in both mother and offspring.

1.1 Maternal Factors Affect Offspring Obesity

1.1.1 Maternal Obesity

By current estimates, the proportion of women who are of both childbearing age and overweight or obese (BMI >25) is two thirds (11). Understanding how maternal obesity, rather than dietary composition or overfeeding, influences developmental programming has proven cumbersome, as studies utilizing animal models typically initiate specific feeding protocols that span the duration of pregnancy and lactation. A study conducted by Shankar et al. (12) utilized pre-conceptually obese dams given a standard chow diet throughout pregnancy whose pups were cross-fostered with lean mothers to model effect of maternal weight on offspring. When offspring of both lean and obese dams were fed the same chow diet as their mothers, no body weight difference existed between pups of either obese or lean mothers. When pups of obese mothers consumed a high fat diet, however, substantial increase in body weight occurred, more so than pups of lean mothers fed a high fat diet. This indicates a critical role that maternal obesity may have on fetal programming and development of offspring obesity.

Maternal Pre-pregnancy BMI as well as percent ideal body weight are both significantly associated with increased risk of large for gestational age (LGA) babies and adolescent obesity (13-14). In human studies, elucidating the role of maternal adiposity in fetal outcomes is challenged by genetic and environmental variations of clinical trial participants such as different dietary composition and intake patterns between households and family history of disease. Employing a sibling study, Smith et al. (15) followed 111 children born to women before and after undergoing anti-obesity surgery to compare body weights, lipid profiles and insulin sensitivity. Results indicated that mothers with approximately 36% body weight loss over a mean of 12 years gave birth to children with overall reductions in macrosomia, leptin and c- reactive protein, and improvements in insulin sensitivity, and lipid profile as compared to children born before maternal weight loss surgery. This result is consistent with a study conducted by Kral et al. (16) of a similar design which saw decreases in prevalence of obesity and overweight status in post-surgery births, tracked through seven years of age, illustrating the drastic alterations to pre-pregnancy weight may have on neonatal outcomes.

Central obesity, identified by an increase in waist circumference and size of visceral adipose tissue depots, poses an increased risk for endocrine and immune dysfunction. Straughen et al. (17) tracked pregnancy-induced modifications to the location of adipose tissue storage, based on weeks of gestation, to determine in what ways the body preferentially stores excess energy during pregnancy. It was concluded that visceral adipose tissue deposition was enhanced as compared to subcutaneous

adipose tissue stores, in pregnancies of both normal weight and obese women, indicating a gestation-based preference for visceral adiposity. In women who have pre-existing central obesity, further accumulation of visceral adipose tissue may increase risk of complication for both mother and baby.

1.1.2 High Fat Diet

Excessive caloric intake and inadequate energy expenditure or "positive energy balance" has largely been targeted by dietitians and medical professionals as the underlying reason for increased weight status of individuals. Dietary studies have identified that meal composition, in addition to quantity of food consumed, are essential factors for understanding the development of obesity (18-19). Diets high in fat and refined carbohydrates are preferentially utilized for energy storage, leading to increases in adipocyte hypertrophy, expansion of visceral and subcutaneous adipose tissue depots and infiltration of adipose tissue into non-adipose organs (20). Parallel studies of humans and animals have indicated that in-utero exposure to over-nutrition results in an increased risk of developing impairments in insulin sensitivity, glucose tolerance and fatty liver beyond infancy (21-22) and is associated with increased visceral adiposity.

Visceral adiposity is a potent effector of satiety and food intake through signaling mechanisms with the central nervous system (23). Evaluation of neurological feeding mechanisms of offspring born to high fat fed dams noted a significant increase in leptin levels in white adipose tissue immediately post-partum followed by decrease in levels accompanied by prolonged leptin resistance that was correlated with loss of sensitivity to

food intake leading to obesity in rats (24). Loss of leptin sensitivity as a feedback mechanism can perpetuate obesity and excessive caloric intake.

Diet "mismatch" is an effective research tool used to evaluate how administration of high fat diet after consumption of normal fat diet- or vice versa- can impact offspring phenotype. A study utilizing a strain of out-bred mice with genetic variance more similar to humans, used a diet mismatch model to explore how timing of diet administration effected outcomes in male mice. Mice were fed a high fat diet either prior to gestation only or were continued on high fat diet through early gestation and then switched to a chow diet. Pups were then cross fostered with lean dams throughout weaning, followed by adherence to either a high fat diet or chow diet until sacrifice. Results concluded that offspring male mice fed a high fat diet experienced a more severe phenotype of adiposity, hyperinsulinemia and hyperleptinemia than control counterparts, even when mothers were exposed to HFD for only a short, developmentally early period of time (25). This programming effect of HFD consumption is supported by a study by Sasson et al. (26) that used a reciprocal embryo implantation model to determine how the exposure period of high fat diet-either preconception or during gestation led to more severe phenotypes in offspring fed a chow diet after weaning. Their work determined that maternal HFD exposure preconception altered placental gene expression and stunted intrauterine growth of the fetus, more so than gestation-based exposure. These studies indicate that HFD may play a vital role in metabolic programming of the neonate at the embryonic stage, potentially independent of maternal adiposity, which may be carried through later life as an inherent risk factor for chronic metabolic disease.

1.1.3 Gestational Weight Gain

The Institute of Medicine has released guidelines stipulating relative amount of ideal weight gain throughout pregnancy, based on pre-pregnancy BMI (27). These guidelines utilize stricter margins for higher weight classes, to prevent fetal energy over exposure in utero. However, these guidelines continue to face scrutiny due to their overarching lack of detail in regard to ideal dietary composition and effective lifestyle change prior to and during pregnancy, leaving many healthcare professionals deficient in evidence-based tools to execute the guidelines.

Failure to adhere to growth curves, evidenced by increased infant weight gain in relation to age, is predictive of overweight or obese status in childhood (28). Multiple studies have indicated that women who are obese prior to becoming pregnant, are more likely than their normal weight counterparts, to be affected by excessive gestational weight gain (29-31), increasing the likelihood that a neonate will be born large for gestational age (LGA). A 3% increase in likelihood of adolescent obesity has been correlated to every 1 Kg increment in excessive gestational weight gain when children were tracked to age seven (32).

Additionally, overweight and obese women who gain excessive gestational weight are at an increased risk for retaining that weight post-partum that may result in an increase in BMI (33-34). This can impact future pregnancies adversely, heightening risk for preeclampsia and gestational diabetes, but can also impact long-term health status after pregnancy. Women who failed to lose the weight gained during gestation enter

menopause at a higher risk for experiencing cardiovascular disease, insulin resistance, and with increased fat mass compared to lean mass (35-36).

1.2 Adipose Tissue Physiology

White adipose tissue is the primary vehicle for storage of energy by the body. It can be divided into two categories- subcutaneous, which lies beneath the skin and serves a protective role and visceral, which infiltrates deeper in the body to surround the viscera and metabolic organs. White adipocytes are characterized by large unilocular lipid droplets which can communicate systemically with other organs as mobilization and storage of energy occurs, making adipose a complex and dynamic organ that responds actively to environmental stimuli.

Adipose tissue has two core mechanisms for maintaining adequate energy storage: hyperplasia and hypertrophy. Hyperplasia is the differentiation of new adipocytes from precursor cells. This function primarily occurs in early development and is localized to the subcutaneous fat depot, however ongoing research has identified that high fat diet feeding and diet induced obesity may trigger the proliferation of new adipocytes for expansion of white adipose tissue in a PI3K-AKT pathway dependent manner (37). An emerging phenotype of obesity, the “metabolically healthy obese” is also thought to have adipose depots with higher rates of hyperplasia and a lower ratio of lipid to cell as compared to traditional models of obesity, indicating that hyper-plasticity of adipose tissue may play a protective role.

Hypertrophy is the expansion of adipocytes with excess lipid. Hypertrophic adipocytes are classically associated with obesity and typically have three identifying

attributes in addition to their increased size: necrosis, increased secretion of inflammation promoting molecules and macrophage recruitment, and insufficient vasculature. Hypertrophy has been implicated as a risk factor for insulin resistance, non-alcoholic fatty liver disease and cardiovascular disease (20).

1.3 Cell Signaling Involved in Fat Deposition

1.3.1 AKT

Protein Kinase B, also referred to as (AKT) is a critical component of cellular synthesis pathways, insulin signaling and cellular growth via protein synthesis. AKT1 is largely involved in cellular growth and homeostasis. AKT2 is the isoform that plays a primary role in insulin signaling due to high affinity binding with phosphoinositides, specifically PIP2 or PIP3. The AKT3 isoform is has the lowest expression and is typically only active in relation to neuronal development (38). AKT requires phosphorylation by phosphoinositide-domain-kinase 1 (PDK1) at Thr308 and PDK2 has been postulated to phosphorylate Ser 473, which is the component that provides stabilization during activation (39). Depending on level of expression, AKT can have potent implications for metabolism and cellular regulation. Downregulation of AKT has been shown to influence development of diabetes and aberrant insulin signaling.

Tumor Necrosis Factor alpha (*TNF-α*), which is largely associated with inflammatory response, also acts on active, phosphorylated AKT and its downstream target PI3K. In doing so, ability to phosphorylate Insulin Receptor Substrate 1 (IRS1) is inhibited, leading to dysfunctional insulin signaling (40-41).

1.3.2 AMPK

AMP- activated protein kinase complex is composed of three subunits α, β, and γ, each with distinctive roles. The β and γ subunits are primarily responsible for identifying shifts in ratio of AMP:ATP in times of reduced energy availability, with the β unit sensing available glycogen stores (42.) This subunit is composed of four distinct CBS domains that create two sites in which AMP can bind, in turn exposing the catalytic domain of the alpha subunit. Phosphorylation of the alpha subunit activates AMPK activity (43). During periods of low energy or hypoxia, AMPK inhibits cell growth by inhibiting mTOR complex 1, but also increases levels of autophagy to ensure that the body has sufficient nutrients available (44). AMPK also plays an important role in regulating lipid metabolism and glycolysis through phosphorylation and consequent inactivation of Acetyl CoA Carboxylase and suppression of Malonyl CoA levels which leads to increased transit of fatty acid for mitochondrial oxidation through CPT1. Phosphorylation and consequent downregulation of AMPK is influenced by high levels pro-inflammatory cytokines *Tnf-α*, *Il-6* and *Il-1β*, whereas inactive AMPK plays an anti-inflammatory role that may be associated with *Il-10* expression (44).

