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Investigating the Pubertal Effects of High Advanced Glycation End Product (AGE) Consumption on Mammary Gland Development

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List of Abbreviations

AGEs – Advanced Glycation End-products

C/EBPβ – CCAAT/Enhancer-Binding Protein beta

CK – Cytokeratin

DAB – 3,3'-Diamino-benzidine

DCIS – Ductal Carcinoma In-situ

DMBA – 7,12-dimethylbenz[a]anthracene

ELISA – Enzyme-linked Immunosorbent Assay

EGF – Epidermal Growth Factor

ER – Estrogen Receptor

ERK – Extracellular Signal-regulated Kinases

EtOH – Ethanol

H&E – Hematoxylin and Eosin

HCl – Hydrochloric Acid

Id2 – Inhibitory of DNA Binding 2

IHC – Immunohistochemistry

MMP-9 – Matrix Metalloprotease-9

MAPK – Mitogen-activated Protein Kinase

NAD(P)H – Nicotinamide Adenine Dinucleotide Phosphate (reduced form)

NF-κB – Nuclear Factor kappa B

NH_4OH – Ammonium Hydroxide

PBS – Phosphate-Buffered Saline

Pd – Pregnancy Day

PFA – Paraformaldehyde

PI3-K – Phosphatidylinositol-3 Kinase

PgR – Progesterone Receptor

RAGE – Receptor for Advanced Glycation End-products

RANK – Receptor Activator of Nuclear Factor Kappa B

RANKL – Receptor Activator of Nuclear Factor Kappa B Ligand

RT – Room Temperature

STAT5 – Signal Transducer and Activator of Transcription 5

TDLU – Terminal Ductal Lobular Unit

TEB – Terminal End Bud

TED – Terminal End Duct

TGFα – Transforming Growth Factor alpha

Abstract

The mammary gland continues to develop postnatally through puberty, pregnancy, lactation and involution. Evidence supports the notion that critical events in mammary development permanently alter developmentally regulated programs which influence the breast microenvironment to increase breast cancer risk.

Advanced glycation end-products (AGEs) are highly reactive metabolites that irreversibly accumulate in tissues as we grow older. The pathogenic effects of AGEs include tissue degeneration, protein dysfunction, aberrant cell signaling and reduced genetic fidelity. AGE accumulation can contribute to pro-inflammatory and -oxidant phenotypes when signaling through the receptor for advanced glycation end-products. AGEs form during normal metabolism but critically, lifestyle factors such as poor diet, a sedentary lifestyle and being obese also contribute to the AGE accumulation pool.

Previous work performed in our lab with mice fed a high AGE diet showed a significant dysregulation of mammary gland development and the formation of hyperproliferative structures. In this study, we further characterized the AGE induced hyperproliferative structures and made an initial assessment of their ability to confer metabolic memory using diet intervention strategies. Given the AGE induced effects on mammary development during puberty, we also examined the effects of chronic AGE consumption on the mammary gland during pregnancy and offspring.

In summary, increased AGE consumption during pubertal growth and mammary development during pregnancy results in significant disruption of normal mammary development. Chronic consumption of a diet high in AGEs leads to disruption of the mammary gland microenvironment, thereby posing an increased risk of developing breast cancer later in life, particularly in susceptible populations.

Background and Introduction

The effects of diet on disease have been limited to studies investigating high- and low- fat diets, with very few studies examining a Western diet and/or a high advanced glycation end-product (AGE) diet. Because of this, a great need remains to explore the effects of a Western diet and/or high AGE diet, which is more prevalent than ever. This body of work examines the effects of consuming a high AGE diet on mammary gland development.

Advanced Glycation End-products

Advanced glycation end-products are formed via a nonenzymatic reaction between reduced sugars and free amino groups of proteins, lipids, or nucleic acids. This is also known as the Maillard reaction. [1] The Maillard or browning reaction occurs in three steps. First, glucose attaches to a free amino acid of a protein, lipid or DNA to form a Schiff base. Next, the Schiff base undergoes chemical rearrangement and forms Amadori products. An accumulation of Amadori products will prompt chemical rearrangements that form crosslinked proteins. In food the final brown products are AGEs. Other methods of forming AGEs include oxidation of glucose, peroxidation of lipids and the polyol pathway. Dicarbonyl derivative formation from glucose oxidation and lipid peroxidation via oxidative stress can interact with monoacids and form AGEs. Additionally, the polyol pathway features glucose that is first converted to sorbitol and then to fructose. Fructose metabolites are converted into reactive species that interact with monoacids to form AGEs. [2]

Food products are not lacking in AGEs. They are naturally present in uncooked foods. Cooking these foods results in the formation of additional AGEs. In particular, cooking foods with high heat (e.g. grilling, broiling, frying, roasting and searing) expedites new AGE formation. [1-3] Heat treatment of some foods results in the promotion of the Maillard reaction, which adds a desirable flavor, color and aroma. [2] A relationship between AGE content and nutritional composition exists. Foods high in lipids and proteins yield the highest AGE levels. For example, lipid oxidation may release free radicals that catalyze the formation of AGEs during the cooking of fats and meats. About 10% of dietary-derived AGEs are absorbed and correlate with circulating and tissue AGE levels. One third of ingested AGEs are excreted via the kidneys. [2, 3] Few studies about intestinal absorption of AGEs have been conducted. However, Chalova et al. found that *Salmonella Typhimurium* reduced AGE levels by 37% in vitro. [4] In rats fed a high AGE diet, gut microbiota abundance and diversity was shown to be reduced, potentially because of AGE's resistance to digestive enzymes, adversely altering gut microbiota composition. This trend was also true in humans in a study showing that consuming a Western diet (described later) was associated with a reduction in the total numbers and diversity of gut microbiota. [5] Further research is necessary to explore the impact of AGEs on gut microbiota.

Pathologically, increased AGE levels have been associated with cardiovascular disease, sarcopenia, diabetic and renal complications and Alzheimer's disease. [2, 3] Formation of endogenous AGEs is an outcome of

normal metabolism. However, if excessively high levels of AGEs accumulate, enter circulation and tissues, pathogenic events may occur. AGEs are able to promote oxidative stress and inflammation by binding with cell surface receptors or by protein cross-linking, thereby altering protein structure and function, resulting in pathological events. [1]

Western Diet

The Western diet is characterized by an increased intake of red meat, fast foods, high-fat dairy products, fried and baked goods, high-sugar foods and drinks, and a reduced intake of fibers and whole grains. [6] Because of this, the average Western diet provides a copious source of AGEs. Furthermore, the Western diet is associated with processed foods. Food processing treatments promote glycoxidation (i.e. AGE formation). [3] AGE formation and accumulation occurs partly due to the consumption of a Western diet, and therefore the Western diet is associated with increased chronic disease risk, such as obesity and type 2 diabetes. [7, 8] The combination of consuming a Western diet and fostering a sedentary lifestyle contributes to the accumulation of AGEs in the body. In contrast, leading an active lifestyle, has been shown to decrease circulating AGE levels. [2]

AGE Reduction

The accumulation of AGEs in our tissues is inevitable, however there are proactive steps one can take to decrease AGE buildup. When preparing food, it has been suggested to decrease the heat and to cook foods for longer. [9]

Consuming antioxidants, such as polyphenols found in fruits and vegetables, has been shown to reduce potential AGE-mediated inflammation. [10] A calorie-restricted diet has shown to increase lifespan in mice, where reducing food intake also decreased the intake of oxidants like AGEs. [11] Murine studies found that reduced dietary AGEs attenuated insulin resistance and decreased the risk of diabetes. In diabetic mouse models, decreased diabetic complications and improved wound healing were outcomes of reducing dietary AGEs. [2] As mentioned previously, physical exercise is a method to reduce AGEs. Studies performed with rats included one group of rats that received regular moderate exercise while the other group did not perform any exercise. Upon examination at endpoints, the group that completed the exercise had lower levels of circulating AGEs in comparison with the sedentary group. [12] Fewer studies have been performed with humans to examine the effects of exercise on AGE reduction. In a study performed with overweight men, an AGE restricted diet and moderate aerobic exercise yielded decreased levels of circulating AGEs. [13] Finally, pharmacological agents as blockers of AGE crosslinking and receptor for advanced glycation end-products (RAGE) (discussed next) inhibitors have been studied with developed compounds. Metformin has shown to decrease circulating AGEs in diabetic patients, as well as decreasing the activity of NF-κB (nuclear factor kappa B), a downstream transcription factor of the AGE-RAGE signaling axis. [14] Alagebrium (ALT-711) was identified to break AGE crosslinks. [15] Another pharmacological agent, aminoguanidine prevents the formation of AGEs from AGE precursors and has been used as a therapeutic intervention to prevent

the development of diabetic complications. [16] In vitro and in vivo studies have identified RAGE as a potential therapeutic target in cancer, cardiovascular diseases, and neurodegeneration. RAGE inhibitors that bind to the extracellular or intracellular domain have been developed (reviewed in Bongarzone et al.). Using the reviewed developed compounds, studies have shown RAGE inhibition to reduce NF-κB (nuclear factor kappa B) activation, cytokine production and overall RAGE expression. Although the compounds are not readily available for clinical use due to the need for optimization, RAGE inhibitors could mitigate the effects of AGE-RAGE signaling. [17]

Receptor for Advanced Glycation End-products

The receptor for advanced glycation end-products (RAGE) is a 45 kDa transmembrane receptor of the immunoglobulin superfamily, consisting of extracellular, hydrophobic transmembrane and cytoplasmic domains. It exists at low concentrations in healthy human tissues, such as lungs, kidneys, liver, cardiovascular, nervous, and immune systems. It is a receptor for AGE and other proinflammatory ligands. Like AGEs, RAGE has been studied as a pathological biomarker in diabetes, cardiovascular diseases, and Alzheimer's disease. [17]

Biological effects of AGEs are a product of two mechanisms- independent of the receptor or dependent of RAGE. Independent of the receptor, AGEs damage protein structure and alter extracellular matrix metabolism. The binding interaction of AGEs to RAGE leads to the activation of the mitogen-activated protein kinases

(MAPKs) and the phosphatidylinositol-3 kinase (PI3-K) pathways. Activation of these pathways leads to the subsequent activation of nuclear factor kappa B (NF-κB). Upon translocation to the nucleus, NF-κB will activate the transcription of genes for cytokines, growth factors and inflammation promoters. As a result of NF-κB activation, RAGE expression is increased, creating a positive feedback loop that further stimulates the production of inflammatory promoters. Additionally, AGE-RAGE interaction activates NAD(P)H (nicotinamide adenine dinucleotide phosphate (reduced form)) oxidase. When NAD(P)H oxidase is upregulated, it increases oxidative stress. This increase in oxidative stress by NAD(P)H oxidase will also activate NF-κB. [2]

AGE-RAGE Signaling and Cancer

A pro-inflammatory environment in the presence of oxidative stress resulting from AGE-RAGE signaling is associated with a poor prognosis. Increased AGE levels and RAGE expression is characteristic of various cancers such as prostate cancer, pancreatic cancer, colon cancer and breast cancer. [8, 18, 19] Consistent with a hallmark of cancer, AGEs promote cell growth, migration, proliferation, tumorigenicity and invasion in cancer cell lines. [19-21] In triple negative breast cancer primary cells, AGEs increased the concentration of secreted matrix metalloprotease-9 (MMP-9), which is highly expressed in most cancers and regulates the invasion and migration of cancer cells. In the presence of a NF-κB inhibitor and ERK (extracellular signal-regulated kinases) (MAPK) inhibitor, AGE-stimulated breast cancer cells showed decreased invasion and

migration activity. This suggests a crucial role of ERK (MAPK) and NF-κB in AGE-induced tumor promotion. [19] Furthermore, aberrant NF-κB activation within mammary epithelium has shown to lead to molecular and morphological changes consistent with the earliest stages of breast cancer. Overall, inhibition of NF-κB signaling following inflammation or the initial signs of mammary gland hyperplastic growth may represent an important opportunity for breast cancer prevention. [22]

