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UNIVERSITY OF CALIFORNIA SAN DIEGO

Adiponectin Protects Against Offspring Inflammation Induced by Maternal Obesity

A Thesis submitted in partial satisfaction of the requirements
for the degree Master Science

in

Biology

by

Brianna Dzyuba

Committee in charge:

Professor Jianhua Shao, Chair
Professor James Kadonaga, Co-Chair
Professor Alexandra Dickinson

2024

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The Thesis of Brianna Dzyuba is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2024

Dedication

I would like to dedicate my thesis to my parents who have encouraged me in my educational journey. Additionally, I would like to dedicate my thesis to everyone in the Shao Laboratory who have provided me with guidance and support.

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Abstract of the Thesis

Adiponectin Protects Against Offspring Inflammation Induced by Maternal Obesity

by

Brianna Dzyuba

Master of Science in Biology

University of California San Diego, 2024

Professor Jianhua Shao, Chair
Professor James Kadonaga, Co-Chair

Maternal obesity adversely affects offspring health through prenatal and postnatal metabolism, yet the mechanisms underlying these effects remain unknown. Adiponectin, an adipocyte-secreted hormone suppressed during obesity, may play a key role in understanding these outcomes. To investigate this, we studied the impact of maternal obesity on postnatal growth and the role of maternal adiponectin in these processes at postnatal day (P) 10 using

C57BL/6 wild-type (WT) and adiponectin gene knockout (*Adipoq*^{-/-}) mice fed a high fat (HF) diet. We found maternal HF feeding induced postnatal catch-up growth in offspring, signified by a greater body weight. This was attributed to an increased villi/crypt ratio in the small intestine (SI), which was associated with elevated intestinal proliferation and inflammation. Offspring from HF-fed *Adipoq*^{-/-} dams exhibited similar rates of intestinal proliferation, more pronounced inflammation, and no change in body weight or villi/crypt ratio, suggesting adiponectin protects against maternal obesity-induced inflammation in offspring. Based on these findings, we studied nutrient supply and mammary gland (MG) development in these dams. Maternal HF feeding increased milk triglyceride (TG) levels in *Adipoq*^{-/-} dams, potentially contributing to inflammation, while in WT dams, it increased milk extracellular vesicle (EV) size. WT dams on a HF diet exhibited a decreased percentage of alveolar epithelial tissue, suggesting impaired MG development, whereas *Adipoq*^{-/-} dams on a chow diet exhibited reduced alveoli and increased adipose tissue, suggesting early involution. These findings indicate that adiponectin protects against maternal obesity-induced inflammation in offspring, influencing both nutrient supply and MG development.

Introduction

Understanding the Impact of Maternal Factors on Infant Health

Obesity is a complex, chronic disease characterized by excessive body fat [1]. It has become a growing epidemic in the United States with 42.4% of the population classified as obese and 9.2% of the population classified as severely obese [2]. From 2005 to 2014, there has been an increasing linear trend in the prevalence of obesity among women [3]. This trend is particularly concerning among women of reproductive age, with 39.7% of women in the United States diagnosed with obesity [2]. The impact of obesity extends beyond mere numbers, significantly affecting various bodily conditions and exacerbating existing health conditions [4]. Obesity is associated with diabetes, cardiovascular disease, cancer, nonalcoholic fatty liver disease, and acute pancreatitis [4]. Of particular concern with the rate of obesity in women of reproductive age is the potential threat to maternal metabolic adaptation to pregnancy and infant health. Human studies have shown that maternal obesity is linked to adverse long-term health outcomes for the offspring, highlighting the importance of studying early-life health as a predictor of life-long well-being [4, 5]. With obesity on the rise, more research must be dedicated to understanding the connection between maternal obesity and infant health.

There are two main types of body fat: brown adipose tissue (BAT) and white adipose tissue (WAT) [6, 7]. While both WAT and BAT store TGs to be utilized by the body when needed, they differ in purpose [6]. Brown adipocytes have numerous mitochondria that consume TGs to generate heat for maintaining body temperature [6]. In contrast, white adipocytes serve as an energy storage depot, accepting fatty acids that are subsequently converted into TGs [8, 9]. These TGs are held in lipid droplets until broken down into their counterparts and utilized by the body for energy [10]. Excessive expansion of WAT, characterized by the formation of new

adipocytes (hyperplasia) and/or through the expansion of existing adipocytes (hypertrophy), leads to obesity [11]. In addition to WAT serving as a fat depot, it is a major regulator of whole-body metabolism and homeostasis through the release of secretory molecules [8]. Some of these secretory molecules include leptin, tumor necrosis factor- α (TNF- α), interleukin 6 (IL-6), and insulin-like growth factor 1 (IGF-1) [12]. One molecule of interest is adiponectin, an adipocyte-secreted hormone that plays a crucial role in modulating insulin sensitivity, glucose levels, and lipid metabolism [9, 10]. Dysregulation of adiponectin function is implicated in numerous metabolic disorders and has been identified as a marker for gestational diabetes mellitus [10, 13]. Among these disorders, obesity stands out, as it is intricately linked with adiponectin. Obesity tends to decrease ADIPOQ gene expression, resulting in low adiponectin levels in the blood (hypoadiponectinemia), which is widely observed in pregnant women with obesity [14, 15]. This opens a compelling area of research into the role of adiponectin in underlying obesity-related outcomes.

One current area of research interest is the developmental origins of health and disease [16]. This theory, also known as fetal programming, considers both prenatal and postnatal environments in understanding infant health, not just at birth but throughout the entire lifespan [16]. Studying fetal programming allows for identifying the source of chronic diseases and enabling the implementation of preventative care for infants [17, 18]. Human and mouse studies on cardiovascular, neural developmental, and psychiatric phenotypes in infants have associated these pathophysiologies with fetal programming [16]. Rodent studies have also revealed that a HF diet during pregnancy and lactation resulted in greater offspring body weight and adiposity by the end of lactation [19]. Based on these findings, along with the prevalence of obesity in the United States and the observation that many obese pregnant women also experience

hypoadiponectinemia, we have chosen to investigate the role of maternal adiponectin in maternal obesity-impaired offspring development [2, 15]. Both human and mouse studies have shown a correlation between obesity and adiponectin levels concerning infant development [20, 21]. However, the examination of adiponectin's role in maternal obesity and its influence on infant health remains to be established. Furthermore, while the consequences of maternal obesity on offspring is widely recognized, its impact on maternal practices such as breastfeeding remains unknown [20, 21]. This underscores the need for further investigation into the breast milk composition of obese mothers, with a particular focus on lipid and exosome content, as their dysregulation during obesity has been demonstrated [22]. Additionally, a significant proportion of the MG is composed of WAT, the primary secretor of adiponectin [9, 10, 23]. Therefore, in addition to investigating offspring nutrient supply, the development of the MG may provide insight into the impact of the intrauterine environment on infant health.

Maternal Obesity and Maternal Hypoadiponectinemia Impact on Offspring

Exploring the adverse health outcomes of offspring born to obese mothers, human and mouse studies have found that these infants have an increased risk of cardiometabolic diseases, neurological problems, and early immune diseases within their lifespan [17, 18, 20]. Maternal obesity has been linked to greater adiposity in the offspring during childhood, adolescence, and adulthood [18, 24, 25]. This increased BMI during childhood is associated with adverse cardiometabolic profiles, such as insulin resistance [26]. Interestingly, a mouse study investigated the link between an adverse cardiometabolic profile in infants and parental obesity [26]. The findings revealed a strong association between offspring health and maternal BMI, rather than paternal BMI, suggesting that the intrauterine environment was responsible for these outcomes [26]. Additionally, birth cohort studies have found an association between maternal

obesity and childhood intelligence quotient and executive function [18]. These studies reported that children born to obese mothers have a lower average intelligence quotient and take longer to complete executive functions when compared to children born to normal-weight mothers [18]. These neurological deficits have also been linked to offspring behavior, with preliminary studies showing an increased risk of autism spectrum disorder seen in children of obese mothers [27]. Furthermore, maternal obesity in humans has been correlated with early immune diseases, such as asthma [28]. These findings illustrate that the intrauterine environment can exert a multisystemic influence on the health of the offspring. However, while the effects of maternal obesity on offspring health in children and adults are well-documented, its impact on neonates and infants remains widely unknown.

Currently, several avenues are being explored to elucidate the mechanisms underlying these outcomes. One avenue is looking at epigenetic modifications. The largest human study in this field of research found that maternal obesity influences the epigenome in offspring; sites that have increased methylation associated with maternal obesity were positively correlated with increased fat deposition in the offspring, while the inverse was observed for sites that have decreased methylation associated with obesity [29]. Additionally, ongoing research is looking at the role of inflammation in obesity-impaired offspring health [30]. Studies have reported elevated levels of pro-inflammatory cytokines in mothers with pre-pregnancy obesity, suggesting the association between maternal obesity and offspring disorders may be attributed to early changes in maternal inflammatory markers [30, 31].

Additionally, the impact of maternal adiponectin levels on infant health warrants further exploration. While research on the link between maternal hypoadiponectinemia and infant health isn't as extensive as that on maternal obesity, studies have found that levels of maternal

adiponectin correlate with infant body weight and early immune diseases [32–34]. Human studies have shown an inverse relationship between maternal blood adiponectin levels and infant birth weight [32]. Moreover, pups born to *Adipoq*^{-/-} dams exhibited alopecia around P20-P25 [34]. This finding is interesting because, similar to asthma seen in the offspring of obese mothers, alopecia is also an inflammatory condition. Despite the observed links between maternal adiponectin levels and infant health, the underlying mechanism remains largely unknown and understudied.

Furthermore, offspring nutrient supply may provide insights into the link between maternal obesity and adverse offspring health. Breastfeeding is the standard practice for infant feeding due to the observed correlation between breastfeeding and improved infant health [35]. Human studies have shown that breastfed infants have been found to have lower rates of respiratory tract infections, gastrointestinal infections, obesity, and type 2 diabetes [36–39]. However, women with obesity frequently encounter challenges with lactation, and research has shown that the composition of breast milk in obese mothers is altered compared to normal-weight mothers [40, 41]. While the adverse health outcomes seen in infants born to obese mothers are well documented, none of these studies consider the mediating role that breast milk may have. Additionally, previous research has shown that breastfeeding by obese mothers resulted in increased body weight and higher levels of insulin and leptin in the offspring of lean dams [42]. This underscores the potential negative impact that breastfeeding may have when the mother is obese.

Mammary Gland Development

The human breast develops in three distinct phases: embryonic, pubertal, and reproductive. There is currently little information on human breast development, resulting in the

rodent MG being utilized as a model [43]. Both the rodent and human MG develop isometrically until just before puberty [44, 45]. Murine MG development begins at embryonic day (E) 10.5 with the formation of bilateral milk lines in the ectoderm [46]. This results in five pairs of placodes, columnar-like cells that arise from the migration and aggregation of ectodermal cells toward surface clusters at the milk line [46]. Mammary epithelial cell differentiation is directed by the mesenchyme underlying the milk line through reciprocal epithelial-mesenchymal interactions [46]. The epithelium influences mesenchymal maturation, with the mesenchyme condensing to form layers of fibroblast-rich cells surrounding the rudimentary epithelial mammary ductal structure [47]. The placodes expand by E14 forming a ball of cells that lower into the underlying mesenchyme forming a stalk connecting the resulting mammary bud to the epidermis [48–50]. By E16, a line of epithelial cells extends from the newly formed mammary bud into a collection of preadipocytes, known as the fat pad, where it begins branching off to yield a system of 10-15 branches [50]. Concurrent with this phase, two additional morphological changes occur. Intercellular spaces form within the epithelial cells extending from the milk line to form the ductal lumen [51]. Additionally, the skin overlying the primary mesenchyme remodels to form the nipple, marked by a thickening of the epidermis, suppression of hair follicles, and invagination of keratinocytes [52].