1.4 Key Modulators

1.4.1 Ppar-γ and C/ebpα

Ppar-γ and *C/ebpα* are considered to work in tandem as the primary regulators of adipogenesis. These two components rely on each other to execute the critical roles in the terminal stages of adipogenesis and allow for transcription of adipogenic genes (45). In cell studies and animal models, *Ppar-γ* has proved to be the driving force of terminal

adipogenesis, working with Fatty Acid Binding Protein 4 to recruit lipid into adipocytes. Knockdown models of *c/ebpα* result in overcompensation of *Ppar-γ* to continue adipogenesis, however complete knockout models of *c/ebpα* results in an inability of adipocytes to develop and form white adipose tissue, as well as an inability of the liver to perform gluconeogenesis indicating the essential role of both *Ppar-γ* and *c/ebpα* as a functional unit for adipogenesis (46).

1.4.2 Srebp1c

Srebp is a family of three isoforms *Srebp1a, Srebp1c* and *Srebp2. Srebp1a* is involved in multiple pathways including cholesterol and triglyceride synthesis, whereas *Srebp1c* and *Srebp2* are more transcriptionally limited to fatty acid synthesis (47). *Srebp1c* is involved in sterol biosynthesis as well as LDL and fatty acid synthesis. *Srebp1c* is highly expressed in adipose tissue and influences adipocyte differentiation by acting upon *Ppar-γ* and *c/ebpα*, although the complete mechanism for this action is unknown (48). In mouse models, overexpression of *Srebp1c* results in lower levels of white adipose tissue, but significant infiltration of fatty acids into the liver and other tissues, which may influence development of lipotoxicity (49).

1.4.3 Acacβ

Acetyl-CoA Carboxylase has two distinct isoforms *Acc1 (acaca)* and *Acc 2 (acacβ). Acc1* is involved in commitment of fatty acid synthesis by catalyzing the conversion of Acetyl CoA to Malonyl-CoA. (50). During times of low energy, *Acc1* is phosphorylated and inactivated; a function that can be executed by AMPK to limit synthesis of fatty acids. In contrast, *Acc2* promotes beta oxidation of fatty acids. Total

knockout models of *Acc2* in mice display reduced fat mass and increased levels of fatty acid oxidation, which has potentiated the role of *Acc2* in future pharmaceutical interventions of obesity (51).

1.4.4 Fasn

Fatty Acid Synthase is a complex composed of multiple enzymes which plays a primary role in synthesizing long chain saturated fatty acids such as palmitate, in the presence of Acetyl CoA and Malonyl CoA. *Fasn* is expressed predominately in the liver, concordant with the high level of lipogenic activity that occurs there. However, in obesity, *Fasn* expression is increased in visceral adipose tissue and the liver and contributes to increased rates of adipogenesis. *Fasn* has been implicated as a driver of obesity, and its upregulation in adipose tissue has been correlated with hyperinsulinemia and dyslipidemia in human patients (52).

1.4.5 CD36 and Fabp

CD36, also referred to as fatty acid translocase is a membrane bound protein that has lipid specific scavenging properties. CD36 is expressed in differentiated adipocytes and is partially responsible for uptake of oxidized LDL and long chain fatty acids (53). In models of CD36 deletion, there is a simultaneous decrease in fatty acid uptake and increases in circulating level of plasma triglycerides and insulin sensitivity in muscle tissue, indicating its role in metabolic homeostasis and fatty acid utilization.

CD36 is expressed in a variety of tissues. CD36 expression on monocytes is correlated with increased digestion of pro-apoptotic cells which is hypothesized to play a significant role in triggering signaling of fibrotic pathways (54).

Fatty acid binding protein (FABP) has many isoforms functioning throughout the body. The adipocyte specific protein FABP-4, or AFABP, functions in tandem with CD36 to transport lipid into cells. AFABP expression increases during lipogenesis and during consumption of high fat diet, indicating its role in lipid transport (55).

1.5 Obesity as a Disease of Inflammation

1.5.1 Triggers of Inflammation

Adipose tissue has the capacity to initiate and respond to local and systemic inflammation by secreting cytokines, adipokines and activating macrophage response. In pregnancy, adipose tissue plays a critical role in evolving the innate and adaptive immune response of the developing fetus, by communicating with the placenta (56). In obesity afflicted pregnancies, the onslaught of pro-inflammatory molecules can negatively affect fetal growth and developmental outcomes and increases maternal risk of miscarriage, preeclampsia, and gestational diabetes. Visceral adiposity induced by high fat diet plays a role in both central and peripheral inflammation. Wild type mice that were recipients of GFP-positive mice donor bone marrow transplants fed high fat diet for 15 or 30 weeks had increased adipocyte hypertrophy and adipose tissue inflammation, but also had increased recruitment of bone marrow derived leukocytes in the central nervous system, which induced inflammation (57).

In regard to obesity induced inflammation, two primary theories exist as to what serves as the initial trigger of this occurrence: hypoxia, and lipotoxicity.

1.5.1.1 Hypoxia

Rapid expansion of adipose tissue results in decreased blood flow and consequently limited transportation of oxygen. Studies have indicated that a concurrent increase in angiogenesis and angiogenic gene expression needs to occur to enhance expansion of adipose tissue. Obese subjects fail to have the associated angiogenic response post prandially as compared to lean subjects characterized by reduced vascularization and decreased transcription levels of angiogenic genes (20). In mouse studies of wildtype vs *ob/ob* mice, levels of adipose tissue oxygenation were significantly lower in obese mice consistent with measurements of tissues which operate in a hypoxic state such as the retina (58). Hypoxia has significant metabolic implications, as it results in lowered levels of oxidative phosphorylation, to conserve oxygen, and the increase in GLUT translocation to compensate for upregulated anaerobic glycolysis. Altered oxidative phosphorylation in the mitochondrial may result in generation of reactive oxygen species which may further enhance local inflammation (59).

1.5.1.2 Lipotoxicity

Obesity strains adipose tissue to store an excess of energy. Due to the unilocular nature of adipocytes, some researchers have theorized that individual adipocytes have a threshold capacity for storage of lipid. Burden to expand beyond this threshold results in structural failure of the cell and spillage of free fatty acid into surrounding compartments. Concurrent with “lipid spillage” there is a reduction in fatty acid transport to adipose tissue, decreased ability to form new adipocytes to accommodate

storage demand, and increased trafficking of fatty acids to non-adipose tissues such as muscle and liver (20,60). Metabolic complications of lipid toxicity are primarily seen in development of conditions effecting other organs such as non-alcoholic fatty liver disease, however lipotoxicity can also result in increased recruitment of macrophages (61), which will be discussed in the next section.

1.6 Macrophage Function and Interaction with Adipocytes

Healthy adipose tissue is equipped with resident macrophages which assist with clearance of dead cells and cellular debris through phagocytosis, which helps ensure proper turnover and health of tissue. Macrophages can also buffer lipolytic activity by preventing excessive lipid release into the bloodstream, ensuring metabolic homeostasis remains intact. These macrophages are broadly referred to as M2 macrophages or alternatively activated macrophages due to their “anti-inflammatory” properties (60).

Under conditions of obesity and high fat feeding, adipose tissue has a marked increase in both number and type of macrophages in visceral adipose tissue, in both human and mice models. Obesity induced inflammation and mitochondrial stress can result in cellular damage and consequent spillage of lipid droplet into surrounding tissue, or lipotoxicity. This stimulates macrophage response for containment of lipid spillage, resulting in pockets of macrophage accumulation called crown like structures (CLS) (62). Macrophages recruited for inflammatory response undergo a phenotypic switch, from M2 to M1 or “classically activated”, which is considered to be significantly more inflammatory. Pro-inflammatory macrophages, or M1 macrophages, induce cellular

apoptosis, monocyte-derived macrophage recruitment and expression of cytokines *Il-6*, *Il-1β* and *Tnf-α*. (63-64).

Multiple studies have indicated that macrophages present in adipose tissue from HFD obesity have different characteristics from their lean counterparts in regard to fat metabolism and secretion of pro-inflammatory cytokines (60, 65-66). One such macrophage is CD68+, a M1 macrophage, which is identified by either F4/80 in mice or EMR1 in humans. These identifiers are cell-surface glycoprotein markers of bone marrow derived macrophages (66) present in obese adipose tissues. Increased presence has been associated with HFD intake and endotoxemia in obese pregnancies (67).

For example, a classification of placentas from obese mothers noted a fourfold increase in inflammation induced insulin resistance, and a significant heterogenous increased in resident CD68+ macrophages versus placentas from lean mothers (68). Given the role that the placenta has in maternal-fetal interface, increased presence of pro-inflammatory macrophages can be a strong indicator of a dysregulated inflammatory phenotype.

1.7 Important Cytokines Involved in Inflammation

1.7.1 Il-6

Il-6 is synthesized quickly and efficiently in response to tissue damage, infection and injury through its receptor complex with GP130. *GP130-Il6* complex activates signaling pathways of inflammation such as Janus Kinase (JAK/STAT), and PI3K (69). Cytokines *Il-6*, *Il-1β* and *Tnf-α* function together to trigger neutrophil accumulation in

times of acute inflammation. The increased density of neutrophils activates *Il-6 trans signaling* which leads to tissue specific chronic inflammation (70).

1.7.2 Il-1β

Il-1β is largely produced by activated monocyte derived macrophages, similar to those found in adipose tissue. *Il-1β* is essential for acute phase response of inflammation, and signals for production of acute phase reactants like C-reactive protein in the liver. Autophagy is a critical physiological function which breaks down cellular components to provide energy in times of depletion, such as during fasting or extended exercise. *Il-1β* interacts within autophagosomes to promote macrophage recruitment in the liver and adipose tissue. When pathways of autophagy are not activated, *Il-1β* is then secreted from the autophagosome to trigger an external inflammatory effect. This relationship has furthered research into *Il-1β*'s role in apoptosis and how its release may be a committed signal for cell death, however this work is still ongoing (71).