Mammary Gland Development

The murine mammary gland is a secretory organ composed of epithelial cells, adipocytes, endothelial cells, and fibroblasts and immune cells. The mammary epithelium is comprised of basal and luminal cells. Myoepithelial cells make up the basal epithelium, which generate the outer layer of the gland. Ducts and secretory alveoli are formed by luminal epithelial cells. The luminal epithelium also contains cell populations defined by their hormone receptor status. The mammary gland functions to secrete milk for the nourishment of the newborn. The milk is delivered via a network of branching ducts that fill the majority of the fat pad of the gland. [23] The mammary gland is distinct compared to other organs as most of its development occurs postnatally. Additionally, it is distinctive as it is continuously remodeled following puberty due to the cyclical influence of reproductive hormones. Overall, there are three stages of mammary gland development: embryonic, pubertal, and reproductive. [24] The stages of pubertal to reproductive (adulthood) development are examined as windows of breast cancer susceptibility due to the massive remodeling of the breast

microenvironment. [25, 26] While the mouse and human mammary gland may structurally differ, the mouse mammary gland is used as a model system to explore developmental mechanisms as it can be used with in vitro and in vivo applications. [23, 24]

Pubertal Mammary Gland Development

Pubertal mammary gland development is marked by vast proliferation under the influence of hormones and growth factors. Terminal end buds (TEBs) are club-shaped structures at the tips of growing ducts which appear around 3 weeks of age in mice. Duct growth through the fat pad is directed by the TEBs. Terminal end bud bifurcation generates the primary ductal structure. Once TEBs reach the end of the fat pad, they regress into terminal end ducts (TEDs) in adulthood. Lateral side branches, or secondary branches, are formed from the primary ducts until a tree-like structure of ducts fills the majority of the fat pad. Later, tertiary branches are formed under the influence of estrous cycling in response to progesterone signaling. [23, 27] In humans, secondary branches give rise to terminal ductal-lobular units (TDLUs) which are comprised of lobules and ductules. The TDLUs are proposed to be the functional equivalent of the TEB in the mouse mammary gland. [28] TEBs in mice and TDLUs in humans are often the main site of cancer initiation. [29] This similarity remains true due to high proliferation (60-90%) and apoptosis rates (5-15%), invasive ability, and ability to recruit stromal cells, such as fibroblasts and macrophages, in the TEBs and TDLUs. [27, 29]

Estrogen

In general, pubertal mammary gland development is hormone-dependent on estrogen, progesterone and prolactin. [30] The ovarian hormone, estrogen, plays a role in promoting the proliferation of normal and neoplastic breast epithelium. Locally, estradiol stimulates DNA synthesis and promotes TEB formation. The activities of estrogens are mediated by the estrogen receptor (ER) that, upon activation, activates transcription of specific genes containing the estrogen response elements. The presence of ER is essential for responsiveness to estrogen action. [29] Estrogen receptor exists in two forms: ERα and ERβ, where the use of ERα knockout mice showed that ERα is crucial for the development and expansion of ducts into the mammary fat pad. [29, 31, 32] Estrogen's action through ERα is facilitated by a paracrine mechanism to promote the proliferation of surrounding cells. Amphiregulin, necessary for ductal elongation, is one paracrine mediator of estrogen. Epidermal growth factor (EGF), transforming growth factor alpha (TGFα), and heregulin are also necessary for pubertal ductal elongation, suggesting that activation of their receptors by estrogen is critical for mammary gland development. [31]

Progesterone

Progesterone, an ovarian steroidal hormone that acts in conjunction with estrogen and through its receptor (PgR), is responsible for regulating normal mammary gland development. [29] Progesterone is responsible for side-branching and alveologenesis required to create a lactation-competent gland. Combined with

prolactin, progesterone promotes the differentiation of alveoli, which synthesize and secrete milk during lactation. Like ER, the PgR exists in two forms: PGR_{iA} and PGR_{iB}. Knockout studies with each of these forms showed that without PGR_{iB}, side branching and alveologenesis is significantly reduced, therefore PGR_{iB} is required for mammary gland development. [23, 33] Estrogen and progesterone rely on paracrine signals to elicit biological responses. [30] In both mice and humans, $ERα^+$ cells also express PgR, and estrogen has been shown to act through ERα to increase PgR expression. This is of importance because 70% of breast cancers express ERα and more than half of $ERα^+$ breast cancers express PgR. Understanding both receptor's roles in breast cancer will beneficially serve the development of therapeutic strategies. One paracrine mediator of progesterone activity is RANKL (receptor activator of nuclear factor kappa B ligand). [31] Data has suggested that the paracrine signal tells neighboring PgR-negative cells to proliferate, thus demonstrating RANKL's responsibility in driving cell proliferation in progesterone-dependent breast cancer. [23] Other downstream targets and paracrine mediators of PgR are WNT-4 and cyclin D1. WNT-4 has been shown to be upregulated in mammary epithelial cells in response to progesterone, while cyclin D1 is required for progesterone-induced proliferation during mammary gland development. [31]

Diet and Mammary Gland Development

The effects of a high fat diet on mammary gland development has been heavily explored. [34-38] However, little is known about the effects of AGEs on

mammary gland development. During puberty, FVB mice fed a high fat diet had an increased number of TEBs and enhanced ductal elongation, characteristic of accelerated pubertal mammary gland development. [38] In contrast, C57BL/6 mice fed a high fat diet into adulthood showed impaired ductal development yielding decreased ductal width, decreased branching frequency and overall a higher percent of incomplete developed ducts. [34] Despite strain differences, disruption of pubertal mammary gland development persists due to a high fat diet. Nonetheless, consumption of a high fat diet during puberty alters breast development, therefore potentially modifying the risk for breast cancer. As previously noted, AGE-RAGE signaling leads to a pro-inflammatory environment which alters roles for immune cells such as macrophages and eosinophils. They contribute to inflammatory processes in the mammary gland and can promote tumorigenesis. [39] Tumors in FVB mice fed a high fat diet had increased intra-tumor macrophages compared to control groups. [38] Diet intervention after a high fat diet has shown that after reaching obesity, mice weights return to that similar to a control diet weight. [35] However, despite implementing dietary restriction, consuming a high fat diet during puberty resulted in increased proliferation and macrophage presence in mammary glands before tumor formation. [36] Subjecting mice fed a high fat diet to DMBA (7,12-Dimethylbenz[a]anthracene), a carcinogen used for mammary tumorigenesis, enhanced tumorigenesis and decreased tumor latency. Consumption of a high fat diet alone did not show significant tumorigenesis and exhibited latent tumor formation. DMBA-induced effects on carcinogenesis are amplified when a high fat diet is consumed. [37] These

pathological consequences may be linked to oxidative stress, non-enzymatic glycation of proteins (i.e. AGEs), epigenetic changes, and chronic inflammation, laying the foundation for the “metabolic memory” theory. [40] This theory calls for early prevention, detection and treatment of signs of disease states, including breast cancer. [41]

Metabolic Memory

The theory of “metabolic memory” was first examined in patients with diabetes. These studies assigned two groups of patients a standard treatment plan and an intensive treatment plan. Half way through the study the patients receiving the standard treatment were switched to intensive treatment. While the patients that received the intensive treatments from the onset demonstrated reduced diabetic complications, the patients that switched from standard to intensive treatment showed a high incidence of complications. This suggests that early intensive intervention may be a likely explanation for the divergence in patient outcomes. This evidence supports the concept that early glycemic environment is remembered or imprinted leading to disease complications and disease progression. This is known as metabolic memory. [40-43] Oxidative stress, non-enzymatic glycation of proteins, epigenetic changes, and chronic inflammation are thought to confer metabolic memory. We hypothesize that the increased consumption of AGEs may promote genetic imprinting to confer metabolic memory which may lead to pathological consequences in later life, such as increased risk of breast cancer. [40]

Breast Cancer

Breast cancer is the most common cancer in American women, with an estimated 268,670 women that will be diagnosed with breast cancer in 2018. [44] Risk factors for breast cancer include age, geography, reproductive history, socioeconomic status, body weight, age at puberty, age at first birth and diet [25, 45]. While some of these risk factors are uncontrollable (e.g. age (in years), socioeconomic status), it is important to minimize risk for those factors that can be controlled (e.g. diet and lifestyle). Diet, defined here as the type of food that a person consumes, is a known modifiable risk factor that has been shown to play a role in the development of breast cancer. [45] As such, awareness of the molecular and structural consequences of food consumption (diet) during active stages of mammary gland development could lead to reduced breast cancer rates. Hyperglycemia is a risk factor in breast cancer progression, as breast cancer risk is increased by 20% in diabetic patients. [46] Furthermore, diabetic breast cancer patients had lower survival rates than euglycemic patients. [47] Hyperglycemia reduces the efficacy of chemotherapeutic drugs in malignant breast epithelial cells. Evidence suggests epigenetic modifications, a contributing factor to metabolic memory, may activate oncogenic pathways under hyperglycemic conditions. [41]

Breast Cancer 'Windows of Susceptibility'

The 'lifecycle' of the breast can be divided into five 'windows of susceptibility' of breast cancer: in utero, pubertal, pregnancy, postpartum involution, and age-related involution (Figure 1). The populations at risk and

duration of time that the populations are at risk are defined by these stages. Each of the five 'windows' share characteristics of tissue remodeling driven by tightly regulated events that involve the mammary epithelium and stroma. Puberty is marked by development of ductal side branches and small alveoli that fill the mammary fat pad. The nascent alveoli are ready to respond to pregnancy hormones. Pregnancy drives proliferation and differentiation of the alveoli formed during puberty. It is only at full term pregnancy when terminal differentiation of the gland is achieved. Disruption of tissue remodeling may increase the future risk of developing breast cancer. Because the 'windows of susceptibility' can be temporally defined, it is easy to target breast cancer prevention efforts. However, administering a chemoprevention drug during in utero or pubertal development may be controversial. [25] It is during these developmentally sensitive times where modifiable breast cancer risk factors can be adjusted to decrease the risk of developing breast cancer later in life.

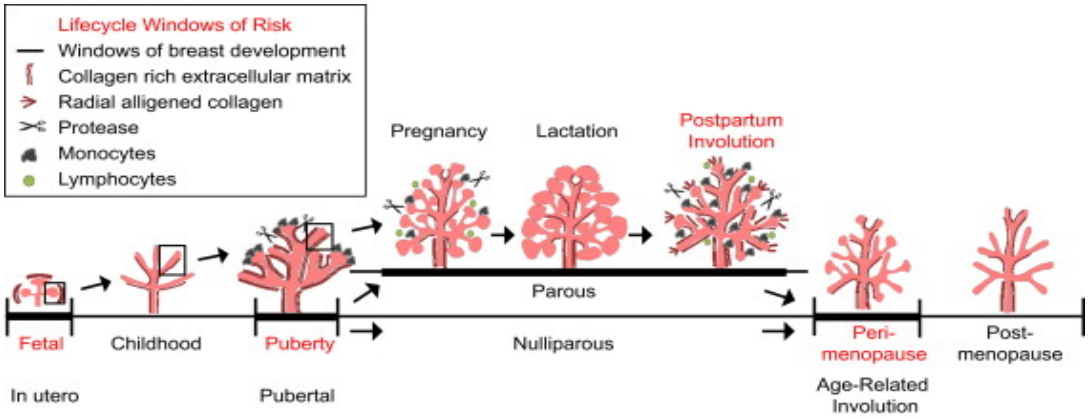


Figure 1. Developmental 'Windows of Susceptibility'. Mammary gland development schematic of the 'windows of susceptibility' for breast cancer. Taken from "Developmental windows of breast cancer risk provide opportunities for targeted chemoprevention." by H. Martinson et.al. 2013, Experimental Cell Research, 319(11):1671-8.