The gland at this point is a rudimentary ductal system, growing proportional to body development. Allometric growth begins at the onset of puberty due to ductal elongation and secondary branching, with an increase in breast size due to a deposit of adipose tissue in the breast [53, 54]. Terminal end buds (TEBs) begin to penetrate the fat pad, driven by the proliferation of a single layer of cap cells at the tips of the TEBs [55]. The primary ductal structure begins to form through TEB divergence, regulated by the surrounding stroma

consisting of a collection of fibroblasts, pre-adipocytes, adipocytes, blood cells, inflammatory cells, and the extracellular matrix [55, 56]. These cap cells differentiate into myoepithelial cells encircling inner luminal cells [55]. During embryogenesis, ductal morphogenesis is constructed without hormonal influences, however, during puberty this process is heavily directed by hormones [57]. Globally, the main hormones stimulating TEB formation and ductal branching are growth hormone and insulin-like growth factor 1, while estrogen plays a local role in this process [57, 58]. Additionally, positive and negative growth factors play a crucial role in establishing the ductal structure, with positive factors promoting growth and negative factors ensuring sufficient space between branches for pregnancy-induced alveolar filling [59–61].

The gland undergoes a significant transformation to prepare for lactation during pregnancy. This begins with a progesterone-induced increase in secondary and tertiary branching, providing ductal arbors for alveolar buds that cleave and differentiate into distinct alveoli, which synthesize and secrete milk during lactation [62, 63]. This process is mainly mitigated by progesterone tasked with creating a lactation-competent gland in tandem with prolactin charged with the differentiation of alveoli [63, 64]. By late pregnancy, the fat pad is mostly composed of alveoli surrounded by a network of capillaries [65]. The machinery for breast milk production begins during the early stages of pregnancy [66]. Some milk-producing genes and enzymes are activated, causing mammary epithelial cells to differentiate and produce some breast milk components [67]. However, these components are reabsorbed back into the blood as suckling is not occurring [68]. The second stage of lactogenesis is initiated by the expulsion of the placenta which triggers a steep reduction in progesterone and estrogen [69]. As their levels diminish, milk production is initiated, aided by prolactin, which regulates milk synthesis [69]. Upon weaning, the lack of suckling results in milk stagnation in the mammary

epithelium, initiating involution of the milk-producing epithelial cells and reverting them to a simple ductal structure [70, 71]. This involution phase is reversible as suckling will reestablish the milk supply [71, 72]. After 48 hours of weaning, the second irreversible phase begins [71]. This consists of a breakdown of the extracellular matrix and a second round of apoptosis, resulting in the removal of a majority of the secretory epithelium, and its subsequent replacement with adipocytes [71, 73, 74].

A key player, yet one vastly understudied, in the maturation and development of the breast structure, is adipose tissue. The majority of tissue in the breast is WAT, marked by large adipocytes that contain single lipid droplets, tasked with controlling energy homeostasis through the storage and release of lipids [23, 75]. As the MG prepares for lactation, there is a drastic reduction of adipose tissue and a simultaneous increase in alveoli [76]. The mechanism underlying this reduction is largely unknown in humans, but a recent study on swine MG development may provide insight into this process [76]. Throughout pregnancy, adipocytes dedifferentiate into preadipocytes and fibroblast-preadipocytes leading to the decrease in adipocyte area seen during the early stages of lactation [76]. This transformation may be influenced by hormones inhibiting adipocyte differentiation, keeping adipocytes in a poorly differentiated state with high proliferative capacity in preparation for remodeling after lactation [76]. Additionally, adiponectin may play a role in this stage of MG development [77, 78]. Previous bovine cell culture studies have shown that adiponectin induces mammary alveolar cell proliferation in a dose-dependent manner, with optimal proliferation occurring at 20 ng/mL [78]. However, concentrations higher than this decreased proliferation rates [78]. During bovine lactation, adiponectin mRNA levels are significantly reduced compared to levels during non-pregnancy and post-weaning [77]. These studies suggest that adiponectin reduction may promote

epithelial proliferation during lactation, while adiponectin increase induces epithelial apoptosis in involution. Mouse studies have shown that after weaning, involution begins resulting in the cessation of MG epithelial cell proliferation and the start of programmed cell death driven by macrophages [79]. Simultaneously, adipocyte expansion is occurring primarily through hypertrophy [80]. The function of these adipocytes during pregnancy is still largely unknown, but the current theory is that these cells release lipids to alveoli during lactation, aiding in milk production [80]. The release of lipids is accompanied by a decrease in adipocyte size and dedifferentiation into preadipocytes [80]. Any surplus of lipids gets trafficked back to the adipocytes [80].

Synthesis and Secretion in the MG

Given the important role of adipocytes in MG development and function, it is important to look at TG metabolism within the MG. TG droplets in the MG mature into cytoplasmic lipid droplets (CLDs) consisting of a hydrophobic core of neutral lipids (triacylglycerols, and cholesteryl and retinyl esters) surrounded by a phospholipid monolayer, and surface-associated proteins [81–83]. These CLDs are accumulated and secreted into milk as plasma membrane bilayer-coated structures in four distinct phases. This process begins mid-pregnancy with the formation of small CLDs in a few immature alveoli [9]. The second and third phases are marked by an increase in size and number isometrically to alveoli maturation near the end of pregnancy, to the extent that milk-secreting cells are filled with CLDs [9]. The last phase occurs during parturition where the CLDs shrink in size and move toward the apical border as they are secreted [9].

Outside of lipids, there are other major nutrients and bioactive components synthesized and secreted by the MG. Breast milk contains a diverse set of human milk oligosaccharides

(HMOs) and glycans utilized by the infant to nurture the intestinal microbiome [84]. HMOs vary drastically due to the activity of two enzymes encoded by the *Secretor* and *Lewis* genes contributing to their poorly understood synthesis and secretion [85]. Additionally, mice lack the complexity of HMOs secreted in human breast milk, making them difficult to study through mouse models [86]. Mouse breast milk only contains 3' and 6'-sialyllactose, whereas human breast milk consists of elongated, branched, and fucosylated oligosaccharides [86]. In addition to establishing gut health, breast milk is vital in establishing overall infant health through its concentration of immunoglobulins and immunological proteins [87]. Breast milk provides the only source of secretory immunoglobulin A (SIgA) for infants until their intestinal plasma cells can produce it on their own [87]. SIgAs play an important role in both humans and mice by preventing infection and inflammation, providing benefits to the intestinal immune system that persists well into adulthood [87]. These SIgAs are produced by plasma cells in the MG which are then transported to alveolar epithelial cells for secretion into breast milk [87]. Additionally, a variety of proteins present in breast milk provide nutritional, antimicrobial, and absorption benefits to the growing offspring [88]. Human milk is whey protein dominant, while mouse milk is casein protein dominant [89, 90]. Throughout human lactation, the whey/casein ratio declines, whereas the protein concentration in mouse milk remains consistent [89, 90]. The majority of proteins are synthesized via lactocytes in the alveoli of the MG, while the rest are taken up from blood plasma [91]. The diverse nutrients, bioactive factors, and beneficial bacteria in breast milk are crucial in supporting infant health.

Effect of Obesity and Hypoadiponectinemia on MG Development and Breast Milk Composition

The first sign of abnormal development of the murine MG begins at E14, signified by a decrease in side-branching and impaired alveolar development [92]. This trend continues into

late pregnancy whereby E18.5, obese dams present an increase in alveolar size, accompanied by an increase in luminal area and reduced epithelial thickness [93]. Additionally, an analysis of the location of lipid accumulation during late pregnancy revealed greater accumulation on the epithelial luminal surface in dams fed a HF diet, leading researchers to conclude advanced milk production and secretion [93]. However, an additional study found that at day 1 of lactation, there was increased lipid accumulation in the alveolar epithelium compared to the alveolar lumen in obese dams, indicating reduced milk secretion [92]. Thus, although there may be an increase in the movement of lipid droplets towards the lumen during late pregnancy, these lipid droplets can't be secreted fully during lactation. These alterations in MG structure and function suggest obesity disrupts MG development, potentially by remodeling the mammary microenvironment.

Additionally, previous human studies have reported difficulty initiating lactation, delayed onset of lactogenesis II, and premature cessation of breastfeeding in obesity [94, 95]. However, the mechanism underlying these complications is largely unknown. Currently, studies are looking into leptin, progesterone, and prolactin as potential factors contributing to the delayed onset of lactation and early involution seen in maternal obesity; yet, due to conflicting results, no definitive conclusions have been reached [96]. Research in mice has indicated that hypoadiponectinemia also impacts MG development, specifically during lactation [34]. A previous study found *Adipoq*^{-/-} dams experienced early involution of the MG, characterized by increased apoptosis and a reduction in ductal/alveolar structure [34]. There was also an increase in leukocyte infiltration, indicating potential inflammation during lactogenesis [34]. Therefore, the existing research suggests that obesity may impact the initial stages of breast milk production and secretion, while adiponectin may influence the later stages during involution. This is

noteworthy as many women who experience obesity during pregnancy also experience hypoadiponectinemia, a factor that was not assessed in the obesity studies.

This effect can extend further throughout infant development due to obesity and adiponectin's impact on breast milk composition. Studies looking into the milk metabolome of obese dams found significant differences in micronutrient concentration compared to controls [97]. HF-fed dams at P21 had significantly less carbohydrates, eicosatetraenoic acid (EPA), and docosahexaenoic acid (DHA), along with increased total fat, arachidonic acid (AA), and leptin content [97]. There were no significant changes in protein content between the two groups [97]. Human milk from obese mothers exhibited lower levels of n-3 polyunsaturated fatty acids (PUFAs), and higher ratios of AA to both EPA and DHA, along with higher ratios of n-6 PUFA to n-3 PUFA [41]. Considering the alterations in lipid content and lipid-associated hormones, in conjunction with decreased lipid secretion, further investigation into lipid processing in obese dams is warranted. Obesity has been shown to impair milk lipid production, potentially due to decreased *de novo* fatty acid synthesis in the lactating MG [22]. This was attributed to the increased availability of already-formed fats due to a HF diet, rendering *de novo* synthesis unnecessary [22]. However, a previous mouse study investigated the difference between a HF diet and obesity utilizing HF-lean and HF-obese mice [22]. HF-obese mice exhibited a significant increase in body weight and fat mass in response to the HF diet, whereas HF-lean mice showed no change [22]. Notably, obesity was found to reduce total lipid milk content without altering the fatty acid composition, but a HF diet had the opposite effect, altering fatty acid composition without affecting total lipid content [22]. The HF-fed dams exhibited elevated levels of long-chain fatty acids, alongside a reduction in the proportion of 16:0 and 16:1 fatty acids [22]. Interestingly, *Adipoq*^{-/-} dam breast milk exhibited increased expression of

proinflammatory cytokines such as TNF- α and IL-6, as well as enzymes that produce proinflammatory lipids such as cyclooxygenase-2 and microsomal epoxide hydrolase [34]. However, there was no significant change in milk lipid content from these dams [34]. Given that the MG is among the largest producers of lipids in the body, the study of milk lipid production and composition emerges as a crucial field for investigation.