1.7.3 Tnf-α

Tnf-α is predominantly secreted from macrophages, rather than adipocytes. *Tnf-α* overexpression as a result of inflammation can activate adipocyte insulin resistance. The cytokine can phosphorylate the serine residue on both Insulin receptor and Insulin Receptor Substrate 1, which tamps down activity of PI3K and may influence AKT activity. *Tnf-α* is also a potent regulator of NF-κB and JNK signaling pathways (72).

1.7.4 Il-10

Contrary to the aforementioned cytokines, *Il-10* is frequently classified as anti-inflammatory. *Il-10* plays an essential role in maintaining inflammatory homeostasis by

mitigating cellular response to exaggerated inflammation. After HFD feeding, increased expression of *Il-10* in adipose tissue has been correlated with presence of M2 macrophages that remains elevated even when insulin sensitivity is maintained (73). Suppression of *Il-10* can result in increased infection, chronic inflammation and chronic disease such as inflammatory bowel disease, asthma and systemic lupus erythematosus (74).

1.8 Cell Signaling for Inflammation

1.8.1 NF-κB

NF-κB is a vital factor in systemic response to inflammation, as well as adaptive and innate immunity. NF-κB has been characterized as a quick response in presence of deleterious cellular stimulation such as reactive oxygen species. Two critical activators of NF-κB response are the pro-inflammatory cytokine *Il-1β* and *Tnf-α*. Induction of NF-κB response is controlled primarily through two pathways: canonical and alternative. Function of the canonical pathway is dependent upon the degradation of the inhibitory component IκB. NF-κB's inhibitory component, identified as IκB1 or IκB2 is prone to phosphorylation and consequent degradation by key stimuli such as reactive oxygen species and proinflammatory cytokines *Il-1β* and *Il-6* (75). Translocation of NF-κB to the cell nucleus results in elevated inflammatory status and insulin resistance which has been seen in murine knockout models of hepatic and skeletal IκB1 (40,63). Alternative activation occurs through production and phosphorylation of the protein p100 as a result of activation to NF-κB inducing kinase (NIK). Both pathways contribute significantly to

the role of NF-κB in inflammation, especially in relation to obesity, chronic disease, and cancer.

1.8.2 JNK

Cellular stress caused by reactive oxygen species (ROS), DNA damage and pro-inflammatory cytokines initiates cellular death pathways of apoptosis in order to maintain health and integrity of undamaged cellular components. C-jun-n terminal kinases, also known as JNK or stress-activated protein kinase (SAPKs) are integral components in cellular apoptosis, inflammatory response, and production of cytokines. The JNK family operates closely with mitogen activated protein kinases, specifically MAP3K and MAP4K, which become activated in response to transmitted stress signals from receptor associated membrane alterations (76). Universally expressed JNK1 and JNK2 are both activated by phosphorylation and translocation to the nucleus, where they can associate with and activate a range of factors such as C-Jun and the p53 pathway which are involved in upregulation of apoptosis, or downstream with inhibitors of cell survival. The Janus Kinase family (JNK1, JNK2, and JNK3) have been seen to be upregulated under chronic inflammatory conditions. During early development, lipotoxicity resulting from increased free fatty acids in the bloodstream of obese women has been associated with placental dysfunction and generation of reactive oxygen species associated with activation of the JNK pathway (77).

1.9 Maternal Diet Intervention

1.9.1 Human Study

The proportion of overweight and obese women of childbearing age in the united states has risen to a staggering number, necessitating effective intervention with long-term adherence. Previously, work has been focused on preventing malnutrition and nutrient deficiencies that are associated with restricted infant growth and birth defects. With the rise of enriched and fortified foods, as well as better understanding of the effects of gestational malnutrition, concern has shifted to the consequences of overnutrition. Gestation based intervention is challenged by potential consequences to fetal growth restriction and complications of extracting true physiological changes brought upon by intervention rather than physical metabolic and hormonal alterations that occur during pregnancy (78).

Researchers have structured intervention protocols with minimally invasive techniques such as water aerobics, specialized physical training sessions, nutrition coaching with registered dietitians, and adherence to Institute of Medicine guidelines for gestational weight gain (79-80). Outcomes have been varied. Many groups have seen little to no improvements in maternal weight control or reduced diagnosis of preeclampsia and gestational diabetes whereas some groups have seen only slight reductions in preventing large for gestational age babies. While these studies provide important attempts at prevention, they still lack conclusive and significant results that can contribute to developing treatment plans for overweight and obese mothers that can also impact offspring health.

1.9.2 Mouse Study

In mouse studies, offspring outcomes have been effectively rescued by maternal diet intervention. A study conducted by Zambrano et al. (81) compared offspring outcomes of pups born to either dams with maternal obesity induced through high fat diet, or dams who underwent maternal diet intervention to a chow diet one month prior to gestation. Offspring born to obese mothers experienced increased levels of serum triglycerides, leptin, insulin resistance, fat mass, and adipocyte size that was either partially or fully recovered by maternal diet intervention. Additionally, a study which evaluated how maternal diabetes impacted cellular stress and apoptosis in murine embryos noted that intervention with metformin in the drinking water of dams alleviated embryonic stress, glucose intolerance, insulin resistance and reduced apoptosis (82).

Previous work from our lab has indicated that a maternal short-term dietary transition prior to pregnancy (1 week) significantly exacerbated dysregulated insulin signaling and glucose tolerance in both hepatocytes and adipocytes, more so than treatment with HFD alone in female offspring (83). The early onset of symptoms in our mice, independent of maternal glucose intolerance or obesity, prompted the question as to how this maternal diet transition time point may contribute to in utero metabolic programming, as well as whether longer gaps in transition prior to gestation may play a feasible role in reducing metabolic complications in offspring.

These studies illustrate the impact that maternal diet intervention can have on reducing or exacerbating negative offspring outcomes. High fat diet intake, excessive circulating energy in utero and maternal obesity have all been implicated in triggering

oxidative and cellular damage, which increases pro-inflammatory signaling cascades. Reducing offspring adiposity might be an essential mechanism to decrease inflammation and maintain metabolic homeostasis.

1.9.3 Overcoming Barriers

Gaps in knowledge still exist as to timing of maternal intervention, ideal dietary composition, lifestyle changes prior to conception and planning long term adherence. Creating a framework in which health professionals can tackle the diverse issues surrounding the underlying causes of obesity in a manner that is sustainable and effective to patients is critical to ensuring health of future generations. Maternal inflammation, BMI and gestational weight gain are critical factors that influence development of offspring obesity (84). Our research is focused on contributing an expanded understanding of systemic inflammation associated with obesity in offspring, which is critical for mitigating poor outcomes and engaging targeted treatment options. Currently, no concrete recommendations exist as to when specific diet intervention should begin to ensure positive outcomes for both mother and baby. Establishing specific timelines to follow is an important step in closing the current gap in patient care. This research aims to lay a framework for identifying important transition points which discourage onset of disease for future offspring, providing better quality of life and enhanced quality of care. Elucidation of key regulatory mechanisms and modeling of in utero programming provides more advanced knowledge about obesity and development, therefore offering opportunity at success when applied in future clinical trials.

CHAPTER II

HYPOTHESIS AND SPECIFIC AIMS

2.1 Overall Hypothesis

Different transition period of maternal diet intervention differentially impacts metabolic and inflammatory complications in male mice offspring induced by maternal high fat diet.

2.1.1 Specific Aim 1

To determine if and how different durations of intervention in maternal diet prior to pregnancy affect physical attributes of obesity and indicators of adipogenesis. Based on previous work from our lab (83), I hypothesize that a long-term, but not a short- or medium-term, diet intervention will allow for recovery of offspring from development of obesity and improved adipose tissue function. I will evaluate differential effects on male offspring adiposity by evaluating physical indicators of adiposity such as ratio of fat to lean mass, adipocyte diameter, and body weight (Aim1a), and cellular control of adipogenesis and lipogenesis by evaluating genes regulating fatty acid transport and metabolism (Aim1b).

2.1.2 Specific Aim 2

Identify how important signaling mechanisms underlying chronic inflammation in visceral white adipose tissue are affected by different durations of maternal diet intervention. I hypothesize that a long-term transition will be protective from over-activation of adipose inflammation and macrophage infiltration in offspring. I will evaluate

cellular characteristics of adipose tissue inflammation such as percent infiltration of F4/80 macrophages, presence of crown like structures and *Il-6*, *Il-1β* and *Tnf-α* cytokine expression. I will further evaluate the JNK and NF-κB signaling cascades which have been identified as critical underlying mechanisms in establishing chronic systemic inflammation in obesity, to determine if activation of these pathways is altered in response to maternal diet.

CHAPTER III

MATERIALS AND METHODS

Female C57/BL6J mice, 12 weeks of age, were selected for study and assigned either a normal fat diet (10% kcal from fat) or a high fat diet (60% kcal from fat), which was adhered to for 12 weeks. Breeding pairs administered a high fat diet continued this diet through gestation and lactation or were transitioned to a normal fat diet at either a one-week, five-week or nine-week time window prior to pregnancy followed through gestation and lactation. Male mice offspring were selected for their distinct obesogenic phenotype upon treatment with HFD. From each litter, a minimum of two male mice were randomly selected, with a minimum of three distinct litters being involved in each treatment group. A separate group, birthed of the breeders who adhered to the NF diet, were continuously fed the NF diet through 12 weeks, and utilized as a reference control group (Figure 1). All other offspring were given a high fat diet for 12 weeks after weaning, to promote weight gain before their consequent sacrifice. Mouse experiments were completed according to a protocol reviewed and approved by the Institutional Animal Care and Use Committee of the University of North Dakota and Texas A&M University, in compliance with the USA Public Health Service Policy on Humane Care and Use of Laboratory Animals.