Ductal Carcinoma In-Situ

Mammary gland ducts are composed of an outer myoepithelial cell layer producing the basement membrane and an inner luminal epithelial cell layer. Ducts are surrounded by a microenvironment composed of extracellular matrix and various stromal cell types. Cell and microenvironment interactions potentially modify the proliferation, survival, polarity, differentiation, and invasive capacity of mammary epithelial cells. [24] Formation of potentially cancerous lesions are initiated by subtle changes such as stromal expansion and epithelial proliferation. These changes are magnified as cancer progression occurs and eventually give rise to malignant and metastatic lesions. [48] Ductal carcinoma in situ (DCIS) of the breast is a group of pre-invasive lesions characterized by varying degrees of risk and potential for transformation into invasive carcinoma. [49] DCIS constituted for 83% of in situ cases diagnosed during 2010-2014. [50] When the myoepithelial layer and underlying basement membrane is physically breached, progression of DCIS to invasive cancer occurs. Normally, the myoepithelium plays an active role in tumor suppression. However, when this function is lost, it results in the transition from preinvasive to invasive cancer. [51]

Intermediate filaments are a component of the cellular cytoskeleton. Intermediate filament protein expression, specifically cytokeratin (CK), suggests the epithelial cell type, state of tissue growth, differentiation, and functional status of the tissue. In normal breast tissue, luminal epithelial and myoepithelial cells exhibit different and distinctive keratin phenotypes. Luminal cells express CK 7, 8,

18 and 19, while CK 5, 14 and 17 are found in myoepithelial/basal cells. A high proportion of invasive breast carcinomas characteristically express only the luminal epithelial cell cytokeratins (CK 7, 8, 18, 19). Progression to a highly metastatic phenotype shows loss of CK 18 expression. In tumors, positive expression of basal markers, CK 5/6 and 17, are associated with poor overall survival. [52] While these expression patterns may be true in breast tumors, disruption of cytokeratin expression in luminal and basal cells may be indicative of a pre-DCIS phenotype. [48, 52, 53]

Identification of biomarkers that can predict DCIS and subsequent invasive cancer could aid in mitigating an individual's risk for resulting tumors. Assessment of proliferation is one major factor involved in treatment decisions in breast cancer patients. Ki-67 is a nuclear protein associated with cellular proliferation, and its utilization as a biomarker of DCIS identification and progression has been proposed. [49, 54] The most common analysis method to detect Ki-67 positivity is immunohistochemistry. Inwald et. al. showed that more aggressive tumors have a higher percentage of cells positively stained for Ki-67. Therefore, the use of Ki-67 may be proposed to be utilized in prognosis and treatment of breast cancer. [55]

Research Plan

Overall Rationale

Fostering an unhealthy, sedentary lifestyle leads to the accumulation of AGEs in the body. AGEs are highly reactive metabolites that irreversibly accumulate in tissues as we grow older. The pathogenic effects of AGE accumulation also include tissue degeneration, protein dysfunction, aberrant cell signaling and reduced genetic fidelity. AGE accumulation can also contribute to pro-inflammatory and pro-oxidant phenotypes when signaling through RAGE. AGEs form during normal metabolism but critically, lifestyle factors such as poor diet, a sedentary lifestyle and being obese also contribute to the AGE accumulation pool. [1-3, 56] The permanence of AGE adducts and their ability to mediate chronic and persistent inflammatory and oxidative stresses is particularly compatible to the concept of metabolic memory. [40-42]

Previous work in our lab has shown that pubertal mice fed a high AGE diet had significant dysregulation of mammary gland development. Results from this previous work showed delayed mammary ductal extension, increased ductal branching and aberrant TEB morphology. The basal myoepithelial cell layer surrounding mammary ducts and TEBs was irregular and epithelial cell proliferation was increased. (Figure 2) Elevated AGE levels accompanied increased RAGE expression. [57]

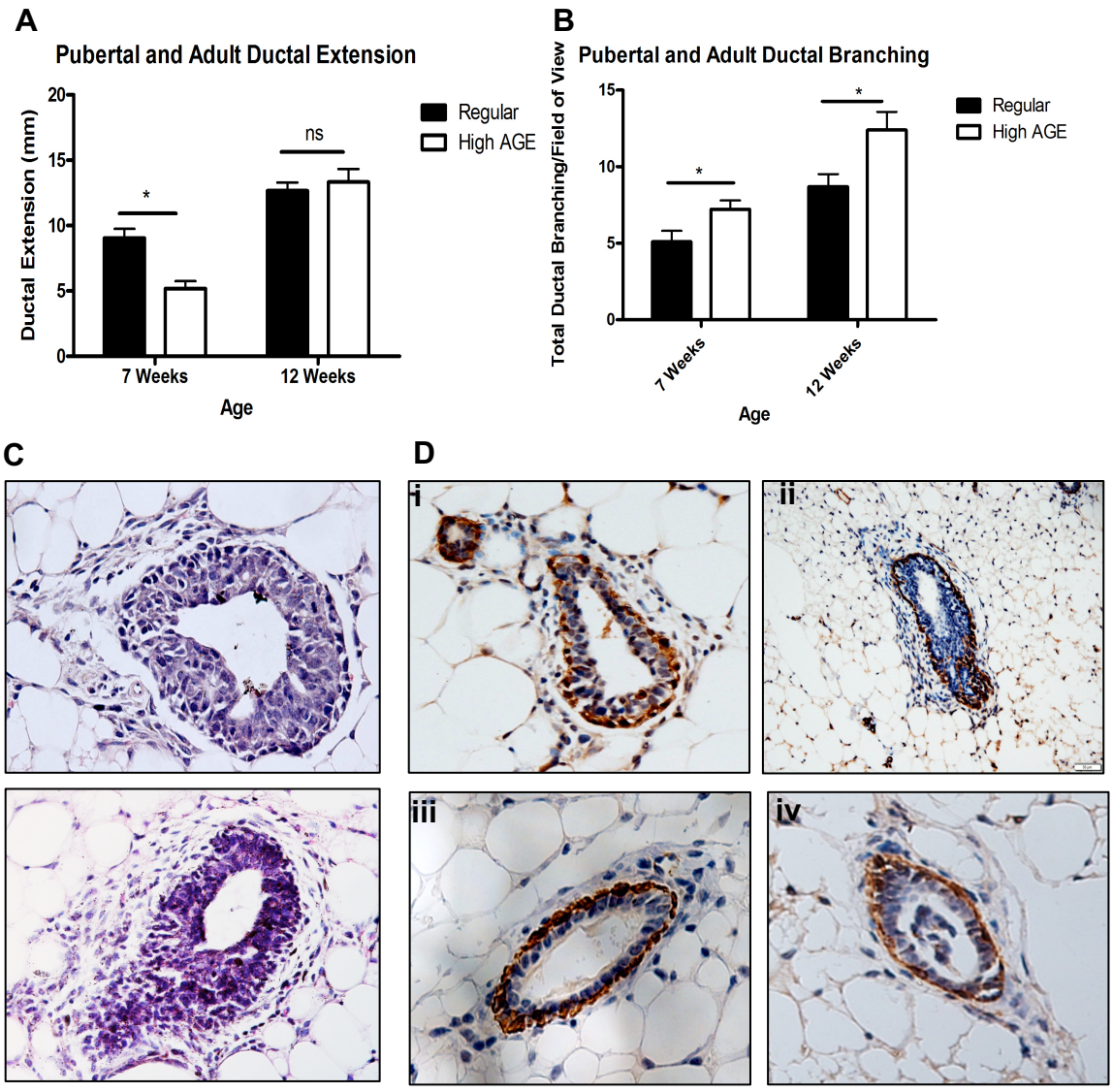


Figure 2. Preliminary Data. Previous work in our lab has shown that a diet high in AGEs results in a delay in ductal extension during puberty which recovered in adulthood (A) and resulted in increased total ductal branching per field of view (B). Morphological changes in TEBs from mice fed a high AGE diet are shown by representative H&E stained glands (C). TEBs from high AGE fed mice were surrounded by a thicker more cellular stroma compared to regular. The irregular basal myoepithelial layer of TEBs (i & ii) and ducts (iii & iv) are shown in representative images of SMA IHC stained mammary gland tissue sections from mice fed a high AGE (ii & iv) diet compared to a regular diet (i & iii) (D). The high AGE diet resulted in disruption of the myoepithelial layer in TEBs and ducts of the developing pubertal glands.

Puberty and pregnancy are sensitive times of mammary gland development on account of the massive tissue remodeling that occurs during these times. Environmental and lifestyle influences that are present during these developmentally sensitive windows can result in long term structural, functional, and/or cellular changes. These effects may pose an increased risk of breast cancer later in life. [25] Diet has been shown to be linked to an increase risk of breast cancer. [36-38, 45] The prevalence of the Western diet, a convenient option for many in a fast-paced world, has increased over time. Therefore, the consumption of foods high in AGEs has increased too. [58] Results obtained from previous work in our lab and other published studies examining the effects of diet on mammary gland development prompted these additional studies to further define the effects of a high AGE diet on mammary gland development.

Hypothesis: Chronic consumption of a high AGE diet leads to hyperproliferative ducts and dysregulation of mammary gland development, resulting in a permanent change in mammary gland function via metabolic imprinting.

Specific Aim 1: To test the hypothesis that hyperproliferative structures formed due to a high AGE diet are independent of circulating hormone levels.

Mammary gland development is hormone-dependent on estrogen and progesterone, the levels of which fluctuate depending on which stage of the

estrous cycle mice are in. [31] The estrous cycle normally lasts 4-5 days and can be divided into four stages: proestrus, estrous, metestrus, and diestrus. Circulating levels of estrogen peak at estrus, while circulating levels of progesterone rise during metestrus and diestrus. [59] Proliferation of mammary epithelial cells peak during diestrus when circulating estrogen levels are low, while circulating progesterone levels are the highest. [31, 59] Due to the changes in hormone levels and proliferation observed during the estrous cycle of mice, we sought to determine whether the observed AGE induced proliferation was hormone-dependent by determining the estrous phase in mice. To assess the impact of a high AGE diet on hormone signaling, we evaluated circulating estradiol and progesterone levels, and expression of ERα in mice fed a high AGE diet in pubertal and adult time points compared to a regular diet.

Experimental Design

AGE Specific Diet

The high AGE diet utilized throughout this research design has been shown to have an increased carbohydrate content to a regular mouse diet (TestDiet DIO 58G9) (Table 1). Furthermore, the high carbohydrate food (TestDiet DIO 58G7) is autoclaved at 120°C for 15 minutes to promote the Maillard reaction to increase the formation of AGEs. [57] This method simulates the process of cooking food and represents an exogenous source of AGEs. This food is referred to as the high AGE diet for the purposes of this study.

Table 1. Nutritional Information of Control and Experimental Diets

	Protein %	Fat %	Fiber %	Carbohydrate %
Regular	24.2	34.7	5.5	27.8
High AGE	20.8	23.6	5.8	41.2

Development of Mouse Model

Female wild-type FVB/n mice were weaned at 3 weeks of age. Experimental (high AGE) and control (regular) diets were started upon weaning. Diets were continued until the desired endpoints of 7 weeks (pubertal) and 12 weeks (mature virgin) of age were reached. At the study endpoint, mammary glands (thoracic and inguinal) were extracted from the mice for paraffin embedding and whole mounting. The inguinal glands were used for analysis, as the lymph node provides a geographical marker for reference. Serum was collected at each endpoint via cardiac puncture for future assessment.

Paraffin Embedding

After extraction, mammary glands were placed in 4% PFA (paraformaldehyde) overnight at 4°C. This was followed by a series of dehydration washes starting with one hour in 70% ethanol (EtOH), two hour-long 95% EtOH washes, two hour-long 100% EtOH washes, and followed by a two-hour wash in toluene. The tissues were incubated overnight at 60°C in a 50/50 mix of toluene and paraffin. The next day, the tissues were incubated twice in paraffin at 60°C for two hours. Upon completion, the mammary glands were paraffin embedded dorsal side down.