Additionally, breast milk contains various bioactive lipid molecules, including extracellular vesicles (EVs), which play a crucial role in maternal-fetal communication [98]. EVs originating from cells, are particles secreted to serve as carriers facilitating communication between cells [98]. The three main subclasses of EVs are exosomes, microvesicles, and apoptotic bodies [99]. Exosomes make up an abundant proportion of breast milk, responsible for transporting lipids, proteins, and nucleic acids, playing a vital role in maternal-fetal biocommunication [100]. Previous mouse studies have shown that exosomes can alter cell development, changing cell fate from normal to pathological in tumor progression, suggesting their negative impact on disease states [101]. Due to exosomes being cell-derived lipid bilayer particles, they can survive the infant intestinal environment and be taken up into the intestinal epithelial cells [100]. Studies have found that milk-derived exosomes play a vital role in maintaining and protecting epithelial cell integrity in the SI, providing further evidence of their impact on infant health [102]. Additionally, these exosomes contain microRNAs (miRNAs) which are short, non-coding RNAs that regulate gene expression by binding to complementary sequences of messenger RNA (mRNA) and repressing their translation [103]. These miRNAs pose an interesting topic of exploration as these exosome-derived miRNAs may regulate offspring gene transcription and tissue development. Studies have shown that obesity increases exosome-derived miRNAs associated with prediabetes and insulin resistance [104]. Furthermore,

plasma exosomes transfected with artificial miRNA overexpressed during obesity induced glucose intolerance, insulin resistance, and central obesity when injected into lean mice [104]. Thus, milk EVs might play an important role in regulating whole-body metabolism in offspring.

Hypothesis/Aim

Obesity is becoming increasingly prevalent in the United States, posing a significant concern for women of reproductive age due to its impact on infant health. While early development is known to predict long-term health outcomes, the consequences of maternal obesity on infants remain poorly understood. Therefore, we aimed to investigate the effects of maternal obesity on infant growth. Additionally, adiponectin is suppressed during obesity, underscoring its potential role in maternal obesity-induced impairments in infant health. Thus, we sought to explore adiponectin's role in the adverse health outcomes seen in infants of obese mothers. Considering breast milk is the sole nutrient source for infants, understanding the role of nutrient supply in these outcomes may provide further insight into the maternal-fetal connection. Moreover, since the MG produces breast milk and contains many adipocytes, understanding adiponectin's function in MG development may shed light on the impact of the intrauterine environment on infant health. Thus, our study explores the implications of maternal obesity and adiponectin on breast milk composition and MG development. By examining the interconnections between maternal obesity, adiponectin, breast milk composition, and MG development, we aim to explore the mechanisms underlying the adverse health outcomes seen in infants born to obese mothers. We hypothesize that adiponectin reduction in dams mediates the adverse effects of maternal obesity on offspring development through milk production and MG development. To test this hypothesis, we first determined the effect of a maternal HF diet on WT and adiponectin gene knockout (*Adipoq*^{-/-}) offspring body weight and intestinal development

at P10. We then analyzed dam nutrient supply and MG development at P10 to determine their contribution to these outcomes.

Methods

Experimental Animals

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *Adipoq*^{-/-} mice were created by Dr. Philipp Scherer's laboratory (UT Southwestern Medical Center, Dallas, TX) and were backcrossed into C57BL/6 background for at least 10 generations in Dr. Shao's lab [105]. Mice were housed under standard conditions and fed either a chow (Charles River; #D12450B, 10 kcal% Fat) or a HF diet (Research Diets Inc.; #D12492, 60 kcal% Fat). The HF diet was provided to female mice at least three months prior to mating and continued throughout pregnancy and lactation. *Adipoq*^{-/-} females mated with *Adipoq*^{-/-} males, and C57BL/6 females were mated with C57BL/6 males to generate *Adipoq*^{-/-} and WT offspring, respectively. Pregnancy was confirmed by the presence of a vaginal plug and was marked as embryonic day (E) 0.5. Dams were sacrificed and MG tissue was collected at pre-pregnancy, embryonic days 5.5, 9, 13, and 18, as well as postnatal days 1, 10, 18, and 24. Dams that were sacrificed at postnatal day 24 had pups weaned at postnatal day 21. Pups were sacrificed, and SI tissue was collected on postnatal day 10. All experimental procedures involving mice were conducted under the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Diego.

Milk Collection

Milk samples were collected at postnatal day 10. To stimulate milk secretion, dams received an injection of Oxytocin (40 mg/kg, i.p., Sigma-Aldrich; Cat #O4375) in PBS (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium phosphate, 1.8 mM monopotassium phosphate). 10 minutes post-injection, general anesthesia was induced with a

combined injection of Zetamine (100 mg/kg, i.p., VetOne; Cat #B1C2) and Xylazine (10 mg/kg, i.p., AKORN Animal Health; Cat#XYAAC). After 5 minutes, manual pressure was applied to the nipples to facilitate milk expression. Milk was collected using a pipette and deposited into collection tubes (QIAGEN; Cat #154046981) (**Figure 1**).



Figure 1: Visual representation of the breast milk extraction process.

TG Concentration

TG concentration was determined using a Triglycerides Reagent Set (HORIBA Instruments Inc.; Cat #321301-015). In an assay plate, breast milk (20 ul) was added to each well of a 96-well plate, followed by the addition of the triglyceride reagent (200 ul) and PBS (100 ul). The solutions were mixed in the plate using a shaker (Lab Line Instruments, Inc.; Model #4826), followed by incubation at 37 °C for 5 minutes in a dry bath incubator (Labnet International Inc.; Model #D1200). The plate was then loaded into a plate reader (EPOCH BioTek Instruments, Inc.; Serial #13030610), and the TG concentration in each milk sample was determined with a calibrator.

Protein Concentration

Protein concentration was determined using a Protein Assay Dye Reagent Concentrate (BIORAD; Log #5000006). In an assay plate, breast milk (100 ul) was loaded into each well, followed by the protein assay mix (200 ul). The plate was briefly shaken on a shaker and incubated at 100 °C for 10 minutes. The plate was loaded into the plate reader, and the protein concentration in each milk sample was determined with a standard curve.

EV Extraction

EVs were extracted from mouse milk samples via ultracentrifugation using a modified human milk EV extraction protocol [106]. Briefly, freshly collected breast milk (50-100 ul) was diluted with 5-fold PBS in a collection tube. The samples were centrifuged at 3,000g for 30 minutes at 4 °C to remove fat globules and cream. The fat globule/cream fraction was extracted and centrifuged at 10,000g for 30 minutes to remove cellular debris and somatic cells. The supernatant was transferred to a tube and EDTA (500 mM, Thermo Fisher Scientific; Cat #040150) was added (0.33 ml of EDTA per ml of supernatant). The samples were ultracentrifuged (Beckman Coulter; Sys. ID #3032645) at 100,000g for 70 minutes, and the pellet was resuspended in Hepes (200 mM, Thermo Fisher Scientific; Cat# 018375). After 10 minutes at 3,000g the supernatant was collected. The EV-enriched solution was filtered and ultracentrifuged at 100,000g overnight and the EV pellet was resuspended in Hepes. The EV samples were imaged and analyzed at the Electron Microscopy Core at the University of California, San Diego.

Pup Body Weight Measurements

Pups at P10 were weighed using a scale (Ohaus; CS Series).

H&E Staining

The majority of the MG and all of the SI tissue utilized for H&E staining were fixed in 10% formalin (3.7% formaldehyde, 10 mM phosphate buffer, pH 7.4), processed, and paraffin-embedded (Leica Biosystems; Cat# 2003159). Dam MG tissue collected at P18 was fixed in 4% Paraformaldehyde (PFA) (2.7 M) and embedded in Optimal Cutting Temperature compound (OCT) (Thermo Fisher Scientific; Cat# 4584). Fixed tissues were sectioned at 5 μ m at room temperature for paraffin-embedded tissue or in a cryostat (Leica Biosystems; Model #CM1520) at -20 °C for OCT-embedded tissue. Fixed samples were either deparaffinized or thawed for 5 minutes. Nuclei were stained with Mayer's Hematoxylin (5% aluminum sulfate, 0.1% Hematoxylin, 0.02% Sodium Iodate, 2% Glacial Acetic Acid) for 1 minute, followed by decolorization using 0.5% Acid Alcohol (70% EtOH, 0.5% HCL) for 1 minute. Tissue sections were dipped in Bluing solution (0.1% sodium bicarbonate in DI water) for 1 minute, rinsed in 95% EtOH for 1 minute, and counterstained with Eosin Y (0.25% Eosin Y, 65% EtOH, 3.8%L Isopropanol, 1% Acetic Acid in DI water) for 30 seconds. Tissue sections were dehydrated and analyzed under light microscopy at x4, x10, and x20 (BZ-X800; Keyence, Laguna Hills, CA). Alveolar epithelial tissue area and villi/crypt ratio (VCLR) measurements were conducted with H&E images and analyzed with ImageJ (64-bit Java 1.8.0-172) software. The percentage of alveolar epithelial tissue was calculated by dividing the area of alveolar epithelial tissue by the total tissue area. VCLR was determined by measuring each villus and the corresponding crypt length.

IHC Staining

MG and SI tissue was fixed in 10% formalin, processed, and paraffin-embedded. Following deparaffinization, tissue sections were blocked with 2% Hydrogen Peroxide (Thermo Fisher Scientific; Cat #162854) in double-distilled water, and then heated in 0.1 mol/L pH 6.0 citrate buffer (65.3 mM in Tween 20) for 20 minutes at 98 °C for antigen retrieval. Tissue sections were blocked for a second time with bovine serum albumin (BSA) (1% BSA, 0.02% Sodium Azide, 0.2% Triton-x100 in PBS, pH 7.4) for 2 hours in a humid chamber (Sigma-Aldrich; #Z67013) at room temperature. After blocking, sections were incubated with anti-Ki67 or anti-F4/80 primary antibodies or BSA (for a negative control) overnight at 4 °C in a humid chamber. Following the primary antibody, anti-rabbit or anti-rat, respectively, secondary antibodies were pipetted onto the tissue sections for 2 hours at room temperature in a humid chamber. Visualization was achieved with 3,3'-diaminobenzidine (DAB) (Vector Laboratories; Cat #ZK1018) at room temperature for 20 minutes for chow diet sections and 10 minutes for HF diet sections, followed by counterstaining with Mayer's Hematoxylin for 1 second. Tissue sections were dehydrated and analyzed under light microscopy at x20. The percentage of Ki67 and F4/80-positive cells was calculated using IHC-stained images and ImageJ software. Ki67 and F4/80-positive cells were divided by the total number of cells to determine the respective percentages.

Statistical Analysis

The data are expressed as the mean $\pm$ the standard error of the mean. All statistical analyses were performed using a Student's t-test or Analysis of Variance (ANOVA), and both

statistical analyses and plotting were conducted using GraphPad Prism (GraphPad Software, LLC; v 8.4.3).