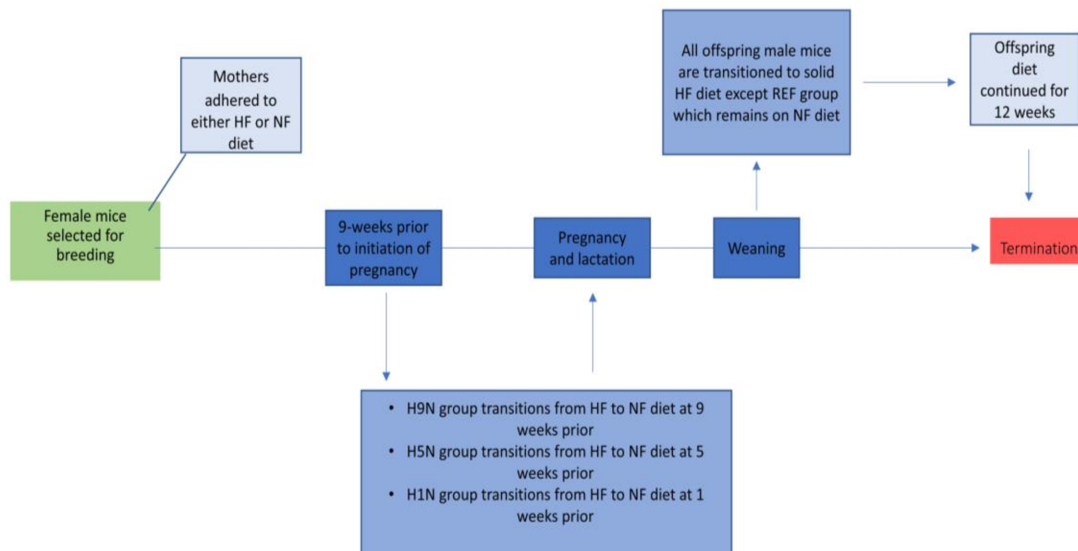


Figure 1: Study Design

Diet was purchased from Research Diets, LLC (New Brunswick, NJ). The normal fat diet (Cat#D12450B) had an energy density of 3.771 kcal/g (10% fat energy, 70% carbohydrate energy, and 20% protein energy). The HF diet (Cat#D12492) had an energy density of 5.157 kcal/g (60% fat energy, 20% carbohydrate energy, and 20% protein energy). The fat source is composed of 92% of lard and 8% of soybean oil.

Mice remained singly housed throughout the study and were weighed once per week. Energy consumption was calculated each week. Both logs were tracked throughout the 12-week post-weaning HFD feeding portion of the study. After the 12-week timepoint concluded, mice were humanely sacrificed with CO_2 asphyxiation followed by cervical dislocation. Fat pads were excised from the visceral region and

weighed immediately, before being equally divided for two tubes and snap frozen in LN_2. Fat pad mass was normalized against individual murine body weight.

Dissected adipose tissue samples of ~1mm were fixed overnight in 10% formalin at 4° Celsius. Tissues were washed briefly in PBS to remove excess formalin and placed into cassettes for processing. Cassette sections were dehydrated in increasing concentrations of Flex-100 and Xylene and then impregnated with paraffin wax overnight. Sections are fixed in molds with paraffin wax and sliced using a Leica Microtome into 5um sections. Tissue sections were mounted on noncharged microscope slides. Slides were stained with Harris-modified Hematoxylin and Eosin-Y solution (alcoholic with phloxine), dried overnight, and mounted with slide-glass using Paramount (85). Microscopy was performed using a Leica D2500 microscope at 10X and 20X magnification. For cell diameter measurement, 100 cells were counted from 2 distinct images (200 cells total/per sample) at 100x magnification using the crosshairs diameter and quantification tool of the Fiji plugin of ImageJ software (NIH). All measurements were taken in units of micrometers.

To determine percent area occupied by crown like structures, each image was converted to 8-bit, with a threshold applied to highlight area occupied by crown like structures, which was subtracted from total image area to determine percent area Fiji plugin of ImageJ. Images containing large white or blank spaces were not used to ensure there was no size bias in measurement. Two images from each sample, n=4-6 per group, were utilized for measurement. Data reported as average % area.

Adipose tissue sections used to determine F4/80 area were mounted on Bond 380 noncharged microscope slides underwent progressive deparaffinization in Xylene and rehydration in serial dilutions of Ethanol. Antigen retrieval was completed using a modified vegetable steamer and a coplin jar of sodium citrate buffer (Na3Citrate 10mM, 0.05% tween 20, pH6.0) for 25 minutes, followed by cooling time and quenching of endogenous peroxidase activity with 3% H_2O_2. VecStain ABC anti-goat reagent kit (Vector Laboratories) will be utilized for the serum, secondary antibody incubation and ABC reagent. Anti-Goat serum will be incubated for 30 minutes at room temperature followed by overnight incubation of F4/80 antibody (Cell Signaling Technology) in a 1:1000 dilution at 4° Celsius. ImmPACT DAB peroxidase substrate kit with chromogen (Vector Laboratories) will be utilized for staining of F4/80 with Mayer's modified hematoxylin used as a counterstain. Analysis will be performed on a color isolated threshold scale using Fiji plugin of Image J (NIH).

Adipose protein was consequently extracted to execute western blot analysis. Approximately 3-5mg of visceral adipose tissue was dissected from total sample, on ice, and placed into a 2.0 mL tube with 200uL RIPA buffer, 50uL Pierce phosphatase inhibitor, and 50uL dissolved Complete Ultra EDTA tabs. Tissue was homogenized on ice using a Homogenizer150 (Fisherbrand) and then left to lyse for 15-30 minutes. Samples were then centrifuged for 30 minutes at 10000 RPM at 4° Celsius. Ultra thin pipette tips, were used to extract the protein layer cleanly from underneath the lipid disk. Samples were centrifuged for 5 minutes at 4° Celsius, to ensure no remaining lipid had contaminated samples.

Protein concentration was determined using a Pierce BCA kit (ThermoFisher). All samples were completed in triplicate and normalized to a standard curve. Protein concentration was finalized by identifying which sample contained lowest concentration and diluting all running samples to the same level for standardization. Respective concentrations of protein, water and Laemmli loading dye, were boiled for 5 minutes at 95° C, vortexed and centrifuged before use. Ten-well agarose gels (7.5%) were utilized, with one lane occupied by ladder and the remaining 9 lanes occupied by sample (3 groups per gel with 3 samples/group). Gels were transferred to PVDF membrane using a Biorad TurboBlot transfer system for 45 minutes at 25aV at 4° C. Membranes were then blocked in a solution of 5% Bovine Serum Albumin and then incubated for 24-48 hours in respective primary antibody (dilution 1:1000) at 4° C (See appendix 1 for list of antibodies). Membranes were washed in TBST 3x10min, incubated with secondary antibody (HRP linked anti—rabbit), washed again in TBST 3x10min, and then imaged using ECL reagents (Millipore) and a Biorad Chemidoc imaging system. Protein quantification was executed utilizing Fiji Plugin of ImageJ software (NIH) and standardized against home-keeper protein GAPDH.

To determine gene expression, we next completed QPCR by first extracting adipose tissue RNA. Adipose tissue samples of approximately 150-200mg were homogenized in approximately 500ul of Trizol on ice in RNASE free tubes. Samples were incubated for 5 minutes at room temperature. Approximately 100ul of chloroform was added and samples were shaken vigorously. Samples were again incubated at room temperature and then centrifuged at 12000RCF for 15 minutes at 4° C. The supernatant

was transferred to a new tube with and equal volume of isopropanol vortexed to mix. Samples were incubated again for 10 minutes and then centrifuged at 12000RCF for 10 minutes at 4° C. This was repeated twice to clean samples and then the final supernatant was removed and the pellet was left to dry in a ventilation room for 5 minutes. RNA concentration was quantified using a Nano Drop 2000. RNA levels were standardized with DDH_2 O to 0.5ug/ul.

cDNA synthesis was completed in 0.2mL tubes utilizing 1uL of RNA template (total RNA concentration 1ug), 15uL RNASE/DNASE free water and 4 uL of readyscript cDNA synthesis mix. Mixture was vortexed, centrifuged and then incubated in a thermocycler (Eppendorf) for 5 minutes at 25° Celsius, 30 minutes at 42° Celsius, 5 minutes at 85° Celsius and then held at 4° Celsius before storage at -20° Celsius. Plates with 384 wells were run on a BioRad plate reader under the following conditions: Step 1 95°Celsius 10 min for initial denaturation of target DNA followed by 40 cycles of steps at 94°Celsius, 58°Celsius, and 72°Celsius, with fluorescence data collection at each 58°Celsius cycle end.

All data was measured through statistical analysis. Measurements for single time points were analyzed by Fishers' least significant difference test so multiple comparisons of differences between groups REF, NF, HF, H1N, H5N and H9N were considered. Fisher's least significant difference test was performed by first carrying out one-way analysis of variance for all treatment groups. For the longitudinal data such as body weight and food consumption, a linear mixed model was used for the analysis of repeated measures with each individual mouse as a random effect. For QPCR data,

reported fold change was calculated using the delta method (86). Values that were significantly different ($p<0.05$) from the NF group are indicated with a * sign, whereas all values significantly different from the HF group are indicated with a # sign. Adipocyte diameter, F4/80 quantification, and CLS calculations were compared using a Fisher's least significant difference using one-way analysis of variance. All analyses were carried out by using SAS JMP software (SAS Institute Inc., Cary, NC, USA).

CHAPTER IV

RESULTS

Initially, postnatal energy intake was determined to determine differences between groups. The REF group had the lowest postnatal energy intake, correlating with its adherence to the NF diet. There was no significant difference in energy intake between the other groups reflected by their adherence to HFD (Fig 2A), indicating that offspring energy consumption was not dependent upon maternal transition time.