Estrous Cycle Phase Determination of Mouse Reproductive Tracts

At the time of mammary gland collection, reproductive tracts (vagina, uterus, ovaries) of 7-week-old and 12-week-old mice fed a regular or high AGE diet were collected. The reproductive tracts were fixed, and paraffin embedded as described earlier. The embedded tissue was sectioned at 4 μm thick and stained with H&E methodology (described below). With the assistance of a veterinarian pathologist, the reproductive tracts were histologically characterized and evaluated for features of each stage (proestrus, estrus, metestrus, and diestrus). Proestrus was characterized by tall cuboidal to columnar uterine epithelium with mitotic figures present (Figure 3, A). Estrus was marked by shedding of the superficial mucoid layers of the vagina and degeneration/necrosis of uterine epithelial cells (Figure 3, B). Metestrus featured mitotic activity and necrosis/debris in the endometrial epithelium with a smaller uterine lumen (Figure 3, C & D). Diestrus

presented a small lumen in the uterine horns that were lined by a low cuboidal or columnar epithelial with few degenerate cells (Figure 3, E & F). [59, 60]

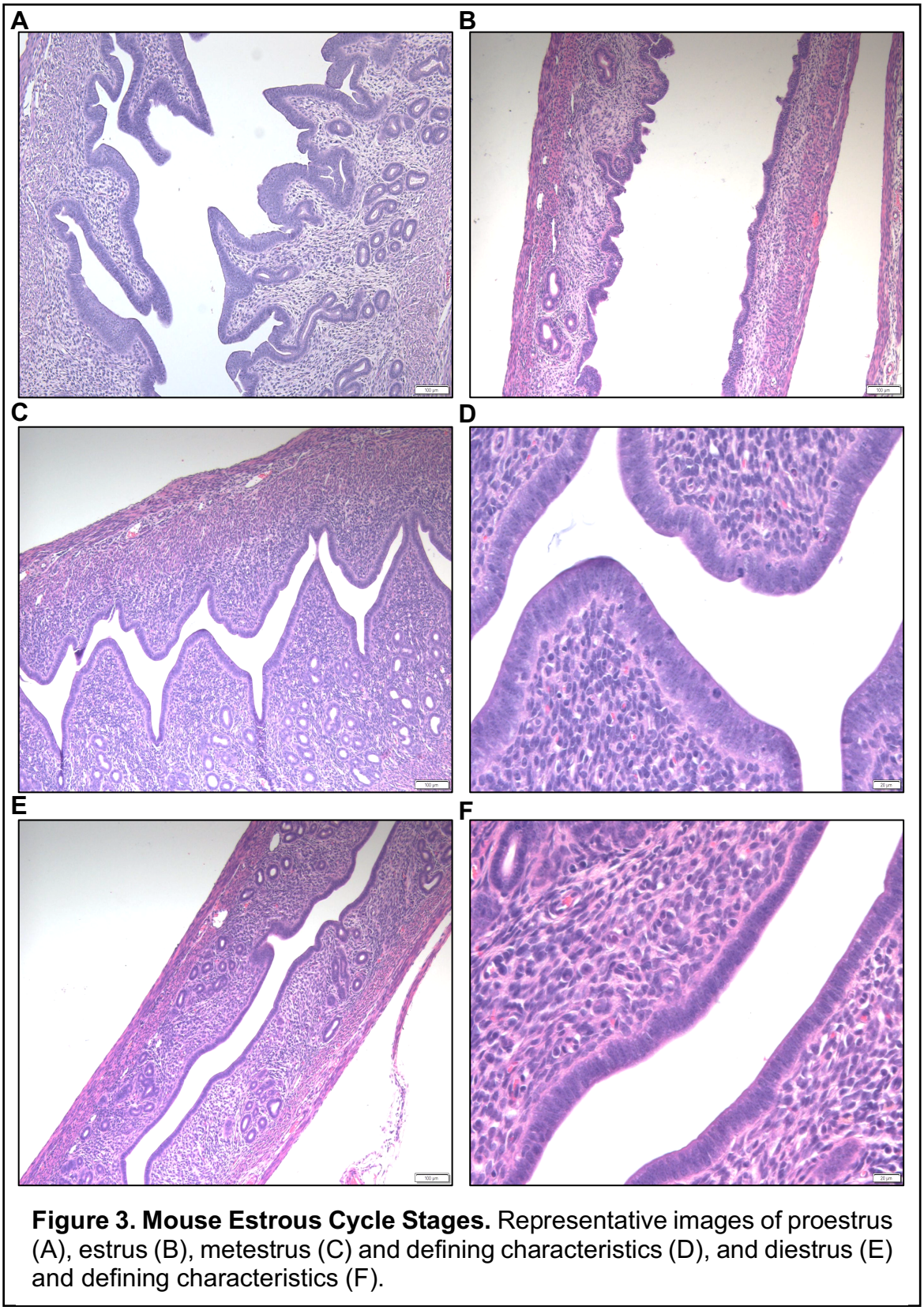


Figure 3. Mouse Estrous Cycle Stages. Representative images of proestrus (A), estrus (B), metestrus (C) and defining characteristics (D), and diestrus (E) and defining characteristics (F).

Ki-67 Immunohistochemistry

To assess proliferation and hyperproliferation in adult inguinal mammary glands paraffin embedded inguinal mammary glands were sectioned 5 µm thick onto a glass slide and utilized for Ki-67 IHC. The glass slides with tissues were incubated for one hour at 60°C to remove paraffin from sections. This was followed by a series of rehydration washes: 20 minutes in xylene (x2), 10 minutes in 100% EtOH (x3), 10 minutes in 95% EtOH (x2), 10 minutes in 70% EtOH, 10 minutes in 50% EtOH and 5 minutes in Milli-Q® water (Millipore Sigma, St. Louis, MO) (x3). Antigen retrieval was performed using citrate-based Vector antigen unmasking solution (Vector Laboratories, Burlingame, CA) and was diluted with Milli-Q® water (Millipore Sigma, St. Louis, MO) at a 1:100 dilution. The glass slides were placed in a pap jar filled with the diluted citrate solution and microwaved on 30% power for 3 minutes. The slides were then put in a vegetable steamer with antigen unmasking solution for 30 minutes at 90°C. The slides in the pap jar were cooled to RT and washed in Milli-Q® water (Millipore Sigma, St. Louis, MO) for 3 minutes (x2). To remove any endogenous peroxidase activity, 3% hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ) was applied to the tissues for 10 minutes. The slides were washed in 1X phosphate-buffered saline containing 0.01% Tween-20 (PBS-T) (Fisher Scientific, Fair Lawn, NJ) for 5 minutes. The slides were placed in a hybridization chamber to prevent evaporation whilst VECTASTAIN® 2.5% horse serum (Vector Laboratories, Burlingame, CA) was applied to the tissue to block non-specific binding and incubated for 20 minutes at RT. The Ki-67 primary antibody (Cell Signaling Technology, Danvers, MA, D3B5 Rabbit MAb, mouse

specific IHC formulation) was diluted by 1:400 in VECTASTAIN® 2.5% horse serum (Vector Laboratories, Burlingame, CA) and applied to the slides in the hybridization chamber to incubate overnight at 4°C. The next day, the slides were washed in 1X phosphate-buffered saline (PBS) for 5 minutes (x3) on an orbital shaker to remove the primary antibody. The VECTASTAIN® secondary antibody solution (Vector Laboratories, Burlingame, CA) was applied to the slides for 30 minutes at RT in the hybridization chamber. The secondary antibody was washed off with three 5-minute 1X PBS washes on an orbital shaker. The tissues were incubated for 5 minutes with SIGMAFAST™ DAB (3,3'-Diamino-benzidine) (Sigma-Aldrich Co., St. Louis, MO) diluted with autoclaved water to activate the specific signal and then placed in Milli-Q® water (Millipore Sigma, St. Louis, MO) to stop the reaction. The slides were washed three times in Milli-Q® water (Millipore Sigma, St. Louis, MO) for 5 minutes. Hematoxylin (Fisher Scientific, Fair Lawn, NJ) was used to counterstain the tissues by applying the staining solution on the tissues for 1 minute. The staining was completed by immersing the slides in Milli-Q® water (Millipore Sigma, St. Louis, MO). Washes of HCl-EtOH for 90 seconds and NH_4OH-EtOH for 45 seconds were used as differentiation solutions to remove overstaining or non-specific staining on the tissues. The slides were washed in Milli-Q® water (Millipore Sigma, St. Louis, MO) for 3 minutes. A 1-minute incubation in Scott's buffer was utilized as a blueing solution for hematoxylin. The slides went through a series of dehydration washes: 3 minutes in Milli-Q® water (Millipore Sigma, St. Louis, MO) (x3), 5 minutes in 50% EtOH, 5 minutes in 70% EtOH, 5 minutes in 95% EtOH (x2), 5 minutes in 100% EtOH (x2) and 10 minutes in xylene

(x2). Glass cover slips were mounted on top of the slides with Permount® (Fisher Scientific, Fair Lawn, NJ). Imaging was completed using an Olympus DP80 microscope (Olympus American, Inc. Center Valley, PA).

Ki-67 Quantification

Images of Ki-67 stained slides were analyzed using ImageJ software by counting positive or brown stained cells in ductal structures. [61] Non-positive or hematoxylin stained cells were counted and totaled to determine the percent of positive stained cells to total cell number. Each percent value from diet groups were averaged together and total average proliferation was reported for each diet group. Hyperproliferative ducts were ductal structures with 50% or more positive staining for Ki-67 (as established by our research team). The presence of hyperproliferative ducts was assessed for each diet group. A student's t-test was used to determine statistical significance of data collected from assessment of total proliferation and hyperproliferation.

H&E

To assess morphological changes in mammary glands and reproductive tracts, paraffin embedded inguinal mammary glands were sectioned 5 μm thick onto a glass slide and utilized for H&E (hematoxylin and eosin) staining. This was followed by a series of rehydration washes: 3 minutes in xylene (x3), 3 minutes in 100% EtOH (x3), 3 minutes in 95% EtOH, 3 minutes in 80% EtOH and 5 minutes in Milli-Q® water (Millipore Sigma, St. Louis, MO). Tissue samples were covered

with hematoxylin (Fisher Scientific, Fair Lawn, NJ) for 1 minute and washed in tap water for 5 minutes. Differentiation was completed by dipping the slides in HCl-EtOH 12 times and followed by a 1-minute wash (x2) in tap water and one 2-minute wash in Milli-Q® water (Millipore Sigma, St. Louis, MO). The tissue was covered by eosin (Fisher Scientific, Fair Lawn, NJ) for 45 seconds, followed by dehydration washes: 5 minutes in 95% EtOH (x3), 5 minutes in 100% EtOH (x3) and 15 minutes in xylene (x3). Glass cover slips were mounted on top of the slides with Permount® (Fisher Scientific, Fair Lawn, NJ). Imaging was completed using an Olympus DP80 microscope (Olympus American, Inc. Center Valley, PA).

Estradiol ELISA

Circulating estradiol levels were assessed in serum collected from 7-week-old and 12-week-old mice fed a high AGE diet compared to a regular diet using the Mouse/Rat Estradiol ELISA kit (Calbiotech, El Cajon, CA). In a 96-well plate, 25µl of standards, experimental samples and controls were dispensed into appropriate wells. Then, 100µl of Estradiol enzyme conjugate was dispensed into each well and mixed by placing the plate on an orbital shaker for 20 seconds. After mixing well, the plate incubated at RT for 2 hours. All liquid was removed from the wells and washed with 300µl of 1X wash buffer (x3). A volume of 100µl of TMB Reagent was added to each well, and the plate was set on an orbital shaker for 10 seconds to mix the reagent. The plate was set at RT to incubate for 30 minutes. The reaction was stopped by adding 50µl of Stop Solution to each well and mixed by setting the plate on an orbital shaker for 30 seconds, carefully watching for color

change from blue to yellow. Immediately after, a plate reader (Bio-Tek Instruments, Inc. Winooski, VT) was used to read the absorbance at 450 nm and results were recorded. A Wilcoxon-Mann-Whitney statistical test was used to analyze the set of data. Internal experimental positive controls were run to confirm efficacy of the assay.

Progesterone ELISA

Circulating progesterone levels were assessed in serum collected from 7-week-old and 12-week-old mice fed a high AGE diet compared to a regular diet using Progesterone ELISA kit (Cayman Chemical, Ann Arbor, MI). The standard for use in ELISA was prepared according to the manufacturer's instructions. In the 96-well plate, 100µL of prepared ELISA Buffer was added to the pre-determined Non-Specific Binding wells in duplicate. In the pre-determined maximum binding wells, 50µL of ELISA Buffer was added in duplicate. A volume of 50µL of the prepared standard was added to their appropriate wells in duplicate. Samples were dispensed at a volume of 50µL in wells. Next, 50µL of Progesterone AChE Tracer was added to each well, except the pre-determined Total Activity and Blank wells, followed by 50µL of ELISA Antiserum into each well, except for the Total Activity, Non-Specific Binding and Blank wells. After all reagents were dispensed, the plate was covered by plastic film and incubated for one hour at RT on an orbital shaker. The wells were emptied and rinsed five times with Wash Buffer. Development of the plate was initiated with the addition of 200µL of Ellman's Reagent to each well, and 5µL of Progesterone AChE Tracer to the Total Activity wells. The plate was

covered with plastic film and incubated for 90 minutes at RT in the dark on an orbital shaker. The plastic film was removed and a plate reader (Bio-Tek Instruments, Inc. Winooski, VT) was used to read it at 450nm. A Wilcoxon-Mann-Whitney statistical test was used to analyze the set of data. Internal experimental positive controls were run to confirm efficacy of the assay.