Results

Maternal HF Feeding Increased Offspring's Neonatal Body Weights

Studies have reported the adverse effect of maternal obesity on offspring metabolism and body weight [107]. However, nearly no studies have analyzed its effects on neonatal fat development, which is essential for the development of obesity in later life [108]. To investigate the impact of maternal obesity on offspring development and the role of adiponectin in these outcomes, we analyzed the body weights of offspring born to either WT or *Adipoq*^{-/-} dams subjected to a HF diet. After prolonged HF feeding, there was a significant increase in maternal body fat, confirming the successful establishment of maternal obesity (data not shown). All pups were breastfed by their biological mothers and shared the same genotype as their respective dams. At P10, we assessed pup body weight to gauge the influence of maternal HF feeding on body composition and metabolic profile. Results showed that the body weights of both male and female WT pups from HF-fed WT dams were significantly higher than that of pups from chow-fed control dams (**Figure 2 A-C**). These results indicate that maternal HF feeding increased offspring body weight at an early age. Unlike WT mice, there was no significant difference in body weights between the pups from HF-fed *Adipoq*^{-/-} dams and chow-fed *Adipoq*^{-/-} dams (**Figure 2A-C**). These results indicate that maternal HF feeding in *Adipoq*^{-/-} dams did not affect offspring postnatal body weight.

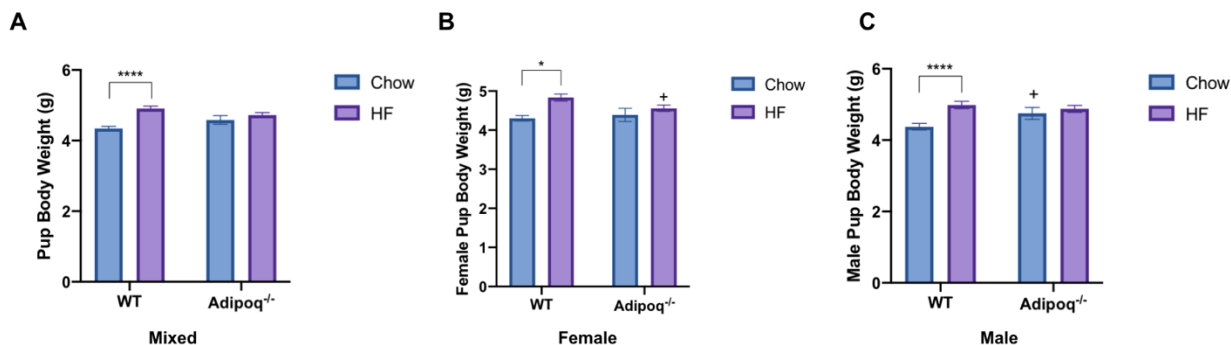


Figure 2: Maternal HF Feeding Increased Offspring Body Weight in WT Mice. Offspring body weight measured at P10. **A:** Both female and male pups, **B:** female pups only, and **C:** male pups only. Data plotted as the mean $\pm$ the standard error of the mean. ANOVA used to determine statistical significance between groups. Notations: * $P < 0.05$, **** $P < 0.0001$ vs. basal, + $P < 0.05$ vs. WT.

Maternal HF Feeding Increased Absorptive Capacity, Inflammation, and Proliferation in the SI of Offspring

The SI is essential in nutrient absorption and still develops during early life [109]. Thus, we measured the villi/crypt ratio using H&E-stained SI tissue sections from pups at P10. Villus atrophy is a nonspecific reaction that can be attributed to a multitude of intestinal injuries such as during periods of starvation or lack of perinatal nutrition [110]. Crypt elongation is a consequence of regeneration or repair, observed before villus atrophy [110]. A greater villi/crypt ratio signifies a greater degree of intestinal development, digestion, and absorptive function [111]. These measurements revealed that WT pups from HF-fed WT dams showed a significant increase in the villi/crypt ratio compared to pups from chow-fed control dams (**Figure 3A**). This suggests that maternal HF feeding increased intestinal structural integrity and absorptive capacity in offspring. However, there was no significant difference in the villi/crypt ratio between pups from HF-fed *Adipoq*^{-/-} dams and chow-fed *Adipoq*^{-/-} dams, indicating that maternal HF feeding did not impact intestinal structural integrity or absorptive capacity in offspring from *Adipoq*^{-/-} dams (**Figure 3A**). Additionally, it is well-documented that obesity induces chronic

inflammation in adult tissue [111]. To assess this, we measured the expression of F4/80, a marker for macrophage infiltration, using IHC in pup SI tissue sections at P10 [112]. Our results showed that WT pups from HF-fed WT dams exhibited a significant increase in the expression of F4/80 compared to pups from chow-fed control dams (**Figure 3B**). This suggests that maternal HF feeding increased macrophage infiltration and inflammation in the SI of these offspring. Furthermore, since obesity stimulates the proliferation of intestinal epithelial cells in adults, we measured the expression of Ki67, a marker for actively dividing epithelial cells, using IHC in pup SI tissue sections at P10 [113]. Results showed that WT pups from HF-fed WT dams exhibited a significant increase in the expression of Ki67 compared to pups from chow-fed control dams, implying that maternal HF feeding increased epithelial cell proliferation in the SI of these offspring (**Figure 3C**). Similar to WT pups, pups from HF-fed *Adipoq*^{-/-} dams exhibited a significant increase in Ki67 and F4/80 expression compared to pups from chow-fed *Adipoq*^{-/-} dams (**Figure 3B and 3C**). This suggests that maternal HF feeding also increased SI proliferation and inflammation in *Adipoq*^{-/-} offspring. Given the significant difference in the basal rates of F4/80 and Ki67 expression in the SI between pups born to chow-fed WT and *Adipoq*^{-/-} dams, we calculated the percent increase for both markers to understand adiponectin's role in maternal HF feeding-impaired offspring growth. Pups from HF-fed *Adipoq*^{-/-} dams had a significantly greater increase in F4/80 expression compared to pups from HF-fed WT dams (**Figure 3B**). However, there was no significant difference in the HF diet-induced increase in Ki67 expression between both groups (**Figure 3C**). This indicates that maternal HF feeding induced more severe inflammation in the SI of offspring from *Adipoq*^{-/-} dams than in offspring from WT dams but induced a similar increase in SI proliferation in both groups.

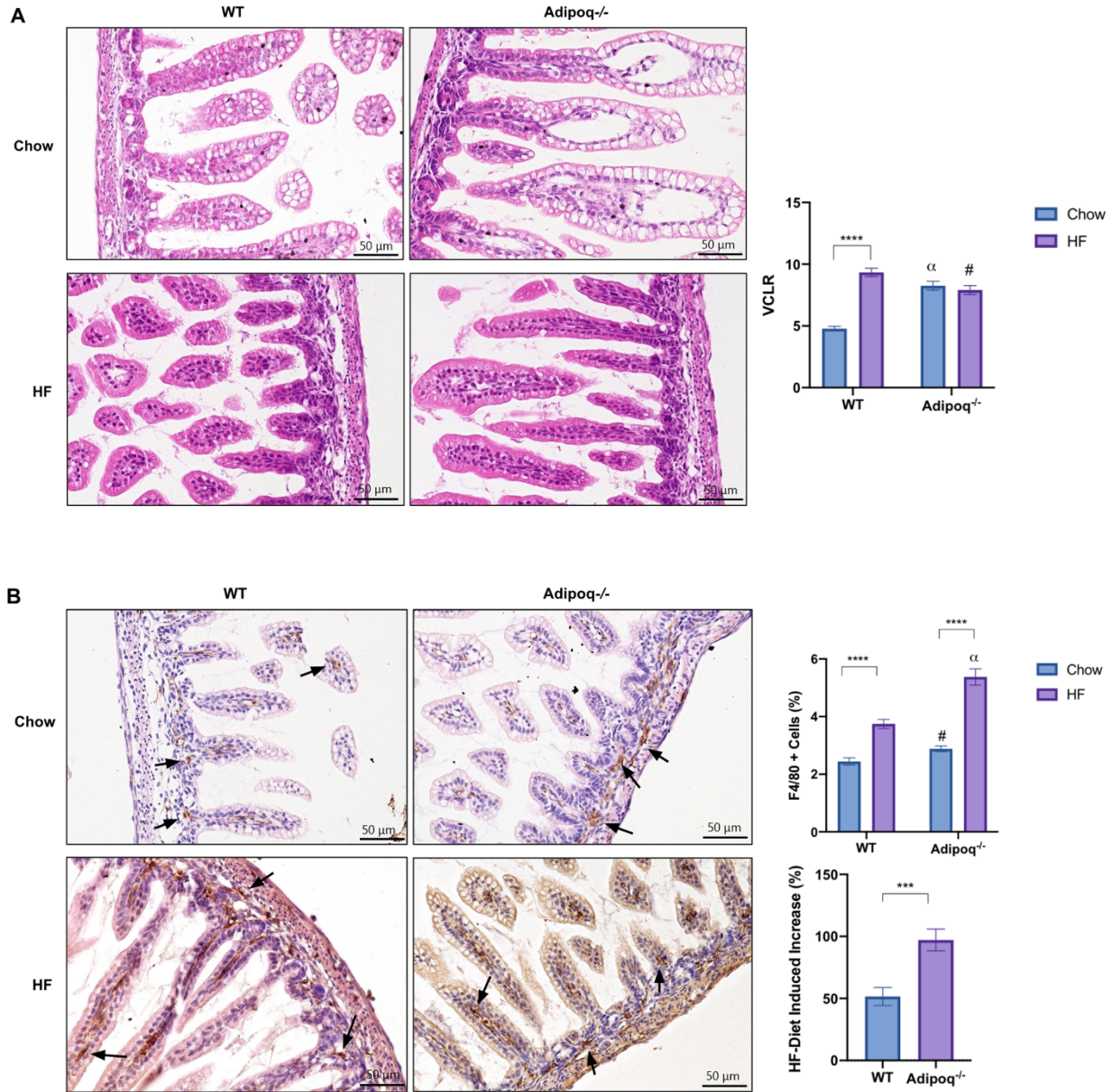


Figure 3: Maternal HF Feeding Increased Absorptive Capacity, Inflammation, and Proliferation in the SI of WT and *Adipoq*^{-/-} Mice. SI tissue samples were collected at P10, fixed in formalin, and paraffin-embedded. **A:** Villus and crypt length measured by ImageJ software with H&E-stained images, then VCLR was calculated; scale bar of 50 μ m. ANOVA used to determine statistical significance between groups. **B and C:** F4/80 and Ki67 protein expression were measured by IHC with anti-F4/80 or anti-Ki67 antibody. The percentage of F4/80 and Ki67 positive cells were calculated using ImageJ by dividing the number of positive cells by the total number of cells; scale bar of 50 μ m. Student's t-test and ANOVA used to determine statistical significance between groups. Data plotted as the mean $\pm$ the standard error of the mean. Notations: ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs. basal, # $P < 0.01$, - $P < 0.001$, $\alpha < P < 0.0001$ vs. WT.