At birth there was no statistically significant difference between body weight in all groups. The REF group maintained the lowest body weight throughout the 12-week postnatal timepoint, correlating with continued adherence to the NF diet. The NF group was slightly but insignificantly elevated as compared to the REF group. The H5N group maintained postnatal weight similar to the NF group and had no significant difference when compared to either the NF or REF group. Both the HF and H9N maintained similar body weights throughout the 12-week postnatal period and were elevated compared to the H5N group. Both HF and H9N had significantly increased body weights compared to the REF group at week 12. The H1N group deviated in body weight beginning at week 5 and maintained a consistent and significant increase in body weight compared to all other groups (Fig 2B). The increased body weight observed in the H1N group did not correlate with a concurrent increase in caloric intake, indicating that the increased body weight was influenced by maternal transition.

To evaluate how maternal diet effected visceral adiposity, we next measured amounts of visceral adipose mass compared to overall body weight (Fig 2C). The H1N group had the highest level relative adipose tissue when visceral adipose tissue mass was normalized against total murine body weight. The relative adipose tissue of the H9N group was significantly reduced compared to the H1N group, although it remained a significantly higher level than that of the NF group. In contrast, while the H5N group had significantly reduced relative adipose tissue mass compared to the H1N, H9N, and HF groups, it was still higher than the REF group (Fig 2C).

Glucose tolerance was measured by performing IPGTT before termination at week 12. Glucose tolerance was determined by evaluating the area under the curve (AUC). Fasting glucose was not significantly different between groups. All groups peaked at approximately 30-60 minutes and were able to return to basal level at 120 minutes with the exception of the H5N group. The NF and HF groups were glucose tolerant at week 12 (Fig 2D and E). The H9N group was not statistically different from the REF group and displayed recovered glucose tolerance compared to the H5N and H1N groups. The H1N and H5N groups displayed significant glucose intolerance ($p<0.01$), as compared to the REF group, however the H1N group was able to return to basal glucose levels after 120 minutes.

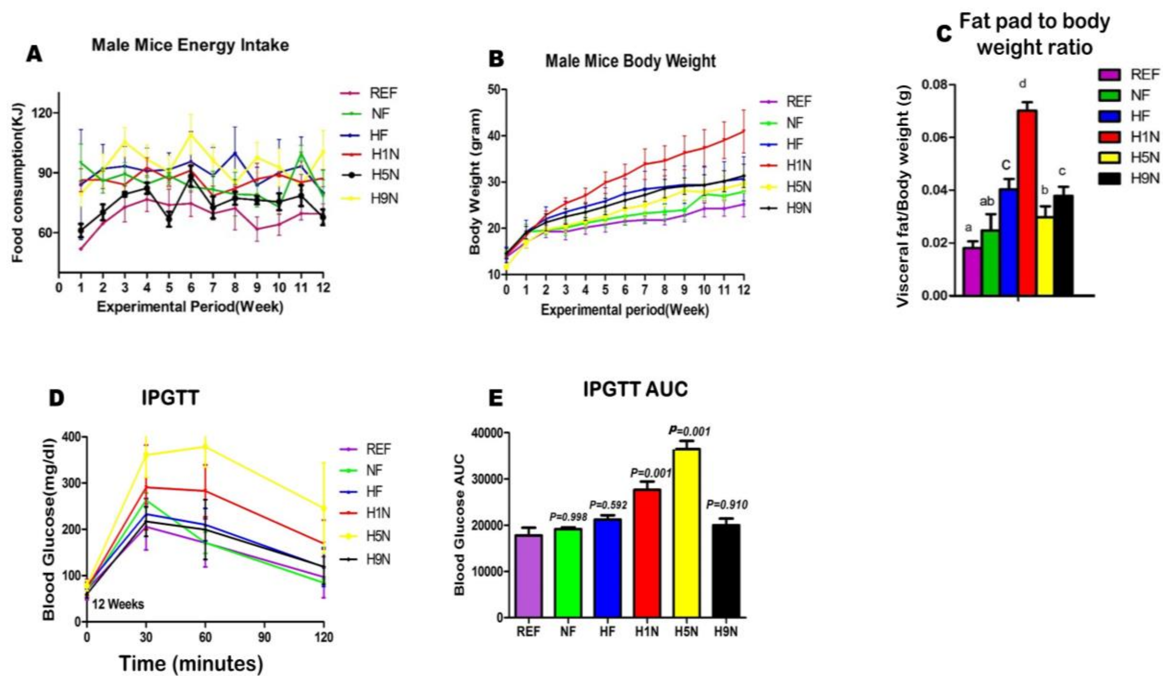


Figure 2- Offspring Energy Intake, Weights and Glucose Tolerance

Legend: (A) Energy intake of the male mice from wean until week 12 showed significantly lower consumption of energy in REF group-the only postweaning group administered NF diet. (B) Body weight of male offspring from wean to week 12. (C) Visceral adipose tissue mass was normalized against individual murine body weight to eliminate outliers. Different letter indicates significance level of *p<0.05* between any two groups. (D) IPGTT at week 12, H1N and H5N group had impaired glucose tolerance. (E) H1N and H5N had significantly increased AUC (*p<.01*) as compared to other groups. All Figure 1 data is reported as Mean±SEM, (n=6-10).

Adipocyte diameter was normalized against the REF group to determine how different duration of maternal diet intervention effected adipocyte size. When compared to the NF offspring, the H1N offspring had significantly increased adipocyte diameter, however this was not significant in relation to any other group. The H5N group also experienced significantly increased adipocyte diameter when compared to the HF group. Although the H9N group had similar adipocyte diameter compared to H5N, there was a slight reduction in adipocyte size which resulted in no significant difference between H9N offspring and any other offspring groups (Fig 3).

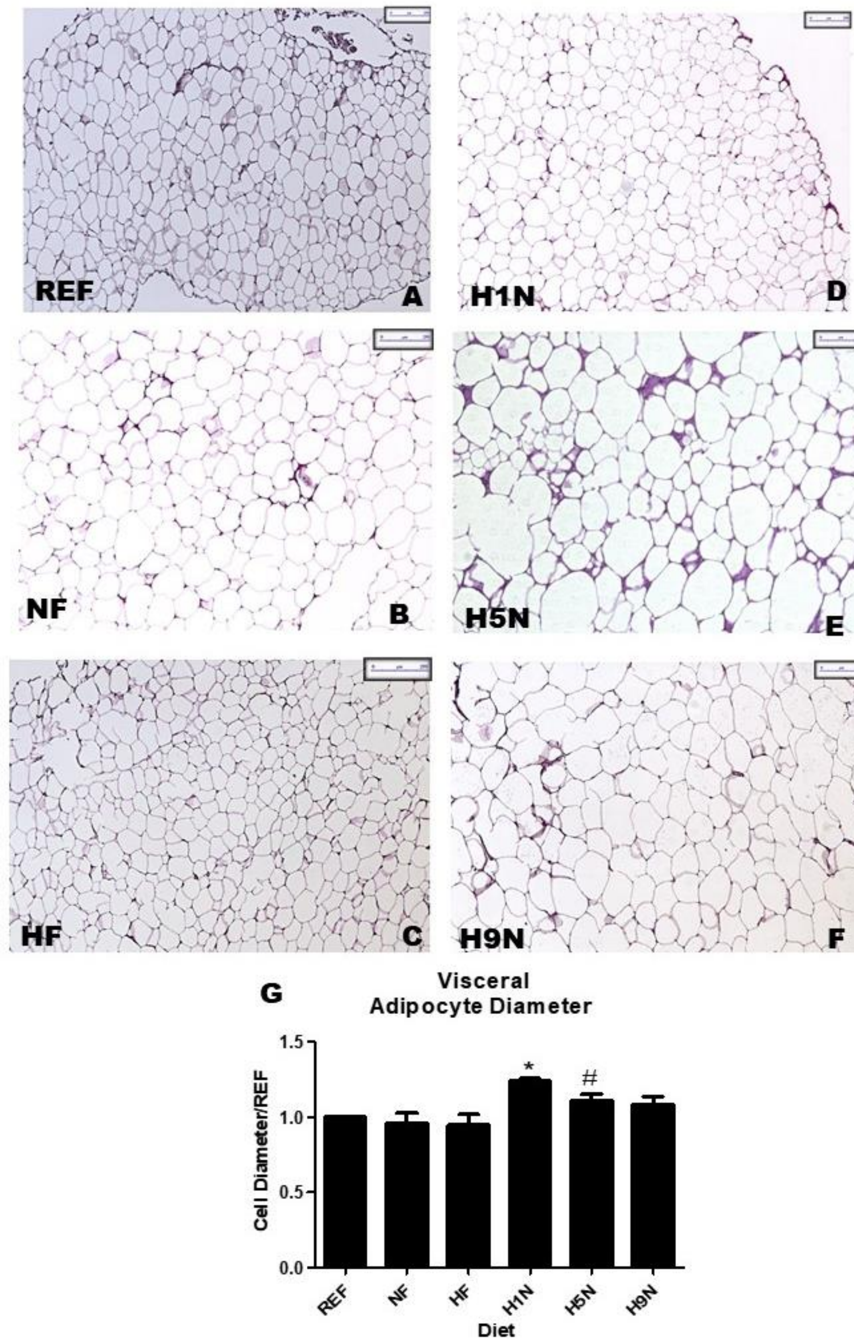


Figure 3- Visceral Adipocyte Diameter

Legend: (A-F) HE staining of visceral adipose tissue. (G) Cell diameter measurements of adipocyte normalized against REF group (n=5-7 ± SEM).

Ppar-γ expression is highly correlated with adipocyte differentiation and is a facilitator for fatty acid and glucose uptake (87). The H1N group experienced significant increase in expression of *Ppar-γ and C/ebpa ,* as did the HF group, when compared against the NF group. The high expression of *Ppar*-γ in the H1N group correlates with the increased fat mass and adipocyte diameter measured in this group, indicating a preferential uptake of excess energy in this group. In contrast, the H5N group has significant reduction in expression of *Ppar*-γ compared to the HF group, although there is a significant increase in levels of *C/ebpa*. Reduced expression of *Ppar*-γ may explain the increased glucose intolerance experienced by the H5N group, however the increased expression of *C/ebpa* may partially explain why the H5N group maintains significantly increased adipocyte diameter, given its role in terminal adipocyte differentiation. In contrast the H9N group maintains expression levels of both genes similar to the NF group and has significantly lowered expression when compared to the HF group (Fig 4 A&B).