ERα Immunohistochemistry

To assess expression of ERα in pubertal and adult mammary glands, paraffin embedded inguinal glands were sectioned 5 µm thick onto a glass slide and utilized for ERα IHC. The glass slides with tissues were incubated for one hour at 60°C to remove paraffin from sections. This was followed by a series of rehydration washes: 20 minutes in xylene (x2), 10 minutes in 100% EtOH (x3), 10 minutes in 95% EtOH (x2), 10 minutes in 70% EtOH, 10 minutes in 50% EtOH and 5 minutes in Milli-Q® water (Millipore Sigma, St. Louis, MO) (x3). Antigen retrieval was performed using citrate-based Vector antigen unmasking solution (Vector Laboratories, Burlingame, CA) and was diluted with Milli-Q® water (Millipore Sigma, St. Louis, MO) at a 1:100 dilution. The glass slides were placed in a pap jar filled with the diluted citrate solution and microwaved on 30% power for 3 minutes. The slides were then put in a vegetable steamer with antigen unmasking solution for 30 minutes at 90°C. The slides in the pap jar were cooled to RT and washed in Milli-Q® water (Millipore Sigma, St. Louis, MO) for 3 minutes (x2). To remove any endogenous peroxidase activity, 3% hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ) was applied to the tissues for 10 minutes. The slides

were washed in 1X phosphate-buffered saline containing 0.01% Tween-20 (PBS-T) (Fisher Scientific, Fair Lawn, NJ) for 5 minutes. The slides were placed in a hybridization chamber to prevent evaporation whilst VECTASTAIN® 2.5% horse serum (Vector Laboratories, Burlingame, CA) was applied to the tissue to block non-specific binding and incubated for 20 minutes at RT. The ERα primary antibody (Abcam, Cambridge, MA, ab75635) was diluted by 1:100 in VECTASTAIN® 2.5% horse serum (Vector Laboratories, Burlingame, CA) and applied to the slides in the hybridization chamber to incubate overnight at 4°C. The next day, the slides were washed in 1X phosphate-buffered saline (PBS) for 5 minutes (x3) on an orbital shaker to remove the primary antibody. The VECTASTAIN® secondary antibody solution (Vector Laboratories, Burlingame, CA) was applied to the slides for 30 minutes at RT in the hybridization chamber. The secondary antibody was washed off with three 5-minute 1X PBS washes on an orbital shaker. The tissues were incubated for 20 seconds with SIGMAFAST™ DAB (3,3'-Diamino-benzidine) (Sigma-Aldrich Co., St. Louis, MO) diluted with autoclaved water to activate the specific signal and then placed in Milli-Q® water (Millipore Sigma, St. Louis, MO) to stop the reaction. The slides were washed three times in Milli-Q® water (Millipore Sigma, St. Louis, MO) for 5 minutes. Hematoxylin (Fisher Scientific, Fair Lawn, NJ) was used to counterstain the tissues by applying the staining solution on the tissues for 1 minute. The staining was completed by immersing the slides in Milli-Q® water (Millipore Sigma, St. Louis, MO). Washes of HCl-EtOH for 90 seconds and NH_4OH-EtOH for 45 seconds were used as differentiation solutions to remove overstaining or non-specific staining on the

tissues. The slides were washed in Milli-Q® water (Millipore Sigma, St. Louis, MO) for 3 minutes. A 1-minute incubation in Scott's buffer was utilized as a blueing solution for hematoxylin. The slides went through a series of dehydration washes: 3 minutes in Milli-Q® water (Millipore Sigma, St. Louis, MO) (x3), 5 minutes in 50% EtOH, 5 minutes in 70% EtOH, 5 minutes in 95% EtOH (x2), 5 minutes in 100% EtOH (x2) and 10 minutes in xylene (x2). Glass cover slips were mounted on top of the slides with Permount® (Fisher Scientific, Fair Lawn, NJ). Imaging was completed using an Olympus DP80 microscope (Olympus American, Inc. Center Valley, PA).

Results

Consumption of a high AGE diet results in significant proliferation in ductal structures. Proliferation is known to be upregulated during the diestrus phase of the estrous cycle. We therefore assessed AGE mediated effects on proliferation in mice that were identified as not being in the diestrus phase. Cellular proliferation in ductal structures of 12-week-old inguinal mammary glands was determined by Ki-67 IHC and quantified (Figure 4). Overall, the consumption of the high AGE diet (n=5) showed a significant increase of proliferation compared to the regular diet (n=4) control group.

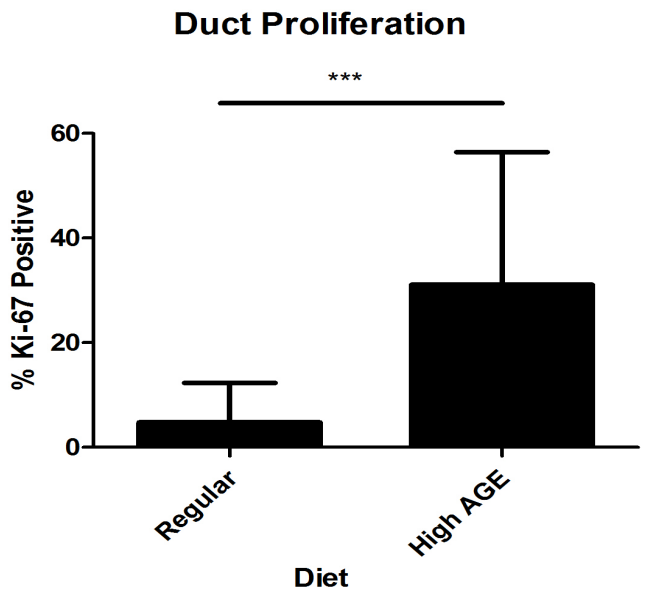


Figure 4. Proliferation of Ducts. Consumption of a high AGE diet (n=5) shows significantly increased proliferation in mammary ducts when compared to mice fed a regular diet (n=4) in Ki-67 IHC inguinal mammary glands. The estrous cycle phase was accounted for in the mice used for this experiment. ***p<0.0001

Hyperproliferative ductal structures are formed as a result of a high AGE diet. As previously mentioned, proliferation and therefore hyperproliferation are known to be increased during the diestrus phase of the estrous cycle. We therefore assessed AGE mediated effects on hyperproliferation in mice that were identified as not being in the diestrus phase. Ki-67 stained ductal structures of the same 12-week-old inguinal mammary glands as used in the previous result (Figure 4) were used to assess hyperproliferation (defined as over 50% positive for Ki-67 staining) in ductal structures. The presence of hyperproliferative ducts in the mice fed a high AGE diet (n=5) compared to a regular control diet (n=4) was significant (Figure 5).

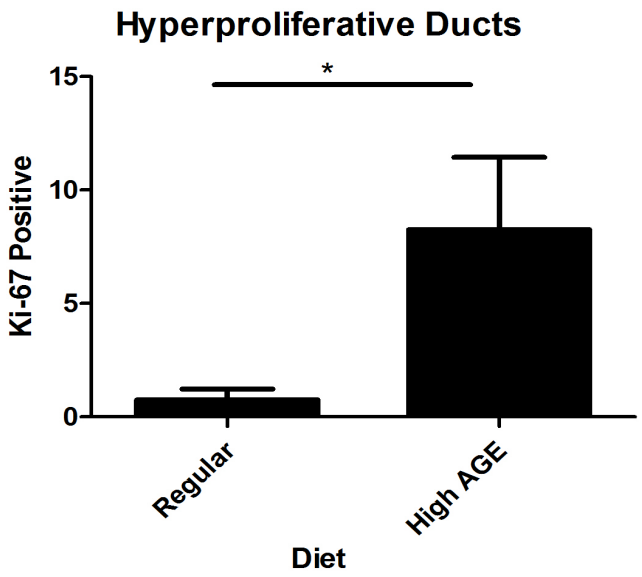


Figure 5. Hyperproliferation of Ducts. Ki67 IHC showed increased hyperproliferation of ducts in mice fed a high AGE diet (n=5) compared to mice fed a regular diet (n=4). The estrous cycle phase was accounted for in the mice used for this experiment. *p<0.05

Circulating estradiol levels are not significantly altered amongst diet and age groups. Circulating estradiol levels peak at estrus compared to the other phases of the estrous cycle. [59] We therefore assessed AGE mediated effects on circulating estradiol levels keeping in mind that any mice identified to be in estrus, may have increased circulating estradiol compared to others. To assess the amount of circulating estradiol in pubertal and adult mice fed the regular and high AGE diets we utilized ELISA analysis. (Figure 6) There appears to be an elevated trend in the 7-week-old regular diet (n=5) and 7-week-old high AGE diet (n=3) fed mice compared to the 12-week-old regular diet (n=5) and 12-week-old high AGE diet (n=6) fed mice. However, overall circulating estradiol levels between puberty and adulthood and diets does not significantly differ.

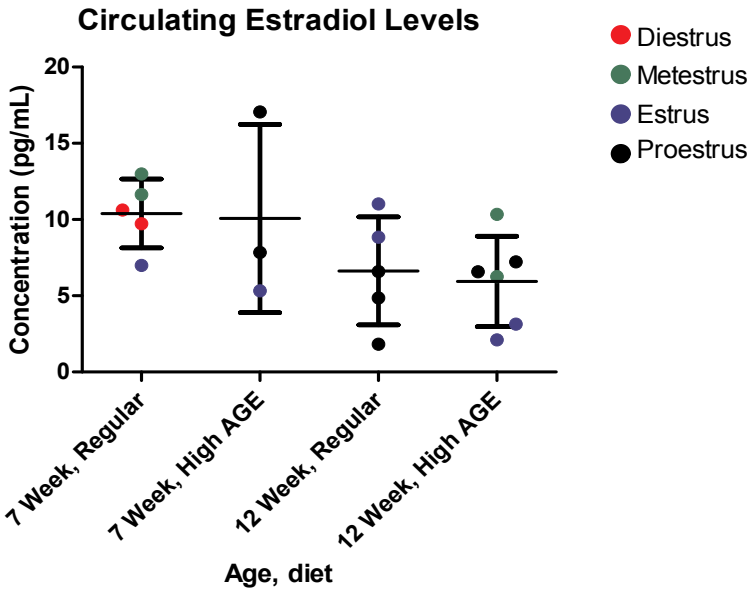


Figure 6. Circulating Estradiol Levels Among Diets and Age. Circulating estradiol levels evaluated via ELISA revealed no significant change of estrogen in the high AGE fed mice at the 7-week and 12-week time point compared to mice fed a regular control diet. All values not significant. The estrous cycle phase was accounted for and labeled in the mice used for this experiment.

Ductal structures in pubertal and adult mice fed a high AGE diet show diminished ERα expression. The presence of ER is essential for the responsiveness to estrogen action. The function of ERα is critical for the development and expansion of ducts into the mammary fat pad. [29] Pubertal (n=3) and adult (n=3) inguinal mammary glands from mice fed a regular (n=3) and high AGE (n=3) were assessed for expression of ERα utilizing IHC methodology. Consumption of a high AGE diet shows downregulation of ERα in pubertal and adult mice. (Figure 7) This evidence supports previous findings in our lab that a high AGE diet disrupts normal mammary gland development. [57] The estrous cycle phase was not accounted for in the mice used for this experiment, however future experiments will address this. The presence and function of ERα relies on circulating estrogen which fluctuates throughout the estrous cycle.