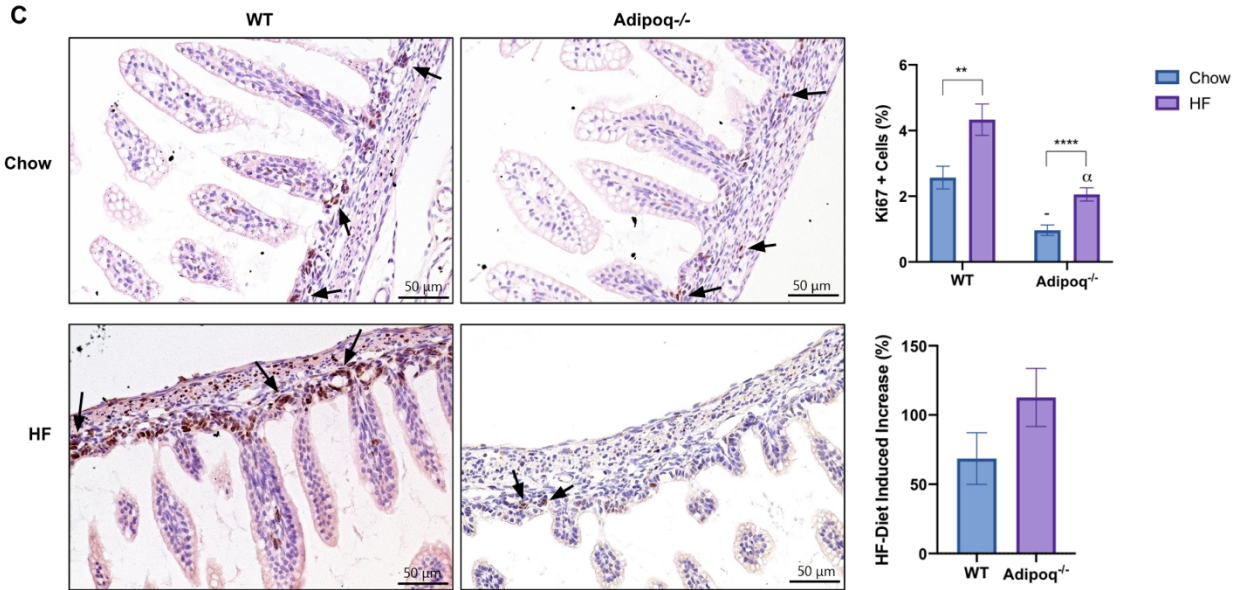


Figure 3 Continued: Maternal HF Feeding Increased Absorptive Capacity, Inflammation and Proliferation in the SI of WT and *Adipoq*^{-/-} Mice. SI tissue samples were collected at P10, fixed in formalin, and paraffin-embedded. **A:** Villus and crypt length measured by ImageJ software with H&E-stained images, then VCLR was calculated; scale bar of 50 μm. ANOVA used to determine statistical significance between groups. **B and C:** F4/80 and Ki67 protein expression were measured by IHC with anti-F4/80 or anti-Ki67 antibody. The percentage of F4/80 and Ki67 positive cells were calculated using ImageJ by dividing the number of positive cells by the total number of cells; scale bar of 50 μm. Student's t-test and ANOVA used to determine statistical significance between groups. Data plotted as the mean ± the standard error of the mean. Notations: ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs. basal, # $P < 0.01$, - $P < 0.001$, $\alpha < 0.0001$ vs. WT.

HF Diet Increased EV Diameter in Breast Milk

Breast milk is vital in infant health, providing nutrients and immune support to the developing infant tissues [84, 86, 87, 89, 90]. However, it is unknown if these beneficial properties are retained during maternal obesity or if they contribute to the adverse effects seen in the offspring of obese mothers. To determine if nutrient supply played a role in maternal HF feeding-impaired offspring growth, we measured TG and protein concentration in breast milk samples using spectrophotometry at P10. Results showed no significant difference in the TG and protein concentration between HF-fed WT dams and chow-fed WT dams (**Figure 4A and 4B**).

Following these results, we explored the bioactive components in dam breast milk by isolating EVs from milk samples using ultracentrifugation techniques at P10. HF-fed WT dams exhibited a significantly greater EV diameter than chow-fed control dams (**Figure 4C and 4D**). This indicates that a maternal HF diet may alter the biological function or content of milk-derived EVs, potentially impacting offspring health. Interestingly, a HF diet in *Adipoq*^{-/-} dams significantly increased TG concentration in breast milk compared to chow-fed *Adipoq*^{-/-} dams (**Figure 4A**). Similarly, there was no significant difference in protein concentration (**Figure 4B**). These results highlight the role that TGs in breast milk may play in mitigating the inflammation seen in pups from HF-fed *Adipoq*^{-/-} dams. Due to the shortage of milk samples from *Adipoq*^{-/-} dams, the milk EVs from these mice were not analyzed.

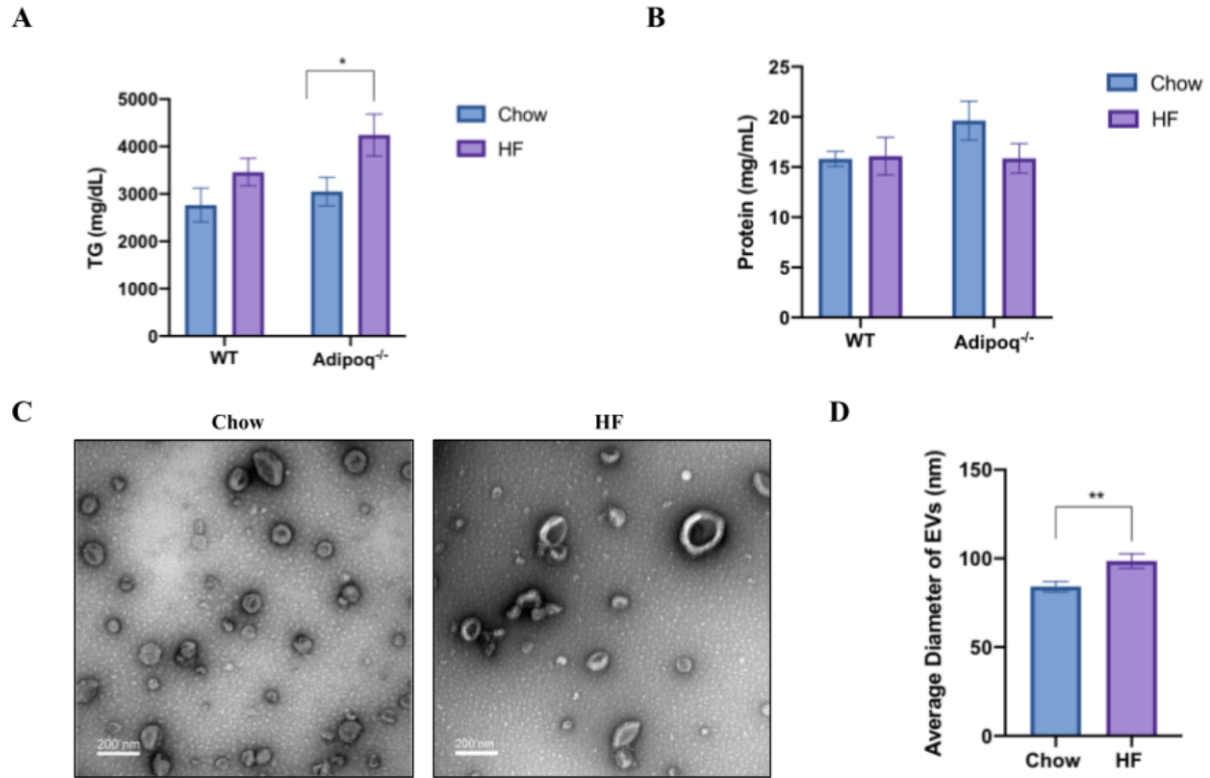


Figure 4: Maternal HF Feeding Increased EV Diameter in Breast Milk of WT Dams. Breast milk composition from dam breast milk at P10. **A and B:** TG and protein concentration determined using spectrophotometry with standard curve. ANOVA used to determine statistical significance between groups. **C and D:** Average diameter measurements of milk-derived EVs, isolated through ultracentrifugation, and analyzed by the Electron Microscopy Core at the University of California, San Diego; scale bar of 200 nm. Student's t-test used to determine statistical significance between groups. Data plotted as the mean $\pm$ the standard error of the mean. Notations: * $P < 0.05$, ** $P < 0.01$ vs. basal.

HF Diet-Induced a Decrease in the Percentage of Alveolar Epithelial Tissue

Given the significant changes that occur within the MG throughout pregnancy and lactation, we sought to characterize MG morphology during different time points, spanning from pre-pregnancy to post-weaning [114]. To examine MG development, we collected MG tissue from WT dams on a chow diet at distinct time points, including pre-pregnancy, E5.5, E9, E13, E18, P1, P10, P18, and P24, and stained them with H&E. Before pregnancy, the MG consists of a rudimentary ductal structure with limited branching, mostly composed of adipose tissue

(**Figure 5A and 5B**). By E5.5, the MG has undergone significant maturation as secondary and tertiary branching is well underway, with the beginning stages of alveoli differentiation visible. Distinct alveoli are visible by E9. By E13, most of the adipose tissue is replaced by alveoli, which continue to expand through E18 and P1, the onset of lactation. At P10, the peak of lactation, the MG is filled with alveoli, while adipose tissue is significantly reduced. As lactation continues through P18, the lumen of the alveoli increases, signifying increased milk secretion. At P24, 3 days post-weaning, most of the MG is composed of adipose tissue, while alveoli are significantly reduced (**Figure 5A and 5B**). The early images depict the progressive replacement of adipose tissue with alveoli throughout pregnancy, a process crucial for lactation preparation. Conversely, the later images depict the reversal of this process, leading to the restoration of the MG's original structure post-weaning.