Srebp1c is highly expressed in adipose tissue and influences adipocyte differentiation by acting upon *Ppar-γ* (48). *Srebp1c* was significantly overexpressed only in the HF offspring when compared to the NF offspring. There was no significant difference between H1N, H5N and H9N offspring who all had expression levels similar to the NF offspring (Fig 4C). *Acacβ* and *Fasn* are two genes encoding two rate-limiting enzymes for fatty acid synthesis. *Fasn* was significantly increased in the HF, H1N and H5N offspring as compared to the NF group. The H9N group had significantly decreased expression when compared to the HF offspring and was expressed similarly to

the NF offspring (Fig 4D). Expression levels *Acacβ* were significantly increased in the H1N and HF offspring compared to the NF offspring. The H5N and H9N groups had reversed expression similar to the NF offspring (Fig 4E).

We further measured the expression of genes in fatty acid transport in adipose. CD36 levels were significantly increased in the HF and H5N groups as compared to the NF group, whereas H9N was significantly decreased compared to the HF group. The H1N group had a similar level of CD36 as the NF group. Additionally, *Fabp-4* overexpression occurred significantly in the HF, H1N and H5N groups compared to the NF group. When compared to the HF offspring, the H9N offspring had significant reduction in expression, similar to the NF offspring.

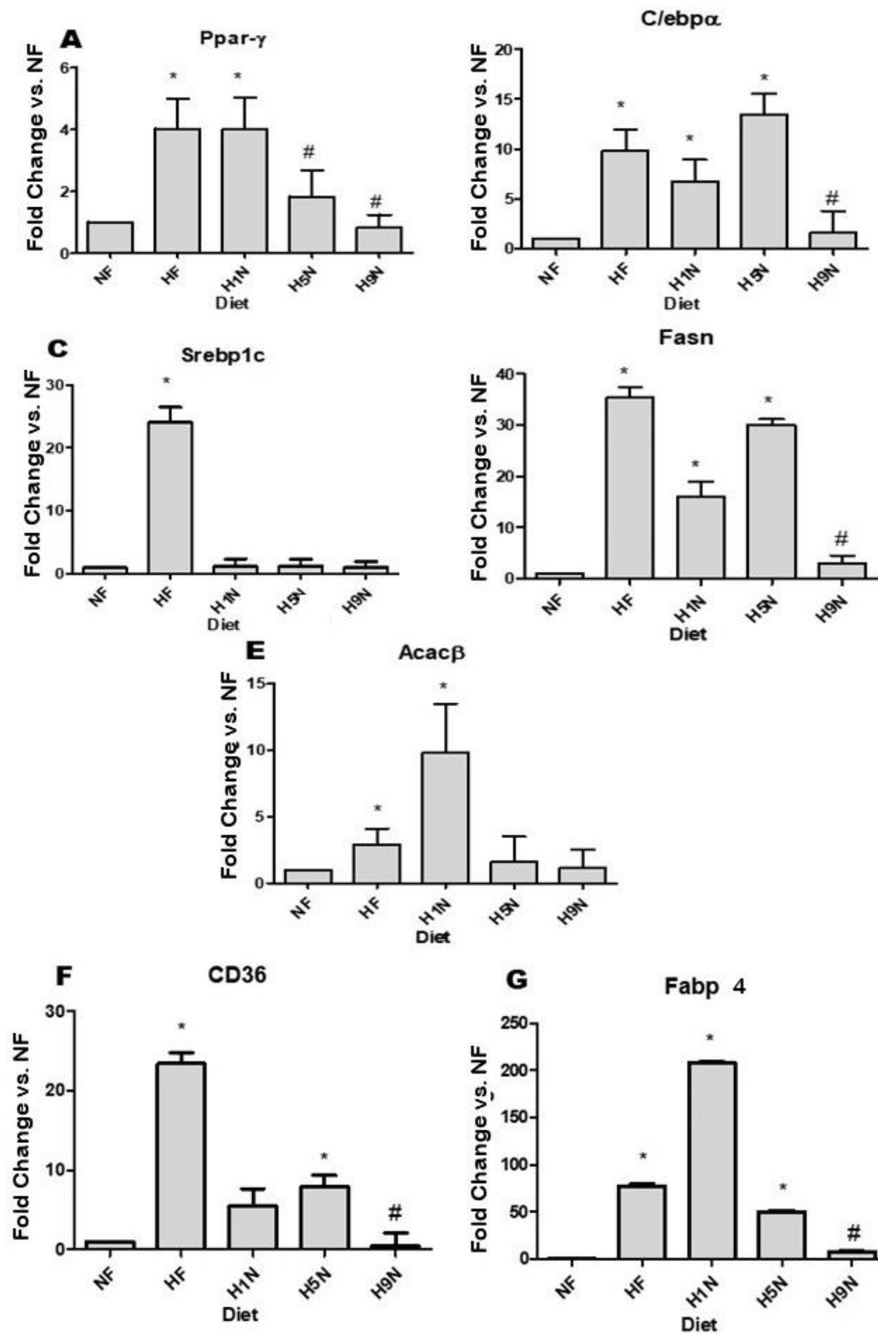


Figure 4-Genes of Adipogenesis

Legend: (A-G) The adipose tissue expression of *Ppar-γ, C/ebpa, Srebp1, Fasn, Acacβ, CD36*, and *Fabp4* were measured by real-time PCR. The expression level was normalized against the NF group. Data is reported as Mean±SEM, n=3-5. The "*" symbol indicates $p<0.05$ versus the NF group. The "#" symbol indicates $p<0.05$ versus the HF group.

CLS are pockets of lipid spillage resulting from cellular or oxidative damage to adipocyte cell membranes. Presence of CLS was not significantly different among the NF, HF, H1N or H5N groups. The H9N group on the other hand had a significantly decreased presence of CLS compared to both the HF and NF groups, indicating a recovery of inflamed phenotype (Fig 5 A, C).

F4/80 is indicative of activated macrophage presence in adipose tissue (82). The H1N and HF group had similar presence of F4/80 and were significantly increased compared to the NF group. The H5N group also had significantly increased presence of F4/80 compared to the NF group, more so than any other group. Expression of F4/80 in these groups was predominantly localized to regions of crown like structures indicating activated pro-inflammatory response of macrophage infiltration. In contrast the NF and H9N group had decreased presence of F4/80 compared to the HF group, and it was primarily localized to the cell membrane, similar to what occurs in resident macrophage populations of healthy adipose tissue (Fig 5 B, D).

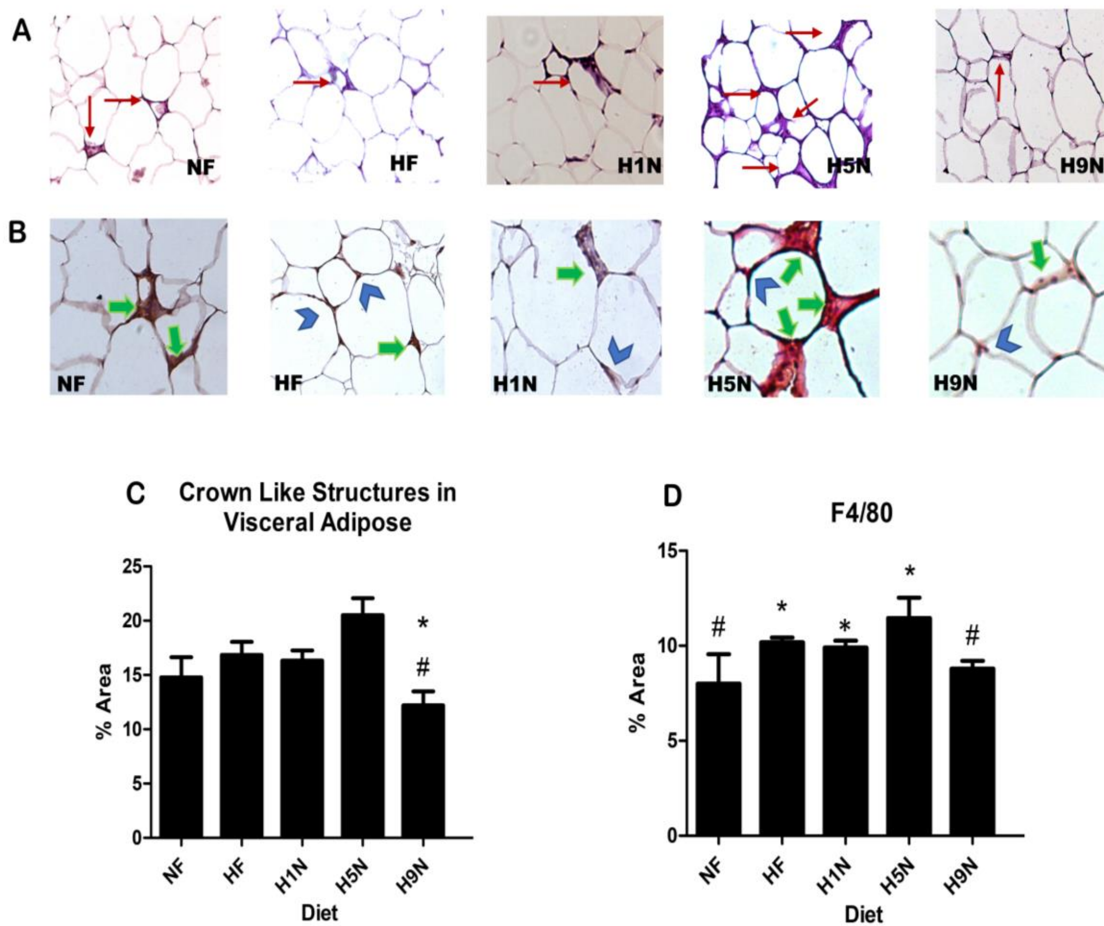


Figure 5-Macrophage Infiltration

Legend: (A) HE staining used to identify CLS (n=5-7). There was no evident presence of CLS or F4/80 in the REF group. Red Arrows indicate CLS. (C) Absolute percent area of CLS present in visceral adipose tissue determined H9N had a significant decrease compared to both NF and HF groups. (B) F4/80 staining used to indicate pro-inflammatory macrophages. Green arrows indicate F4/80 present in CLS. (D) HF, H1N, and H5N groups were significantly increased compared to NF group. NF and H9N group were decreased as compared to HF group.