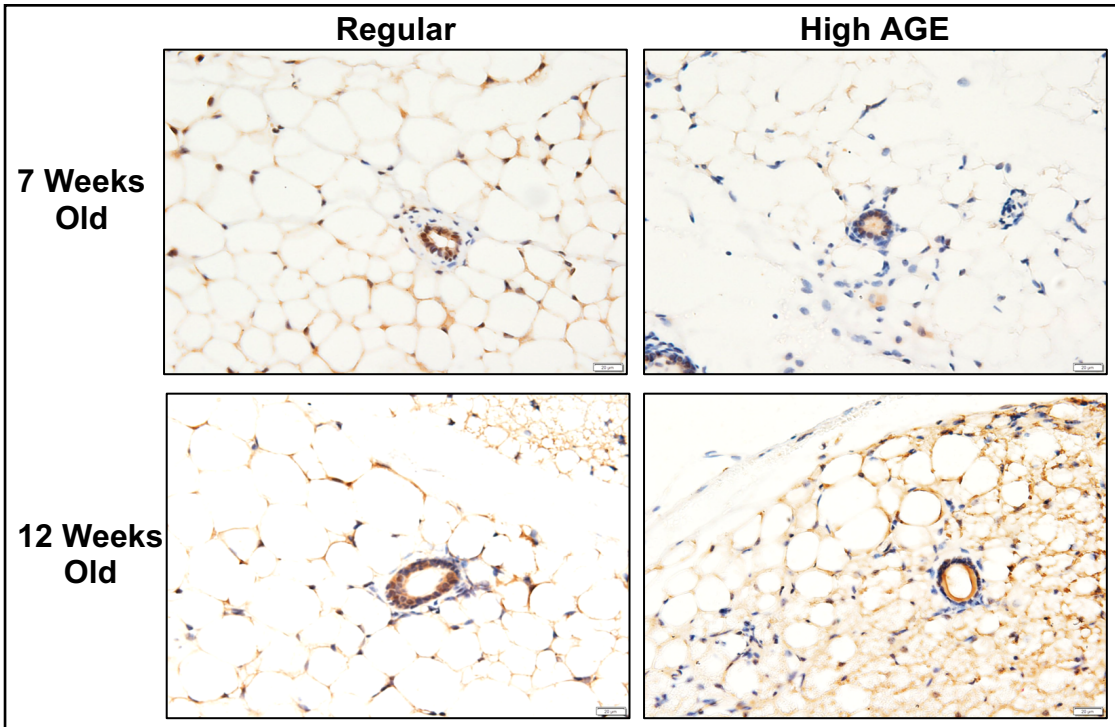


Figure 7. ERα Expression in Puberty and Adulthood. Representative images of ERα IHC, showing the downregulation of ERα in mammary glands from mice fed a high AGE diet at pubertal (7 weeks old) (n=3) and adult (12 weeks old) (n=3) time points compared to a regular diet at 7-week (n=3) and 12-week (n=3) time points.

Circulating progesterone levels are not significantly altered among diet and age groups. Circulating progesterone levels rise at metestrus and diestrus compared to the other phases of the estrous cycle. [59] We therefore assessed AGE mediated effects on circulating progesterone levels keeping in mind that any mice identified to be in metestrus and diestrus, may have increased circulating progesterone levels compared to others. To assess the amount of circulating progesterone in pubertal and adult mice fed the regular and high AGE diets we utilized ELISA analysis. (Figure 8) There appears to be an elevated trend in the 7-week-old regular diet (n=4) and 7-week-old high AGE diet (n=4) fed mice compared to the 12-week-old regular diet (n=4) and 12-week-old high AGE diet (n=6) fed mice. However, overall circulating estradiol levels between puberty and adulthood and diets do not significantly differ.

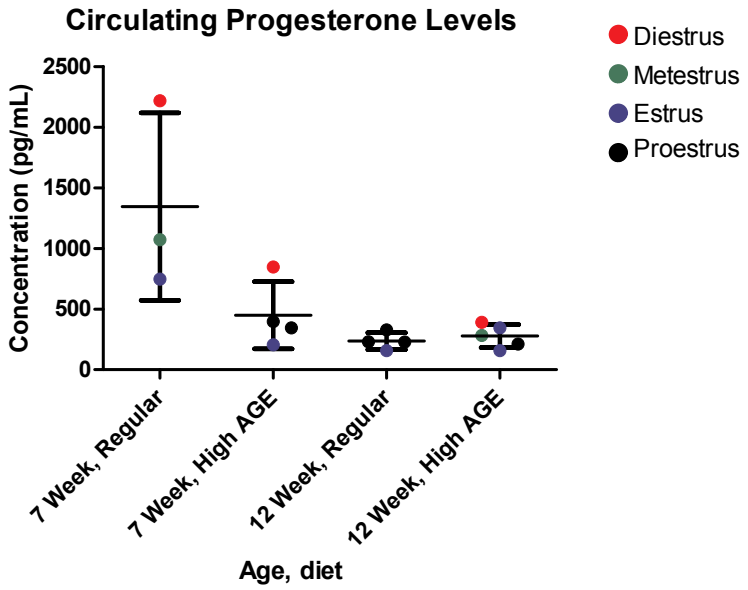


Figure 8. Circulating Progesterone Levels Among Diets and Age. Circulating progesterone levels evaluated via ELISA revealed no significant change of progesterone in the high AGE fed mice at the 7-week-old and 12-week-old time point compared to mice fed a regular control diet. All values not significant. The estrous cycle phase was accounted for and labeled in the mice used for this experiment.

Discussion

Accounting for the reproductive stage of the mice used in the experiments to address Specific Aim 1 allows us to rule out any notion that the effects observed from a high AGE diet, such as hyperproliferation, are due to the reproductive cycle. Compared to mice fed a regular control diet, a significant increase in proliferation and hyperproliferation was observed in mice fed a high AGE diet. Furthermore, results show that circulating estradiol levels do not significantly differ between mice fed a regular and high AGE diet, as well as between pubertal (7-week-old) and adulthood (12-week-old) time points. Assessment of circulating progesterone levels shows no significant change between age and diet groups as well. This allows us to rule out a hormone-dependent mechanism driving the morphological changes observed in mice fed a high AGE diet at 7 weeks of age and 12 weeks of age. To further explore estrogen and its receptor, ERα, IHC analysis was performed on inguinal mammary glands fed a high AGE and regular diet at pubertal (7-week-old) and adulthood (12-week-old) time points. This data showed reduced expression of ERα in pubertal and adult mice fed a high AGE diet compared to mice fed a regular diet, although the estrous cycle phase was not determined in these mice. Further confirmation of these results can be performed by Western Blot analysis with mammary gland tissue samples from pubertal and adult mice fed a regular and high AGE diet with known estrous cycle phases.

Specific Aim 2: To test the hypothesis that AGE induced effects on mammary gland development persist despite diet intervention.

The concept of metabolic memory postulates that an early glycemic environment is imprinted leading to disease complications and disease progression. [40-43] Due to their permanent nature and their regulatory effects on oxidative and immune stress pathways, AGEs are proposed to be a potential mechanism in the development of metabolic memory. Puberty is a sensitive time of mammary gland development, which also serves as a window of susceptibility of breast cancer. AGE mediated effects on metabolic memory may not only affect mammary development, but also lead to increased risk of breast cancer later in life. As detailed earlier, previous work in our lab has shown effects a high AGE diet on mammary gland development. [57] The objective of Specific Aim 2 is to switch diet composition at a defined time during puberty in our mouse model to examine if molecular changes introduced by consumption of a high AGE diet are "remembered" or "imprinted" and have laid the foundation for any future disruption of mammary gland development. [25]

To this end, upon weaning, 3-week-old female FVB/n mice were fed either a high AGE diet or regular diet until 7 weeks of age. At this pubertal time point, the diets were switched. Mice initially fed a high AGE diet were changed to a regular diet until 12 weeks of age, and mice initially fed the regular diet were provided a high AGE diet until 12 weeks of age. (Figure 9) By introducing a regular diet at 7 weeks of age, the high AGE diet may have laid the foundation for any disruption

of mammary gland development which would be indicative of metabolic memory. Since most of the mammary gland tissue remodeling has occurred during the period of time when the regular diet was consumed we anticipate that introduction of a high AGE diet during the end of puberty and into adulthood may have minimal effects.

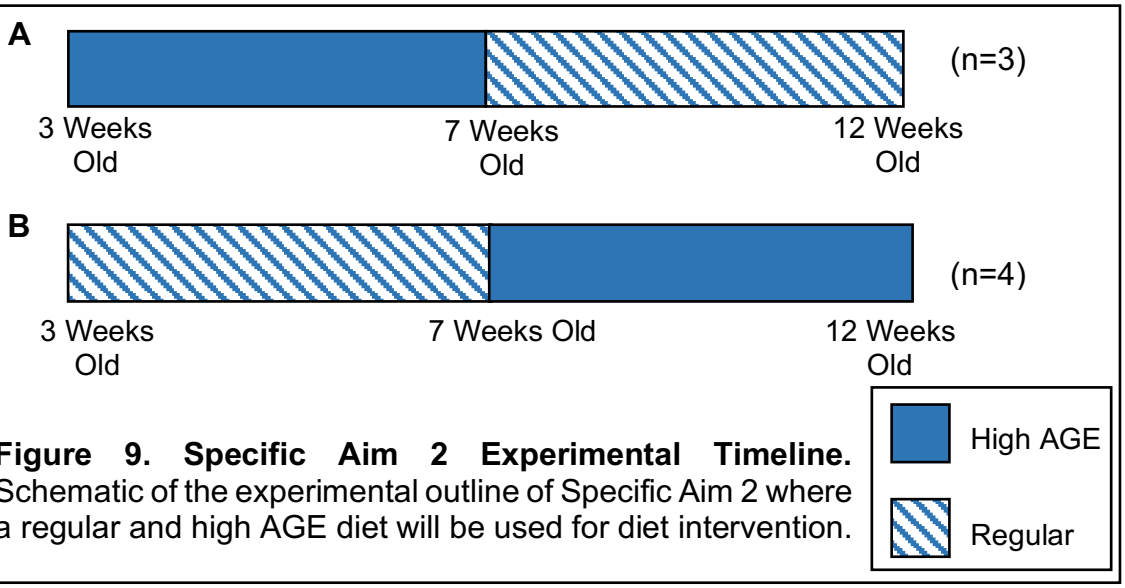


Figure 9. Specific Aim 2 Experimental Timeline. Schematic of the experimental outline of Specific Aim 2 where a regular and high AGE diet will be used for diet intervention.

Experimental Design

Whole Mounts and Analysis

The extracted mammary glands were stretched onto a glass slide and fixed in 4% PFA overnight at 4°C. The next day, the tissues went through a series of room temperature (RT) rehydration washes for one hour each: 70% EtOH, 50% EtOH, 30% EtOH and autoclaved water. The glass slides with tissues were placed in carmine staining solution (0.2% carmine, 0.5% aluminum potassium sulfate (Sigma-Aldrich Co., St. Louis, MO)) overnight at RT. The following day, the tissues were rinsed and incubated in autoclaved water for 1 hour at RT, followed by a series of dehydration washes for one hour each: 30% EtOH, 50% EtOH, 70% EtOH, 95% EtOH twice and 100% EtOH twice. The glass slides were covered with a clean slide, clamped together and immersed in xylene overnight at RT. The next day, glass cover slips were mounted on top of the slides using Permount® (Fisher Scientific, Fair Lawn, NJ). Once dry, the slides were photographed using a photo scanner (Epson Perfection 2450 Photo). Quantitation of the prepared whole mounts was performed by two individuals ensure unbiased results.

TEB number was assessed using ImageJ software and scanned images of the inguinal mammary glands. [61] A field view of 2 mm^2 was established by drawing a square with the software and placed at the top of the gland with the leading edge centered in the field of view. The number of TEBs in each field of view were quantified by moving down the established square marking the field of view until all of the TEBs at the leading edge were accounted for. Average number

of TEBs per field of view were compared between individual mice in each diet group and between different diet groups. The average number of TEBs per field of view in each diet group were recorded.

TEB area was assessed using ImageJ software and scanned images of the inguinal mammary glands. [61] TEBs were identified as club-like structures at the end of ductal branches, predominantly in the leading end of the gland. After setting the scale for measurement, length (from tip to neck) and width (at the widest point) of the TEB was measured. Both measurements were utilized to calculate the area of an eclipse (πab), which is the shape of a TEB. Average area was compared between individual mice in each diet group and between different diet groups. The average TEB area per each diet group was recorded.

Ductal extension was assessed using ImageJ software and the scanned images of the inguinal mammary glands. [61] After setting the scale for measurement on ImageJ, the length from the end of the lymph node to the tip of the furthest TEB was measured. The average ductal extension from individual mice was compared among diet groups and between different diet groups.