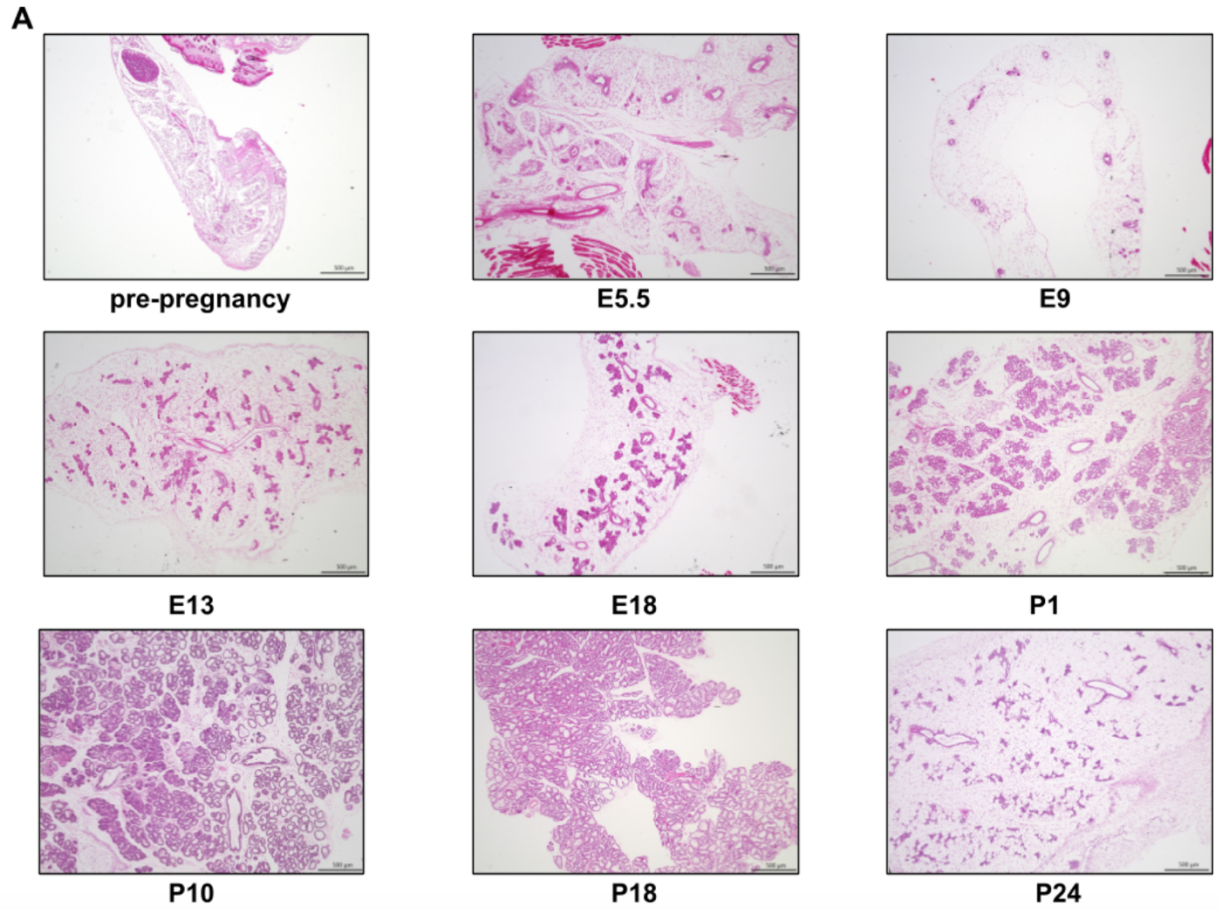


Figure 5: MG Development. MG tissue from WT dams at indicated ages visualized with H&E staining. **A:** Magnification at 4x with 500 µm scale bar and **B:** at 20x with 50 µm scale bar.

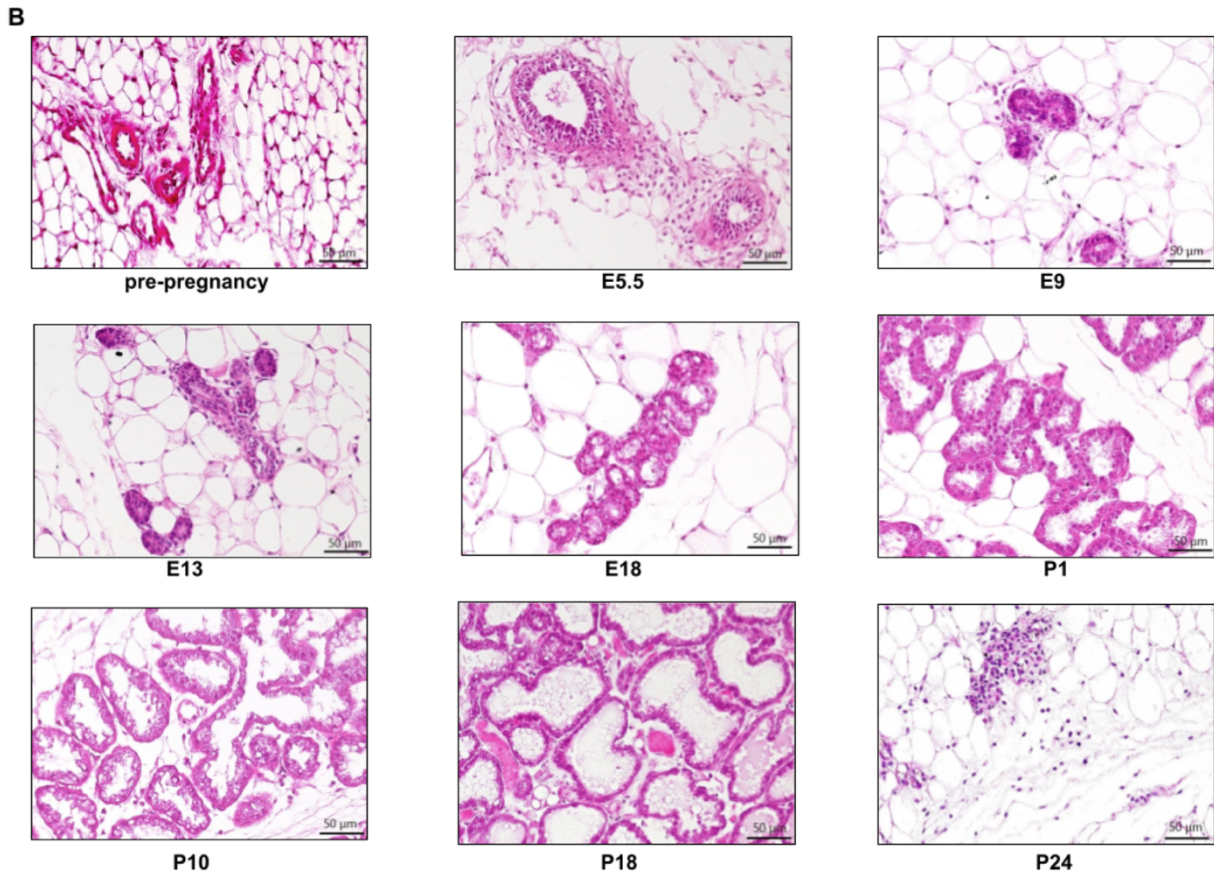


Figure 5 Continued: MG Development. MG tissue from WT dams at indicated ages visualized with H&E staining. **A:** Magnification at 4x with 500 μm scale bar and **B:** at 20x with 50 μm scale bar.

Maternal metabolism plays a key role in shaping offspring health, with the intrauterine environment shaping their development in adolescence and beyond [16]. The MG provides an interesting area of exploration given its dual role: breast milk secretion, facilitating maternal-infant biocommunication, and acting as a significant storage depot of WAT, thereby serving as a major source of local adiponectin [9, 23]. To examine how dam health impacts offspring health and the role of adiponectin on MG development, we investigated MG development in WT and *Adipoq*^{-/-} dams on a HF diet. Given the deficits obese mothers experience with lactation, we utilized H&E-stained tissue to analyze the percentage of alveolar epithelial tissue in the MG at P10 [94, 95]. WT dams on a HF diet displayed a significantly lower percentage of alveolar

epithelial tissue than chow-fed control dams, indicating potential impairments in MG development and lactation (**Figure 6A**). In light of these results, we measured the expression of Ki67, a marker for actively dividing epithelial cells, using IHC in the MG at P10 to determine if reduced epithelial cell proliferation could explain these results [113]. Surprisingly, there was no significant difference in Ki67 expression between HF-fed WT dams and chow-fed control dams (**Figure 6B**). This suggests that the source of the decreased percentage of alveolar epithelial tissue in HF-fed dams is not due to decreased proliferation in MG epithelial cells. However, more studies are required to determine if any proliferation at earlier time points contributed to the HF-feeding-enlarged MG. It is important to note that obesity induces inflammation in adipose tissue of both humans and mice [114]. While we anticipate an increase in inflammation in dams on a HF diet, it's crucial to evaluate inflammation in the MG as it could impact offspring health. Thus, we measured the expression of F4/80, a marker for macrophage infiltration, using IHC in the MG at P10 [112]. Results showed that WT dams on a HF diet exhibited a significant decrease in F4/80 expression compared to chow-fed control dams (**Figure 6C**). These findings imply that a HF diet decreased the rate of inflammation in the MG, contrary to existing literature reporting an increase in inflammation in the human MG [115]. Conversely, a HF diet in *Adipoq*^{-/-} dams had no significant effect on the percentage of alveolar epithelial tissue in the MG but resulted in a significant increase in Ki67 expression compared to chow-fed *Adipoq*^{-/-} dams (**Figure 6A and 6B**). Similarly, a HF diet in *Adipoq*^{-/-} dams resulted in a significant decrease in F4/80 expression compared to chow-fed *Adipoq*^{-/-} dams (**Figure 6C**). These findings indicate that a HF diet in *Adipoq*^{-/-} dams did not alter the percentage of alveolar epithelial tissue but increased epithelial cell proliferation in the MG, contrasting the effect observed in WT dams. Additionally, a HF diet in *Adipoq*^{-/-} dams significantly reduced

inflammation in the MG (**Figure 6C**). Given the significant difference in the basal rates of F4/80 between WT and *Adipoq*^{-/-} dams, we calculated the percent decrease of this marker to understand the role of adiponectin in macrophage infiltration of the dam MG. Results showed that a HF diet in *Adipoq*^{-/-} dams induced a significantly greater percent decrease in F4/80 expression than in WT dams (**Figure 6C**). This indicates that a HF diet induced a more severe decrease in MG inflammation in *Adipoq*^{-/-} dams than in WT dams.

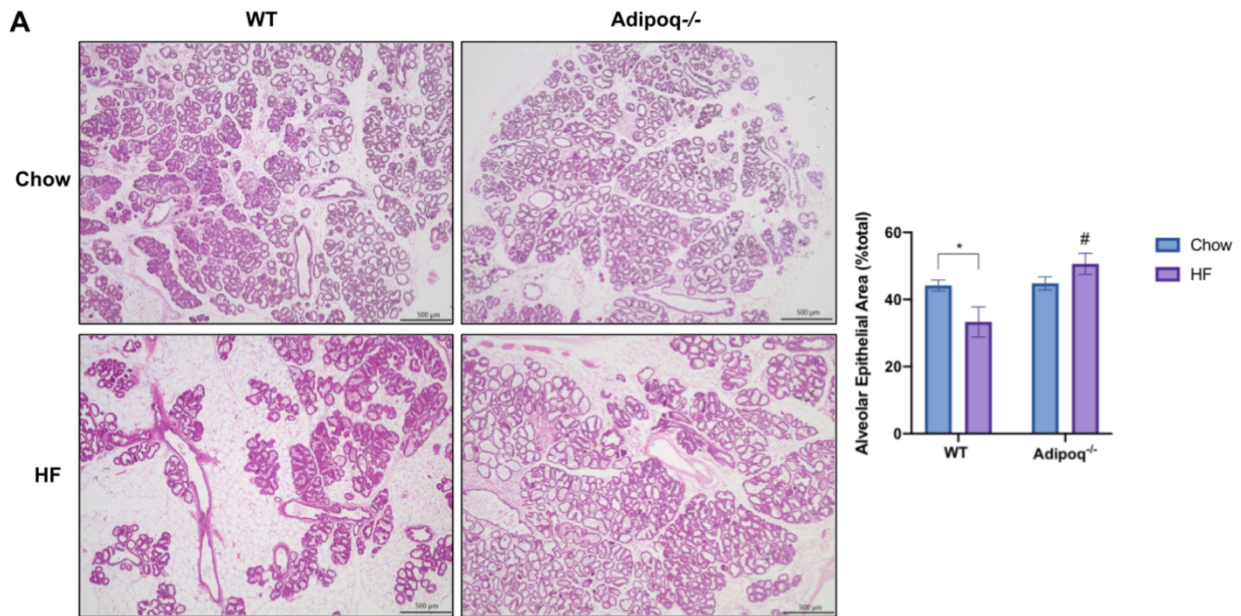


Figure 6: Maternal HF Feeding Decreased the Percentage of Alveolar Epithelial Tissue in WT Mice. MG tissue from dams at P10, fixed in formalin, and paraffin-embedded. **A:** Area of alveolar epithelial tissue measured by ImageJ software with H&E-stained images, alveolar area divided by whole tissue area to determine percentage; scale bar of 500 μ m. Two images were analyzed per tissue section. ANOVA used to determine statistical significance between groups. **B and C:** F4/80 and Ki67 protein expression were measured by IHC with anti-F4/80 or anti-Ki67 antibody. The percentage of F4/80 and Ki67 positive cells was calculated using ImageJ by dividing the number of positive cells by the total number of cells; scale bar of 50 μ m. Student's t-test and ANOVA used to determine statistical significance between groups. Data plotted as the mean $\pm$ the standard error of the mean. Notations: * $P < 0.05$, *** $P < 0.0001$ vs. basal, + $P < 0.05$, - $P < 0.001$, $\alpha < 0.0001$ vs. WT.