We further assessed inflammatory activity by evaluating the cytokines *Il-6*, *Il-1β Tnf-α* and *Il-10*. *Il-1β* is largely derived from activated macrophages to assist in inflammatory response and phagocytosis. *Il-1β* was significantly increased in the HF, and H5N group, compared to the NF group, which agrees with the level of F4/80 macrophage expression. Surprisingly, *Il-1β* levels were also significantly increased in the H9N group as compared to the NF group (Fig 6A), and this was the only cytokine of which H9N experienced any upregulation. The H1N offspring had the lowest expression of *Il-1β*, however it still maintained a significant increase in expression as compared to the HF group.

Il-6 was significantly overexpressed in H1N and H5N offspring compared to both the NF and HF offspring. H9N offspring had a reduction in expression of *Il-6,* however this was not significant compared to any group (Fig 6B). Similarities exist in expression levels of *Tnf-α*, which experienced significant upregulation in both HF and H1N offspring when compared to the NF offspring, although there were significant reductions in both H5N and H9N when compared to the HF group (Fig 6C).

Interestingly, the H1N group had significantly increased expression of *Il-10* as compared to both NF and HF offspring (Fig 6D), while the H5N was significantly increased as compared to the NF offspring. There was no significant difference in the H9N groups when compared to either the NF or HF groups.

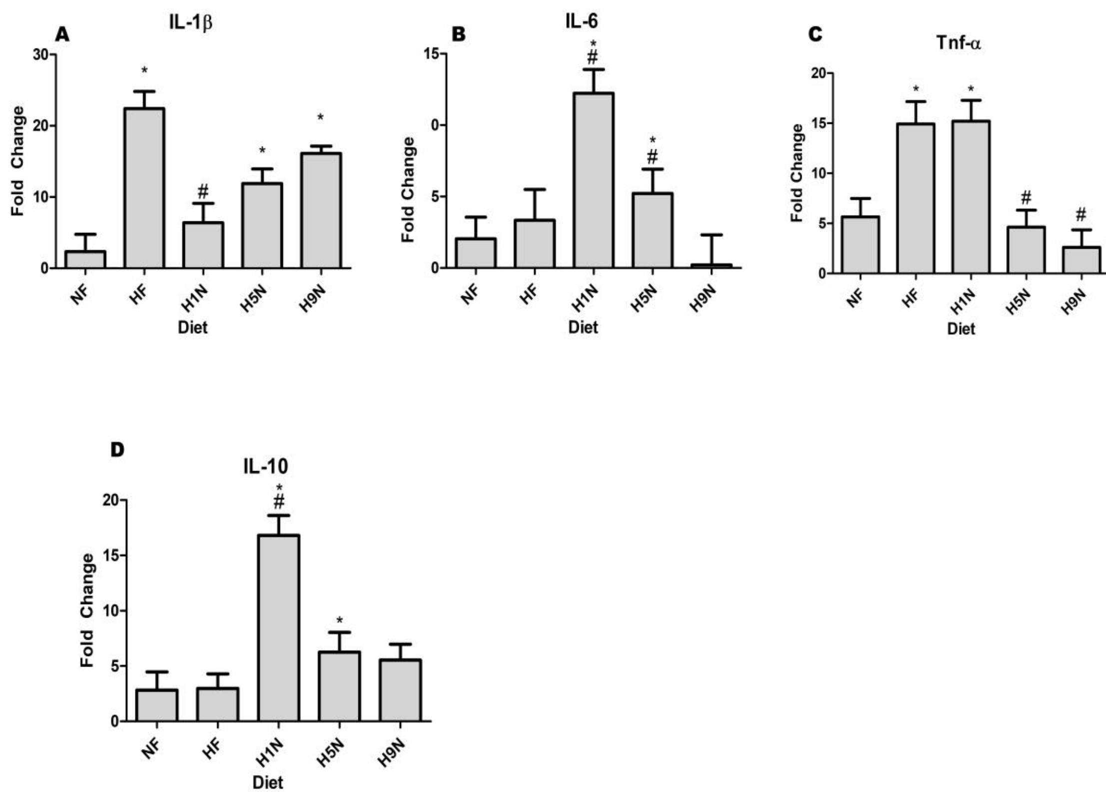
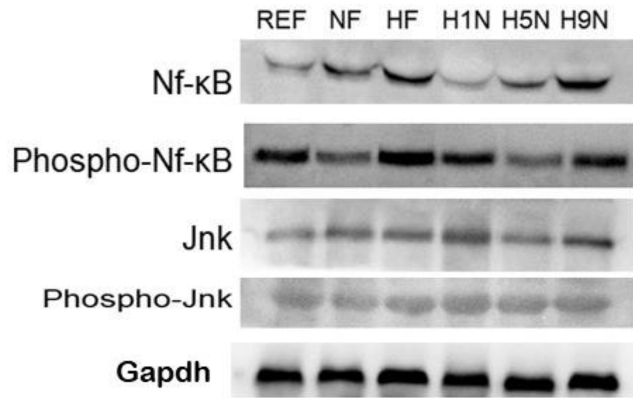


Figure 6: Cytokine Expression in Visceral Adipose Tissue

Legend: (A-D) The adipose tissue expression of cytokines *Il-6*, *Il-1β Tnf-α* and *Il-10*.were measured by real-time PCR. The expression level was analyzed using the fold change of the delta method. Data is reported as Mean±SEM, n=3-5. The “*” symbol indicates $p<0.05$ versus the NF group. The “#” symbol indicates $p<0.05$ versus the HF group.

JNK and NF-κB signaling pathways are activated by phosphorylation which is carried out largely by the cytokines *Il-6, Il-1β* and *Tnf-α.* H1N group experienced significantly upregulated ratio of phosphorylated NF-κB to NF-κB as compared to the HF group or NF group (Fig 7A). There was no significant difference when comparing the H5N group and H9N group to either the NF or HF group. Of the three intervention groups, H9N had the lowest expression level, however this was not significantly different from the NF or HF groups.

Ratio of pJNK/JNK was significantly increased in the H1N group, but not other groups, as compared to the NF group. Interestingly, the H5N group experienced a significant reduction in expression as compared to the HF group, while it was not significantly different from the NF group. pJNK/JNK in the H9N group was not significantly different from any group (Fig 7B).



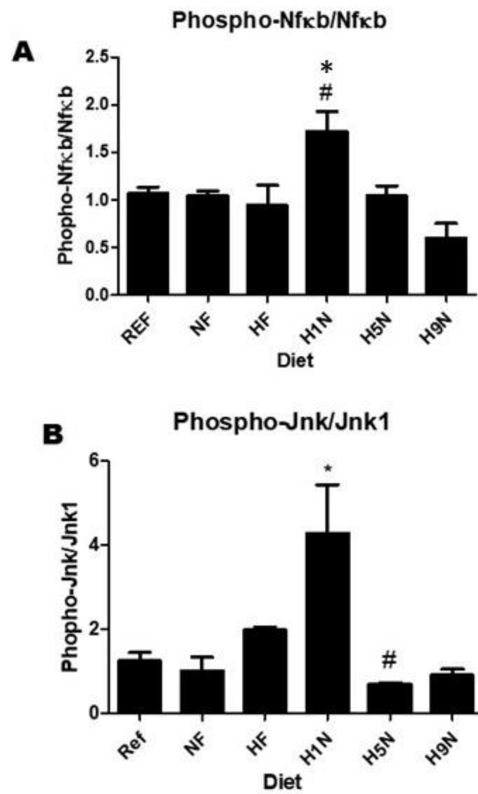


Figure 7- Signaling Pathways of Inflammation

Legend: Ratios of phosphorylated to non-phosphorylated signaling were evaluated to determine pro-inflammatory pathway activation. Data reported as Mean± SEM (n=3).

A primary difficulty of elucidating offspring phenotypes in response to maternal diet are the confounding factors that can affect gestation such as weight gain and hormonal changes, gestational diabetes, and late gestation insulin resistance. By maintaining similar body weight of the mothers across all intervention groups at both the beginning of the study and after 12 weeks on HF diet we were able to eliminate significant influence of confounding variables (Fig 8A). Weight maintained consistent between the H1N, H5N and H9N groups as compared to the HF group, and this was continued with no significant weight gain or loss through the lactation period. This allows for isolation of the effects of dietary intervention. Additionally, there was no indication of glucose intolerance before or after pregnancy (Fig 8B-C) of any groups, which eliminates any potential influence of gestational diabetes and minimizes risk of *in-utero* over exposure to excess energy.

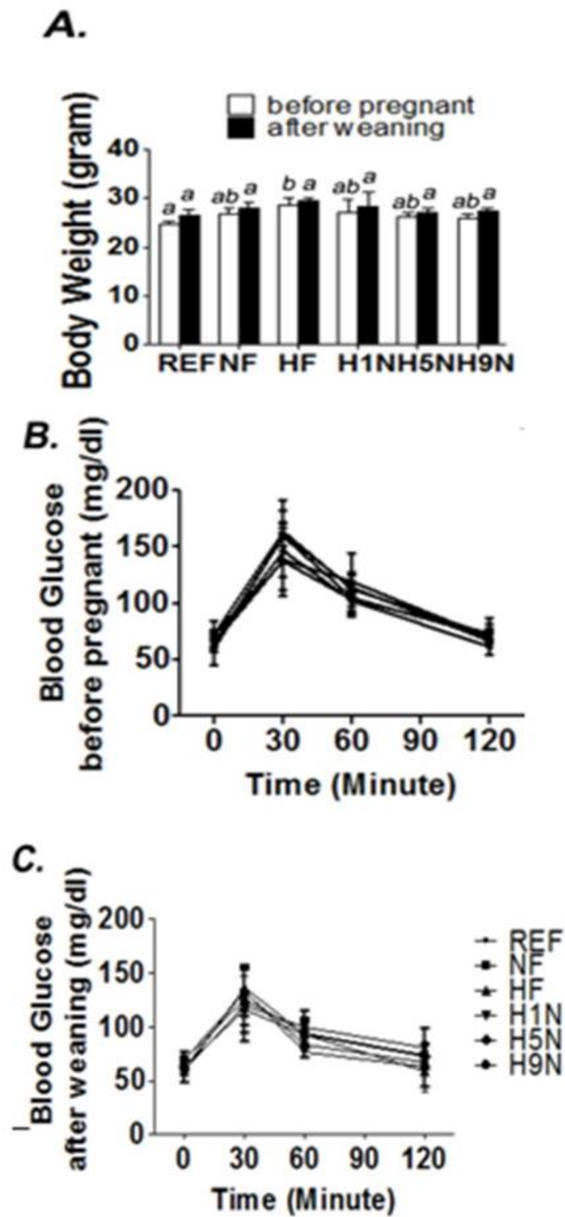


Figure 8- Maternal Weight and Glucose Before and After Pregnancy

Legend: (A) Evaluation of maternal body weight before pregnancy and after weaning resulted in no significant differences. (B-C) Blood glucose levels before and after pregnancy did not differ significantly indicating that this did not contribute to observed offspring phenotypes.