Ductal branching was assessed using ImageJ software and the scanned images of the inguinal mammary glands. [61] A field view of 2 mm^2 was established by drawing a square with the software and placed at the top edge of the gland with the leading edge to the far-right side of the field. The square marking the field of

view was moved two fields back into the ductal tree of the gland. Primary, secondary and tertiary branch points were quantified. Once all branch points in each field were counted, the field of view was moved down until the entire gland had been accounted for. Total branching was calculated by summing all branch points per field of view. The average total ductal branching of each mouse and diet group were compared and recorded.

Ki-67 Quantitation

Ki-67 quantitation was performed as previously described in Specific Aim 1. Statistical analysis of the hyperproliferation data was performed by the Hollings Cancer Center Biostatistics Shared Resource. The new variable percent is defined as the number of hyperproliferative ducts divided by the total number of ducts and reported for each diet group as an average. To compare the group mean difference, an ANOVA analysis was performed and the p-value of 0.0735 shows a borderline significance among the diet groups when compared to the regular control group. In addition, we fit a log-linear model on the aggregate data in which we compare the log of expect counts among the diet groups. There is no significant difference of the log of expect counts between high AGE diet and high AGE-regular diet group, but there is a significant difference of the log of expect counts between regular-high AGE diet and high AGE-regular diet, between regular and high AGE-regular diet, and between high AGE and regular-high AGE diet with the corresponding p-value of 0.0012, 0.0057, and <0.001.

Estrous Cycle Phase Determination of Mouse Reproductive Tracts

Although the estrous cycle phase was not identified in the mice that underwent the diet intervention, Ki-67 IHC staining revealed some glands in each cohort with 100% Ki-67 positivity (i.e. all ductal structures in the gland were hyperproliferative). These mice were eliminated from all analysis as they were most likely undergoing diestrus. Reproductive tracts fed only a regular or high AGE diet were analyzed to determine the estrous phase as previously described in Specific Aim 1. Mice in these cohorts undergoing diestrus were ruled out from analysis.

Results

TEBs persist into adulthood in mammary glands of mice exposed to a high AGE diet. During normal mammary development, at adulthood TEBs usually regress to TEDs. [23, 27] Our earlier studies demonstrated that more TEBs are present at adulthood in mice fed a high AGE diet than in control mice with normal mammary development. [57] We therefore examined the effects of the diet switch on the number of TEBs observed at adulthood. To assess if disruption of TEB formation persists after diet switching, overall TEB number was quantified in the 12-week old diet intervention groups. First, where mice were fed a high AGE diet and switched to a regular diet (n=3), and then where mice were fed a regular diet and switched to a high AGE diet (n=4). These values were compared to control groups of mice fed only a regular (n=5) or high AGE diet (n=4) using whole mounted inguinal mammary glands. While only 50% of the mice mammary glands had evidence of TEBs remaining after consumption of a regular diet, mice fed the high AGE diet since weaning until 12 weeks of age still had evidence of TEB structures in all (100%) of their glands. This was also the case for the mice that underwent the diet switch. Both groups of mice from a high AGE diet to regular diet switch, and a regular diet to high AGE diet switch had TEBs present in all examined glands. (Figure10)

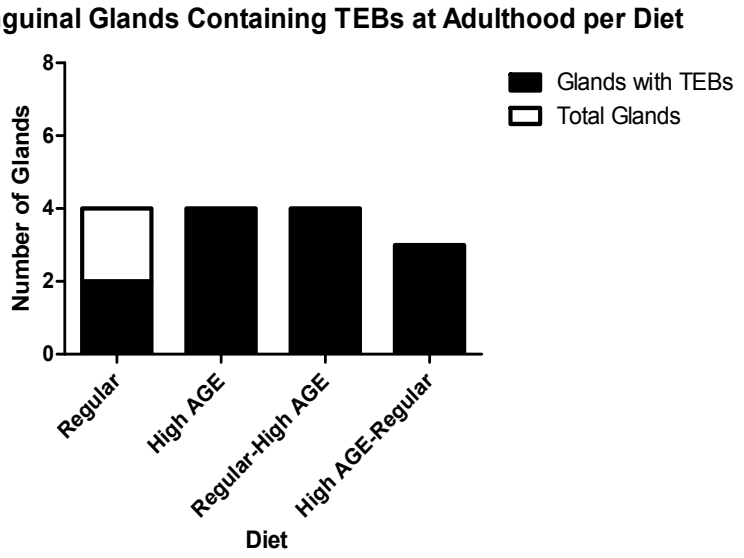


Figure 10. TEB Number at Adulthood Among Diets. Carmine stained whole mount analysis revealed that TEBs persist after exposure to a high AGE diet independent of timing of exposure.

TEB area is not significantly altered in mice fed an intervention diet. Earlier experiments performed in our lab demonstrated that a high AGE diet results in increased TEB area at 7 and 8 weeks of age during active pubertal development. Figure 10 shows that TEBs persist in 12-week-old mice. Therefore, we assessed TEB size at this adult time point. Using whole mounted inguinal mammary glands of 12-week-old mice fed a regular (n=5) and high AGE diet (n=4), TEB area was quantified and compared to that observed when the diets were switched at the 7-week time point from a high AGE diet to regular diet (n=3) and from a regular diet to a high AGE diet (n=4). Overall, TEB area was not significantly increased in mice continually fed a high AGE diet compared to the regular diet. This was also not significantly altered in either of the diet switch groups examined. (Figure 11)

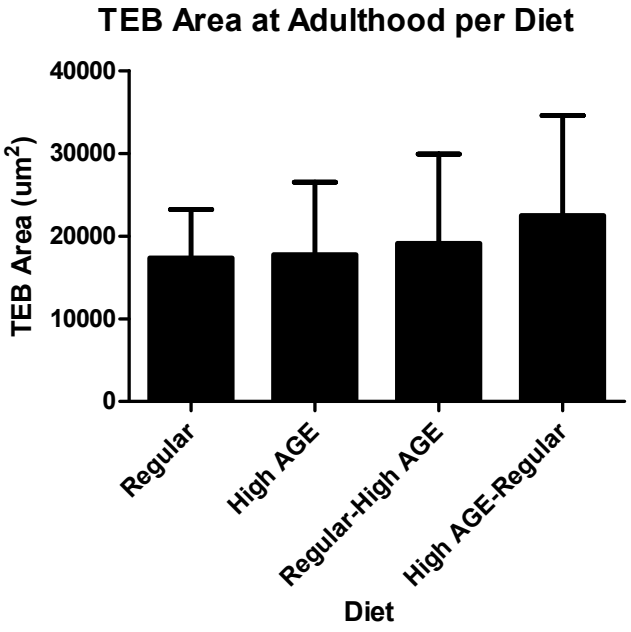


Figure 11. TEB Area is Unaltered Between Diets. Carmine stained whole mount analysis revealed exposure to a high AGE diet caused no significant increase in TEB area at the 12-week-old, adulthood time point. No significant differences between values was observed.

Mice fed the intervention diet showed a decrease in ductal branching. Previous findings in our lab demonstrated that mice fed a high AGE diet showed a significant increase in total ductal branching within the mammary gland at 7, 8 and 12 weeks of age compared to a regular diet. [57] We therefore determined if increased ductal branching persists when the diets are switched at the 7-week time point. Ductal branching was assessed in mice fed the intervention diets and control diet groups using whole mounted inguinal mammary glands. Compared to a regular diet (n=5) and in contrast to a high AGE diet (n=4), we observed a small but significant decrease in total ductal branching when the diet was changed from a regular diet to a high AGE diet (n=4) at 7 weeks of age. In contrast, we saw no significant change in ductal branching in the high AGE to regular diet (n=3) switch. (Figure 12) These data indicate that the continual presence of AGEs is required for the observed effects on ductal branching and indicate that metabolic memory is not a process affecting ductal branching. However, as temporal differences in primary, secondary and tertiary branching were observed in pubertal mice fed a high AGE diet into adulthood, it is likely that a more in-depth analysis of branching is required moving forward to understand the impact of diet intervention on this morphological change.

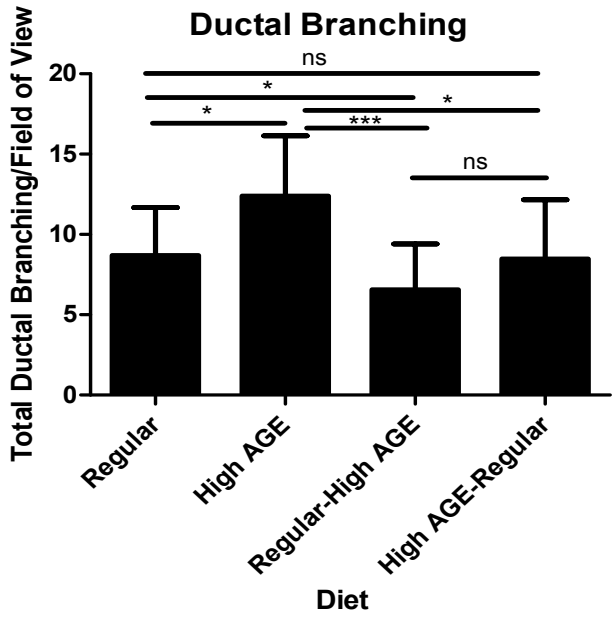


Figure 12. Ductal Branching Among Diets. Carmine stained whole mount analysis revealed that overall ductal branching did not differ between diet intervention groups. However, ductal branching between control diets and intervention diets significantly differed. *p<0.05, ***p<0.0001, ns – not significant

Ductal extension is not significantly changed in mice fed an intervention diet. Utilization of the whole mounted inguinal glands from 12-week-old mice fed control diets and intervention diets allowed for quantification of ductal extension. (Figure 13) Previous data from our lab has shown that a high AGE diet led to a significant inhibition of ductal extension when assessed at the 7-week-old and 8-week-old time points. [57] However, this was concluded to be a delay in extension rather than inhibition as no significant changes in ductal extension were observed at the 12 week old time point in mice fed the regular (n=5) and high AGE diets (n=4). [57] Therefore, to assess whether the intervention diets had any impact on ductal extension at adulthood, we assessed ductal extension in these mice at 12 weeks of age. We observed results similar to the mice fed a continuous high AGE diet as we did in the mice fed a regular and then high AGE diet (n=4) and the mice fed a high AGE diet then regular diet (n=3). No significant changes in ductal extension were observed.

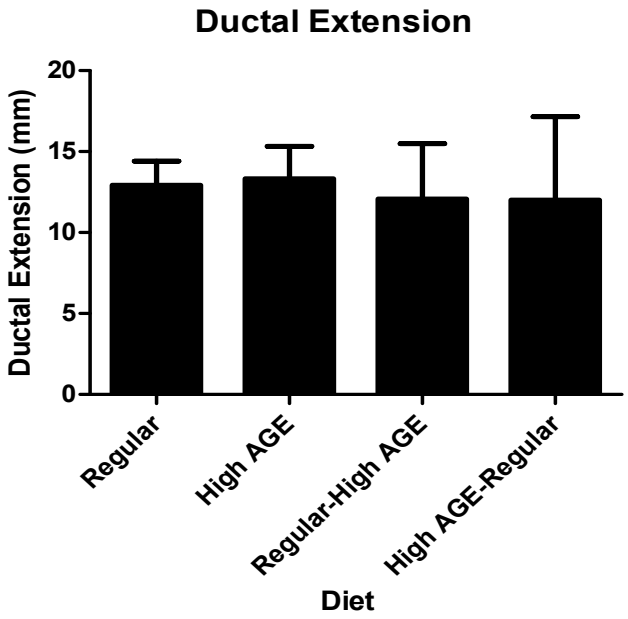


Figure 13. Ductal Extension Among Diets. Carmine stained whole mount analysis revealed no significant changes in mice fed control diets (regular and high AGE) compared to an intervention diet (regular-high AGE and high AGE-regular). No significant differences between values was observed.

Ductal proliferation is increased overall, independent of diet intervention.

Preliminary data in our lab has shown that ductal proliferation was increased in mice fed a high AGE diet at both 7 weeks and 8 weeks of age when compared to mice fed a regular diet. [57] Therefore, to assess whether this persists into adulthood, cellular proliferation in ductal structures of 12-week-old inguinal mammary glands was determined by Ki-67 IHC. Positive staining was counted, and quantified (Figure 13) and representative images are shown (Figure 14). We observed a significant increase in ductal proliferation in mice fed a high AGE diet (n=4) when compared to mice fed the regular diet (n=5), demonstrating that increased ductal proliferation persists into adulthood.