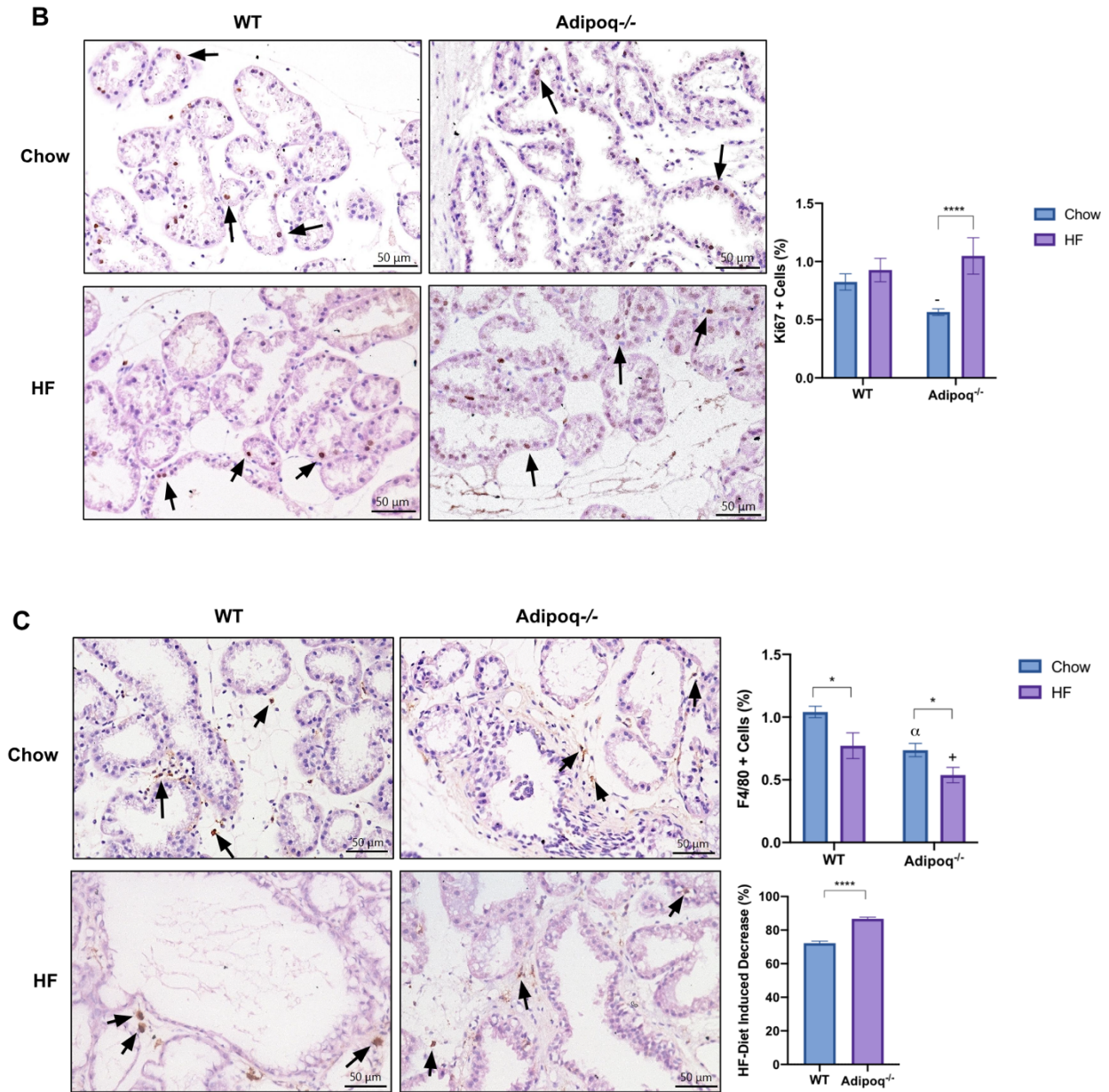


Figure 6 Continued: Maternal HF Feeding Decreased the Percentage of Alveolar Epithelial Tissue in WT Mice. MG tissue from dams at P10, fixed in formalin, and paraffin-embedded. **A:** Area of alveolar epithelial tissue measured by ImageJ software with H&E stained images, alveolar area divided by whole tissue area to determine percentage; scale bar of 500 μ m. Two images were analyzed per tissue section. ANOVA used to determine statistical significance between groups. **B and C:** F4/80 and Ki67 protein expression were measured by IHC with anti-F4/80 or anti-Ki67 antibody. The percentage of F4/80 and Ki67 positive cells was calculated using ImageJ by dividing the number of positive cells by the total number of cells; scale bar of 50 μ m. Student's t-test and ANOVA used to determine statistical significance between groups. Data plotted as the mean $\pm$ the standard error of the mean. Notations: * $P < 0.05$, **** $P < 0.0001$ vs. basal, + $P < 0.05$, - $P < 0.001$, $\alpha < 0.0001$ vs. WT.

Adiponectin Gene Knockout Induced Early Involution of the MG

A study had reported early involution of the MG in *Adipoq*^{-/-} dams during lactation, thus we sought to confirm these findings in our mouse models by examining MG morphology in chow-fed WT and *Adipoq*^{-/-} dams during lactation [34]. MG tissue was harvested at P18 and visualized with H&E staining. In MG tissue from *Adipoq*^{-/-} dams, there was a pronounced reduction in alveoli and an increase in adipose tissue compared to MG tissue from WT dams (**Figure 7**). These results confirm early involution of the MG in *Adipoq*^{-/-} dams.

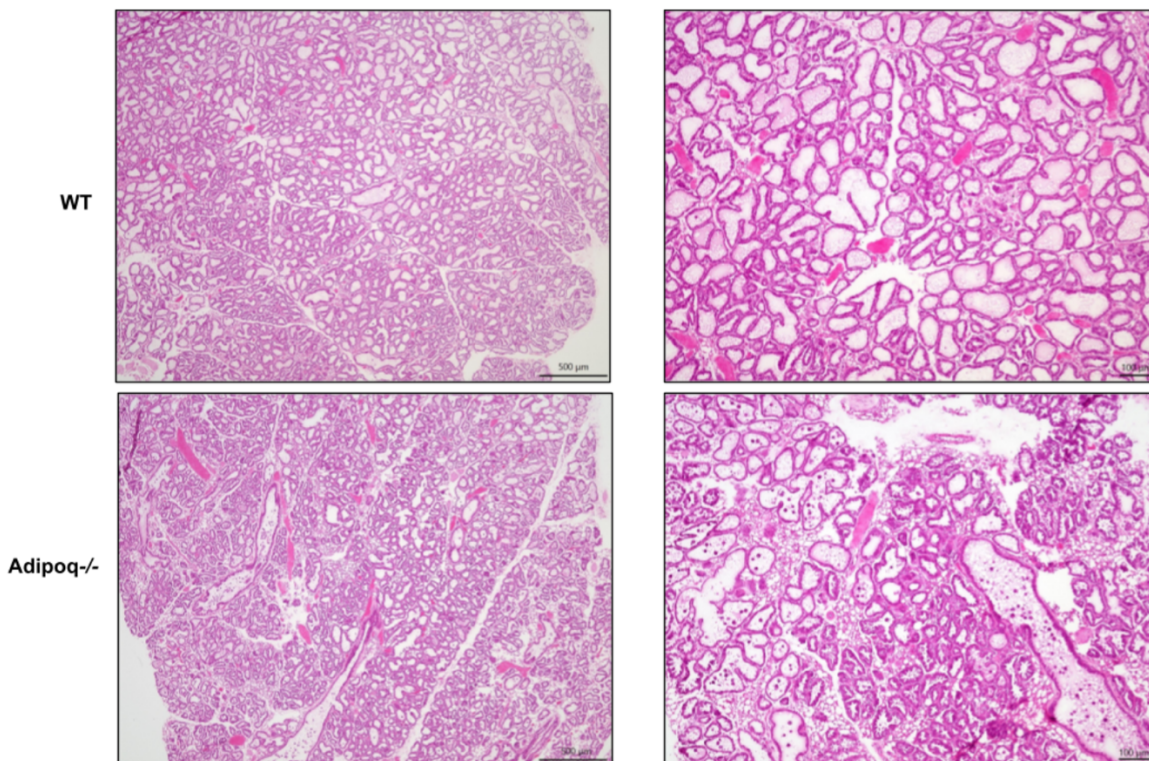


Figure 7: Adiponectin Gene Knockout Induced Early MG Involution. MG tissue from dams at P18, fixed in paraformaldehyde, and OCT embedded. Scale bar of 500 um for left panels; 100 um for right panels.

Discussion

In this study, we aimed to characterize the effect of maternal obesity on postnatal growth and the role of maternal adiponectin in maternal obesity-impaired offspring development. Although previous studies have documented health issues in children born to mothers with obesity, no studies are looking at the role of adiponectin as a mediator in these outcomes, despite well-documented evidence that shows a significant proportion of obese pregnant women also experience hypoadiponectinemia [15, 17, 18, 20, 32, 33]. Additionally, existing studies only focus on childhood and adulthood, leaving the effects of maternal obesity on infants unknown. To address this gap, we utilized *Adipoq*^{-/-} and WT mice on a HF diet to study the role of maternal adiponectin in maternal obesity-impaired infant development and growth at P10. Our study found that pups from HF-fed WT dams exhibited increased body weight. This increase was attributed to increased villi/crypt ratio in the SI, which was associated with elevated intestinal proliferation and inflammation, indicating postnatal catch-up growth in offspring. We observed that pups from HF-fed *Adipoq*^{-/-} dams showed more severe inflammation than pups from HF-fed WT dams, suggesting adiponectin may protect against maternal obesity-induced inflammation in offspring tissue. Additionally, we found that maternal HF feeding increased milk TG levels in *Adipoq*^{-/-} dams, suggesting adiponectin's protective role against offspring inflammation may be mediated through breast milk. While TG levels in WT breast milk were unaffected by a HF diet, the size of milk EVs was increased in HF-fed WT dams, suggesting a HF diet may alter the bioactive components in breast milk. Furthermore, WT dams on a HF diet exhibited a decreased percentage of alveolar epithelial tissue during lactation, indicating impaired MG development, while *Adipoq*^{-/-} dams on a chow diet exhibited a decrease in alveoli and an increase in adipose tissue, indicating early involution of the MG. This study first

demonstrated that maternal obesity impacts infant growth and SI physiology in early infancy. We then found that adiponectin protects against maternal obesity-induced inflammation observed in offspring from obese mothers, potentially mediated through breast milk. Furthermore, we observed that maternal obesity altered the bioactive components of breast milk and MG structure in dams. Lastly, we exhibited that adiponectin is essential for MG development.