CHAPTER V

DISCUSSION AND CONCLUSION

High fat diet induced obesity is associated with onset of chronic inflammation and development of metabolic complications and disease. Previous work from our lab has suggested that the effects of HFD feeding may be attenuated by transitioning the maternal diet prior to pregnancy in a long-term manner, and that short-term transition exacerbates and promotes a negative phenotype in offspring female mice (75). Our current study further validates this outcome in male mice.

Increased adipocyte size is characteristic of hypertrophy and is an indication of obesity and increased fat mass, which is concordant with our results from the H1N and H5N groups. Hypertrophic adipocytes have been correlated with insulin resistance and aberrant insulin signaling, which may have influenced the presence of glucose intolerance that occurred in the H1N and H5N offspring. The H5N offspring display more severe glucose intolerance even though the H1N offspring had the largest visceral adipose tissue mass and body weight, indicating that the transition period of 5-weeks before pregnancy is not long enough for sufficient readjustment in the maternal body. Given the reduced fat mass and body weight, compared to the H1N group, the presence of glucose tolerance is not surprising in the H9N group.

Further evaluation of the metabolic impacts of visceral adiposity indicated dysfunctional adipogenesis and fat transport in the H1N and H5N groups which was recovered in the H9N groups. Specifically, increased expression of *Fasn* is indicative of adipogenesis and obesity when expressed in adipose tissue (52). Expression levels that

occurred in all groups align with expected results based on visceral adiposity and adipocyte size. Expression levels of *Ppar-γ* and *C/ebpα,* which play a role in terminal adipocyte differentiation, were upregulated in the H1N and H5N offspring but recovered in the H9N offspring. Exacerbated adipocyte differentiation has been known to occur under conditions of obesity, as a response to manage the excessive lipid stored in adipose tissue (60), which may have been intensified by increased expression of genes regulating fatty acid uptake in the H1N and H5N offspring. This gene expression is concurrent with increased adipocyte fat mass deposition and adipocyte size in H1N and H5N offspring. Interestingly, the H9N group maintained significantly higher body weight and adipose tissue mass as compared to the H5N group but maintained a "metabolically healthy obese" phenotype with normalized adipogenesis and glucose tolerance, illustrating that the maternal diet transition timepoint has a distinct implication for metabolic programming in the offspring.

Obesity and low-grade inflammation have been systematically linked as a trigger for metabolic complications and development of diseases such as Type 2 Diabetes Mellitus and Cardiovascular Disease. Macrophage infiltration and presence of CLS are potent indicators of inflammation in adipose tissues (88). While not significant, the H5N group had a slightly elevated presence of CLS which was supported by a significant increase in presence of F4/80 as compared to the NF group. Of particular note, expression of CD36 was also increased significantly in the H5N group as compared to the NF group. CD36 also plays a role in phagocytosis and macrophage response in adipose tissue (53) which may be associated with the slight increase in crown like

structures witnessed in this group. However, the H5N group did not have activated Nf-κB and JNK signaling, indicating a different molecular signaling pathway may be involved. Only H9N offspring experienced significant decrease in levels of CLS presence as compared to both the NF and HF groups.

Interestingly, the H1N group had insignificant infiltration of F4/80 and CLS presence in adipose tissue which was contrary to expectations. However, there remained an increased expression of pro-inflammatory cytokines *Il-6, I1-β* and *TNF-α* and consequent activation of pro-inflammatory signaling pathways Nf-κB and JNK, which may have been perpetuated by the increased adipocyte diameter and fat mass of the H1N offspring. Although the H5N offspring had higher levels of macrophage infiltration and CLS, there was not a concurrent expression of pro-inflammatory cytokines, indicating that either there was another pro-inflammatory pathway activated, or that the larger population of macrophages were not as potent in recruiting cytokines as were the enlarged adipocytes of the H1N offspring. The H9N group experienced recovery of macrophage infiltration, CLS, and significantly decreased expression of pro-inflammatory cytokines which is consistent with decreased adiposity and body weight compared to the H1N group. Taken together, the data from macrophage infiltration, CLS, pro-inflammatory cytokines and pro-inflammatory signaling pathways indicates a distinct and significant pro-inflammatory phenotype in the H1N and H5N groups that is recovered in the H9N group.

Overall, the H1N group and H5N group had significant exacerbation of both physical and cellular pathways of adipogenesis and inflammation. In contrast the H9N

group had significantly reduced inflammation and physical attributes of obesity which supports our hypothesis that a long-term transition can effectively reduce symptoms of obesity, recover dysfunctional adipogenesis, and reduce inflammation instilled by maternal high fat diet.

Metabolic memory, theorized as the memory the body retains of a metabolic insult such as prolonged hyperglycemia in diabetes, even after normalization, has been widely researched as an opportunity for aggressive, early treatment for deterring advancement of metabolic diseases (89). In our study, we witnessed the recovery of a distinct metabolic phenotype which effectively reversed the damaging effects of maternal HFD administration. However, the mechanism of this remains unknown. If maternal metabolic memory was time-dependent, there would be a stepwise reduction in phenotype between the H1N, H5N and H9N offspring. Yet, the H5N offspring displayed exacerbated glucose intolerance, and increased macrophage infiltration and CLS presence as compared to all other groups. Literature has noted that HFD intake immediately prior to conception has longstanding influences on activation of maternal lipogenesis genes in the placenta, leptin generation and circulation, and adiposity which effect offspring long term (8, 12, 68), which may provide a more concrete characterization of how alterations in maternal diet influence reprogramming in utero, especially when considering the differences in inflammatory activation between the H1N offspring and H5N offspring.

Our study, for the first time, evaluated how maternal diet transition at different time points prior to pregnancy effects offspring outcomes of inflammation and obesity in

the visceral adipose tissue. Visceral adipose tissue is a driver of metabolic complications such as Type 2 Diabetes Mellitus, Non-Alcoholic Fatty Liver Disease, and Metabolic Syndrome. Understanding how maternal dietary intake impacts offspring risk- either exacerbating or recovering inflammatory phenotype- has potent implications for reducing intergenerational risk of disease. Our work demonstrates the ability of properly timed maternal diet intervention to reduce risk, but also effectively illustrates that maternal diet if poorly timed, can exacerbate negative offspring phenotypes- in some instances more so than without intervention. This demonstrates the crucial need for more research which evaluates the mechanisms by which offspring programming occurs, and the critical windows that can have optimal impact and reduce serious health risk.

Additionally, our work also serves as an evaluation on the viability of a simple dietary intervention without maternal weight loss on influencing offspring outcomes of obesity and inflammation. A widespread barrier to diet adherence and recruitment of women to clinical trials is that interventions are often time consuming and involve drastic lifestyle changes (90). Our intervention provides distinct results in recovering offspring health, independent of maternal weight loss. This provides viability to long term adherence and provides an avenue in which dietitians can become involved in future clinical trials through dietary coaching and education. Focusing on dietary intervention prior to pregnancy helps lay the framework for effective guidelines with feasible implementation capabilities across diverse populations.

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APPENDIX I

ANTIBODIES

Nf-κB ……………………………………………………Cell Signaling Technology #8242

Phospho- Nf-κB……………………………………… Cell Signaling Technology #3033

JNK…………………………………………………..... Cell Signaling Technology #9252

Phospho-JNK…………………………………………Cell Signaling Technology #9255

Gapdh……………………………………………………..Cell Signaling Technology #2118

F4/80……………………………………………………….Cell Signaling Technology #70076

APPENDIX II

QPCR PRIMERS 5'- 3'

Acacb-F	*GAACCGGCTTCCTGGTTG*
Acacb-R	TCCTCCCCTATGCCGAAA-GA
Cd36-F	TGGAGGCATTCTCATGCC-AG
Cd36-R	TTGCTGCTGTTCTTTGCC-AC
Fabp-F	GTGGTCCGCAATGAGTTC-AC
Fabp-R	GCTTGACGACTGCCTTGA-CT
Fas F	GGAGGTGGTGATAGCCGG-TAT
FAS F	TATCAAGGAGGCCCATTT-TGC
IL10 F	GCTCTTACTGACTGGCATGAG
IL10 R	CGCAGCTCTAGGAGCATGTG
IL1b F	GCAACTGTTCCTGAACTCAACT
IL1b R	ATCTTTTGGGGTCCGTCAACT
IL6 F	TGCCTTCTTGGGACTGAT-GC
IL6 R	CTGTTGTTCAGACTCTCTCCCT
PPARg-1F	TGGTTCAAATATGCCACC-AG
PPARg-1R	CCAAGTGCTGGGATTAAA-GG
SREBP-1c F	AGCAGTCACCAGCTTCAG-TC
SREBP-1c R	GGTCATGTTGGAAACCAC-GC
Tnf F	CCCTCACACTCAGATCATCTTCT
Tnf R	GCTACGACGTGGGCTACAG