The correlation between infant health and later development is well-established, thus understanding the impact of maternal obesity on offspring in early development may provide insights into the origins and development of metabolic disorders in adulthood [16]. First, we demonstrated that maternal obesity impacts offspring growth in early infancy. Both male and female WT pups from HF-fed WT dams exhibited a significant increase in body weight at P10 (**Figure 2A-C**). Our previous study and others have reported that maternal obesity induces intrauterine growth restriction (IUGR) in rodents [117–119]. Thus, the increased offspring body weight at P10 indicates postnatal catch-up growth. Importantly, similar to the effects on adult offspring, our results indicate that maternal obesity promotes offspring growth in early life [107]. One of our previous studies also reported that adiponectin inhibits fetal growth [120]. Therefore, we expected to detect higher body weight in HF-fed *Adipoq*^{-/-} offspring. However, in contrast to the HF-induced increase in body weight of WT pups, there was no significant difference in body weight between pups from HF-fed *Adipoq*^{-/-} dams and chow-fed *Adipoq*^{-/-} dams at P10 (**Figure 2A-C**). Although this study does not explain why maternal obesity-enhanced postnatal growth was absent in *Adipoq*^{-/-} offspring, these results suggest that adiponectin is required in maternal obesity-enhanced postnatal growth.

The development and growth of the SI drives postnatal growth in the infant, serving as the main site for digestion and nutrient absorption [109]. Investigating the physiology of the SI in

offspring, we found that maternal obesity increased the SI's absorptive capacity in offspring. WT pups from HF-fed WT dams exhibited a significant increase in the villi/crypt ratio at P10 compared to chow-fed controls (**Figure 3A**). Previous research utilized villi/crypt ratio to determine the structural integrity and absorptive capacity of the SI [111]. Importantly, studies have reported a correlation between increased villi/crypt ratio and increased body weight [121, 122]. Therefore, these changes in SI structure may underlie the significant increase in body weight of WT pups from HF-fed WT dams (**Figure 2A-C**). Our findings suggest that maternal obesity enhances nutrient uptake in the SI of offspring through alterations in SI structure. Given our body weight results in pups from HF-fed *Adipoq*^{-/-} dams and the correlation between villi/crypt ratio and body weight, we expected no significant change in the villi/crypt ratio in pups from HF-fed *Adipoq*^{-/-} dams (**Figure 2A-C**). As expected, we observed no significant difference in the villi/crypt ratio between offspring from HF-fed *Adipoq*^{-/-} dams and chow-fed *Adipoq*^{-/-} dams at P10 (**Figure 3A**). These findings indicate that adiponectin is required for the increased absorptive capacity of the SI, contributing to the postnatal growth observed in offspring of obese dams.

Significantly, postnatal catch-up growth correlates with the development of obesity and metabolic disorders in later life [123]. We found that maternal obesity increased SI villi/crypt ratio in pups in early life (**Figure 3A**). Similar to a previous report, WT pups from HF-fed dams exhibited significantly increased intestinal proliferation and inflammation at P10, which may be attributed to the increase in the villi/crypt ratio underlying postnatal catch-up growth (**Figure 3B and C**) [124]. Given the anti-inflammatory actions of adiponectin and that obesity induces inflammation in adult tissue, we anticipated severe intestinal inflammation in offspring from HF-fed *Adipoq*^{-/-} dams [124, 125]. As expected, our results showed significantly more severe

macrophage infiltration in pups from HF-fed *Adipoq*^{-/-} dams at P10 compared to pups from HF-fed WT dams and controls (**Figure 3B**). However, maternal obesity had a similar effect on intestinal epithelial proliferation in pups from both WT and HF-fed *Adipoq*^{-/-} dams at P10, thus, adiponectin reduction alone cannot fully account for the adverse effects of maternal obesity on offspring development (**Figure 3B and 3C**). Nevertheless, these results suggest that adiponectin protects against maternal obesity-induced inflammation in offspring.

Recognizing the critical role of nutrient absorption in infant development, we examined dam breast milk for its role in infant nutrient supply. Breastfeeding supports infant health through the supply of nutrients and bioactive components [84, 86, 87, 89, 90]. Among these bioactive components are EVs, which are hypothesized to transmit biological information from mother to infant [126]. Previous studies have found a negative association between maternal obesity and breast milk EV-derived miRNAs involved in insulin signaling and adipogenesis [127]. Given this, we expected a decrease in EV size but found that HF-fed WT dams exhibited a significant increase in the average diameter of breast milk-derived EVs at P10 (**Figure 4C and D**). However, due to the extensive number of miRNAs found in EVs, a more comprehensive evaluation of the miRNA profile of these breast milk-derived EVs is needed. Regardless, our results show that maternal obesity alters the size of breast milk-derived EVs. Additionally, previous studies have shown that adiponectin reduces plasma TG levels [128]. Given the proximity of adipocytes and alveoli in the MG, we anticipated adiponectin to similarly modulate TG levels in breast milk. We found that breast milk from HF-fed *Adipoq*^{-/-} dams contained a significantly increased concentration of TGs compared to chow-fed *Adipoq*^{-/-} dams at P10 (**Figure 4A**). Elevated plasma TG levels are associated with chronic inflammation and infection, thus suggesting that adiponectin protection against maternal obesity-induced inflammation may

be mediated through breast milk [129]. Our results indicate that adiponectin promotes an anti-inflammatory state in offspring born to obese dams through breast milk. While we examined TG and protein levels in breast milk, other macronutrients may also impact infant health. However, more studies are warranted to further assess these macronutrients.

MG development controls milk production [130]. We characterized MG morphology spanning from pre-pregnancy to post-lactation. We demonstrated the transformation the MG undergoes to prepare for lactation, marked by the progressive replacement of adipose tissue with alveoli. Post-weaning, the MG rapidly returns to its pre-pregnancy structure as adipose tissue replaces the alveoli (**Figure 5A and 5B**). This dynamic process underscores the MG's adaptability to meet lactation demands and its capacity for structural reorganization. Our histological assessment of epithelial and adipose tissue in the MG aligns with others, showing consistent and clear changes at specific time points during pregnancy and lactation [114]. These findings provide further insight into MG morphology throughout these stages and serve as reference points for identifying deviations that may indicate certain diseases or conditions. Further research into adiponectin's role in driving these morphological changes is needed to fully understand its role in offspring health.

Given the large proportion of adipose tissue in the MG, along with the MG's role in milk production, we sought to analyze MG development during maternal obesity [23]. We observed that obesity during pregnancy and lactation impacts the structure of the MG. WT dams on a HF diet exhibited a significant decrease in the percentage of alveolar epithelial tissue with no significant difference in the proliferation of MG epithelial cells at P10 (**Figure 6A and 6B**). Previous studies have linked adipose hypertrophy and hyperplasia to the pathological expansion of WAT seen in obese individuals [11]. Importantly, increased adipocyte expansion of the MG

has been linked to dysfunctional secretion of proinflammatory factors, free fatty acids, and hormones, compounds associated with MG development and breast milk synthesis and secretion [131, 132]. Therefore, the decreased percentage of alveolar epithelial tissue may indicate altered MG development and milk secretion. Despite previous studies indicating a HF diet induces inflammation in mouse and human tissue, we found that HF-fed WT dams exhibited a significant decrease in macrophage infiltration in the MG at P10 [115, 116] (**Figure 6C**). Many factors can contribute to this discrepancy, including species, diet, and experimental model design. Additionally, there are several other markers for inflammation other than F4/80 that were not explored in this study. Further research into different inflammatory markers and adipocyte expansion is needed to confirm these findings. Regardless, our results show that maternal obesity promotes adipocyte expansion in the MG. Previous bovine studies have shown that adiponectin reduction induces mammary alveolar cell proliferation [78]. Based on these findings and our previous observations in HF-fed WT dams, we anticipated an increase in epithelial cell proliferation and, consequently, an increased percentage of alveolar epithelial tissue. However, contrary to our expectations, HF-fed *Adipoq*^{-/-} dams exhibited significantly increased epithelial cell proliferation in the MG without a corresponding change in the percentage of alveolar epithelial tissue at P10 (**Figure 6A and 6B**). This suggests that concurrent processes such as cell death or remodeling may counterbalance the proliferation, thereby preventing structural changes. Additionally, considering adiponectin's known anti-inflammatory effects and the pro-inflammatory impact of obesity on adult tissues, we expected to observe pronounced MG inflammation in HF-fed *Adipoq*^{-/-} dams [124, 125]. Surprisingly, these dams showed a severe decrease in MG inflammation at P10 (**Figure 6C**). Similar to HF-fed WT dams, there are several other markers for inflammation other than F4/80 that were not explored in this study.

Furthermore, we did not assess a comprehensive list of adipocyte-secreted hormones and proteins which may provide insight into these unexpected results. However, our findings show that adiponectin is required for MG development during pregnancy and lactation.

Adiponectin has been shown to play an important role in regulating MG involution, warranting further research [34]. We found that adiponectin reduction resulted in early involution of the MG. Using MG tissue from chow-fed *Adipoq*^{-/-} dams, we observed a reduction of alveoli and an increase in adipose tissue at P18 (**Figure 7**). Previous studies have signified early involution of the mammary gland at P10 and P15 as a decrease in ductal/alveolar structure [34]. Thus, this reduction in alveoli and increase in adipose tissue at P18 indicates early involution of the MG in chow-fed *Adipoq*^{-/-} dams. Despite these results, other studies have indicated that increased adiponectin, as observed through mRNA levels, promotes epithelial apoptosis during involution [77]. However, this difference suggests that there may be other mechanisms influencing adiponectin levels that were not explored in this study. Nevertheless, our results suggest that adiponectin is required for maintaining normal lactation progression and preventing premature involution.

Taken together, our study reveals that maternal obesity induces postnatal growth which may contribute to later obesity. Additionally, we demonstrated that adiponectin protects against maternal obesity-induced inflammation in offspring, highlighting adiponectin as an area of study to improve offspring health. Furthermore, we found that breast milk may contribute to the adverse infant health seen in the offspring of obese mothers. This suggests that understanding and potentially modifying breast milk composition could optimize infant care in mothers affected by maternal obesity. However, further research is needed to determine whether these effects are attributed to the intrauterine environment, the composition of dam breast milk, or a combination

of both factors. We also demonstrated that maternal obesity adversely affects MG development and the important role of adiponectin in maintaining MG structure and physiology. These findings emphasize the influence of maternal metabolism on infant health. Additionally, they highlight the role of adiponectin in both maternal and offspring well-being.

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