To determine whether a diet intervention can impact the observed increase in ductal proliferation within the 12-week-old mice, we assessed ductal proliferation in the 12-week-old mice fed the intervention diet, fed either a regular diet first and switched to a high AGE diet (n=4) or fed a high AGE diet first and switched to a regular diet (n=3). We observed an increase in ductal proliferation in these 12-week-old mice when either diet intervention was administered when compared to the regular diet. We also observed that ductal proliferation was significantly reduced in the mice fed the intervention diets when compared to mice fed only the high AGE diet with no switch. Proliferation of ductal structures in the two diet intervention groups alone did not significantly differ. (Figure 15) These data suggest that the duration, not the timing of the high AGE diet is critical for driving proliferation within the glands.

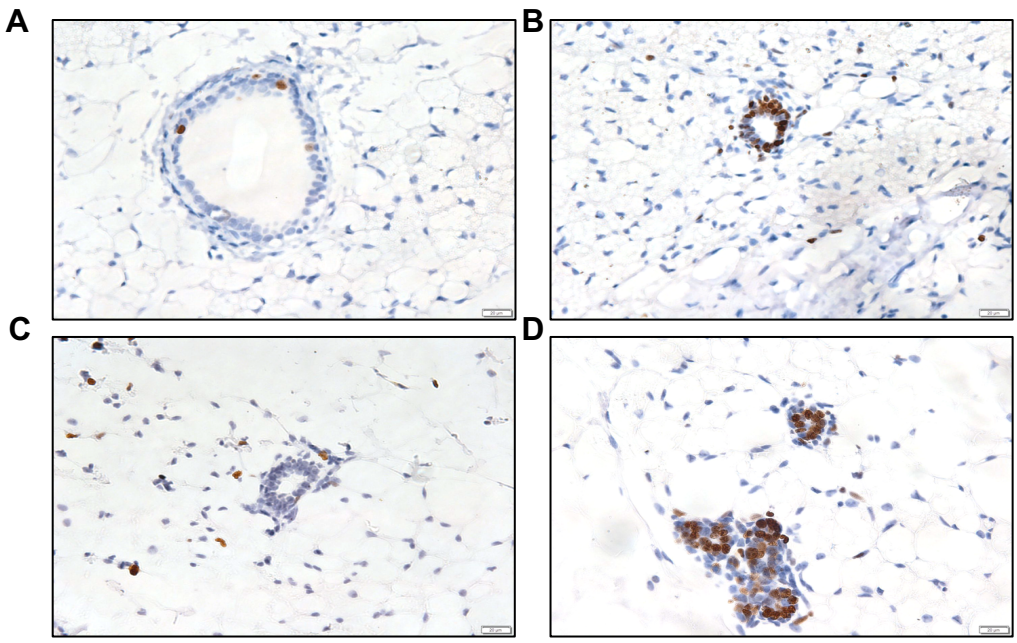


Figure 14. Ductal Structures Ki-67 IHC. Representative Ki-67 IHC images of ductal structures in mice fed a regular diet **(A)**, high AGE diet **(B)**, regular → high AGE intervention diet **(C)** and high AGE → regular intervention diet **(D)** at 12 weeks of age.

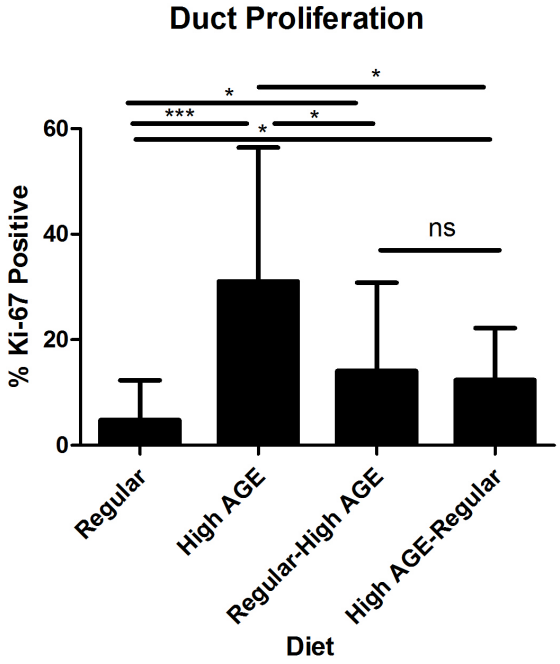


Figure 15. Duct Proliferation Among Diets. Consumption of a high AGE diet shows significantly increased proliferation compared to mice exposed to a regular diet. *p<0.05, ***p<0.0001, ns – not significant

Hyperproliferative ducts persist into adulthood in mice. As ductal proliferation was increased overall, we decided to take a closer look at proliferation within individual ducts as we observed a range of proliferation among ductal structures within the mammary gland. Hyperproliferation is defined as an abnormally high rate of proliferation in cells. In this study, we defined hyperproliferative ductal structures as those containing greater than 50% Ki-67 positive cells per structure (Figure 16). We microscopically examined the entire Ki-67 stained tissue section and scored each one for the total number of hyperproliferative ductal structures and reported this as a percentage of the total number of ductal structures per gland. We observed a significant increase in hyperproliferative ducts in glands from mice that continuously consumed a high AGE diet compared to the mice that continually consumed a regular diet. Next, this was examined in mice fed the intervention diets. We observed that consumption of either intervention diet also significantly increased hyperproliferation when compared to mice fed the regular diet alone. This increased hyperproliferation was comparable to the levels observed in mice continually fed the high AGE diet, suggesting that diet intervention is not able to inhibit the observed increase in hyperproliferation. Of interest, mice initially fed a regular diet (during early puberty) then switched to a high AGE diet (during late puberty) showed the greatest increase in hyperproliferative structures. This was significantly increased over the amount of hyperproliferative structures observed in the mice fed the high AGE diet continually. These data might suggest that the consumption of the high AGE diet leads to an acute response in proliferation within the gland. To examine this further

hyperproliferation in 16-week-old and/or 20-week-old mice should be assessed. Importantly, we show that an increase in hyperproliferation cannot be ablated with a diet intervention either during early or late puberty.

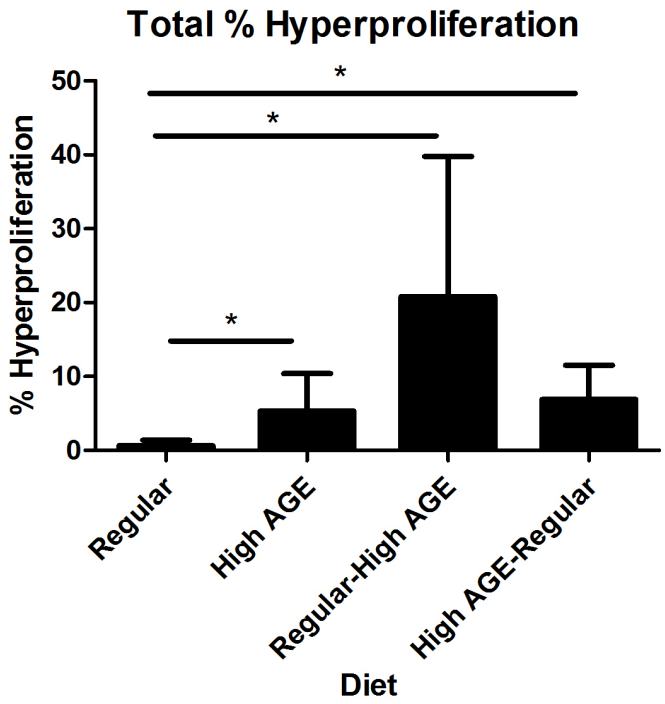


Figure 16. Duct Hyperproliferation Among Diets. Consuming a high AGE diet for any amount of time yields significantly higher proliferation compared to consuming only a regular diet. *p<0.05

Discussion

Assessment of TEB area showed that there was no significant difference in TEB size when present in adult mice fed either an intervention diet or a continuous regular or high AGE diet. However, in both the high AGE and intervention diet groups, TEBs persisted into adulthood. This occurred at a higher rate when compared to the control regular diet alone. This is an unexpected phenotype at 12 weeks of age as TEBs normally regress into TEDs at this time. [23, 27] These data also indicate that the AGE mediated effects of TEB regression may be independent of metabolic memory as they are independent of when the diet switch occurs.

Assessment of total ductal branching showed that mice on either diet intervention plan had decreased ductal branching compared to mice fed a regular or high AGE diet. However, a breakdown of primary, secondary and tertiary branching may need to be assessed in order to investigate this further. Also, ductal extension did not significantly change in mice fed an intervention diet. These findings are consistent with previous findings in our lab that ductal extension does not vary between diet groups at adulthood, since the ductal tree should be fully extended at this time point. [57]

Assessment of proliferation indicates that while consumption of a diet high in AGEs increases both proliferation and hyperproliferation, the timing that the high AGE diet is introduced can have significant effects on the outcome. Compared to the continual consumption of a high AGE diet, proliferation was decreased by the introduction of both intervention diets, although it remained higher than the mice

continually fed the regular diet. This is in contrast to hyperproliferation, which saw the highest levels when the mice were fed the regular diet first before being switched to the high AGE diet during late puberty.

The combined data from Specific Aim 2 suggest that although the consumption of a high AGE diet manifests many detrimental changes to normal mammary gland architecture, not all of these phenotypes are reversible with a diet intervention. Overall, increased AGE consumption at any time can affect change within the mammary gland. Intervention of the high AGE with a regular diet did not diminish the effects of the high AGE diet by adulthood at 12 weeks of age. In addition, intervention of a regular diet with a high AGE diet showed similar effects of a regular diet. However, the presence of a high AGE diet during the latter part of puberty into adulthood still affected the TEB and ductal morphology as observed in the whole mounts. These findings are indicative of metabolic memory where early glycemic environment is remembered or imprinted leading to disruption in mammary gland morphology. [41]

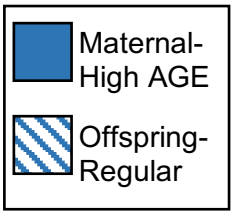
Specific Aim 3: To test the hypothesis that AGE induced effects on the mammary gland affect pregnancy and pup development.

Pregnancy and its associated development is a susceptible time of mammary gland development. During this time environmental factors, such as diet, can increase the risk of developing breast cancer later in life. Studies have shown in animal models that maternal exposure to a high fat diet during pregnancy increases female offspring's mammary cancer risk. [62-64] Additionally, Nguyen et al. found that maternal consumption a high fat diet before and during pregnancy increased mammary cancer risk of daughters (F1) and granddaughters (F2), indicating multigenerational inheritance. [63] Multigenerational inheritance is seen in the F1 and F2 but not the F3 generation, which instead would be indicative of transgenerational inheritance. [65]

Since effects of a high fat diet have been observed via multigenerational inheritance, we wanted to test whether AGE induced effects would be passed down via multigenerational inheritance. Two female mice were placed in a cage with one male mouse for two weeks, and at the same time the high AGE diet was started. The female mice were removed after two weeks and placed in separate cages where the mice continued to consume a high AGE diet. Once the female mice gave birth, the litter sizes were normalized to 6 total pups/dam. The dams nursed their offspring until they were three weeks of age when the offspring were then weaned. During the 3-week period of lactation, the offspring were exposed to the high AGE diet via the milk from their mom. [66] Once weaned, the female mice

were fed a regular diet until the pubertal time point of 7 weeks of age. At this time, the female mice were taken for mammary gland extractions which were then whole mounted, and paraffin embedded for future analysis as previously described. (Figure 17)

Figure 17. Specific Aim 3 Experimental Timeline. Schematic of the experimental outline of Specific Aim 3 where a maternal high AGE diet effects will be tested on their offspring fed a regular diet.



Results

Pups born from a mom fed a high AGE diet had significantly decreased weight compared to pups born from a regular diet fed mom. Previous and current work in our lab has explored the plethora of AGE effects on normal mammary gland development. [57] To further explore these effects with regards to inheritance, we wanted to see whether the AGE mediated effects on the mom would have any effects on offspring. Pregnant mice were fed a high AGE diet before, during and after pregnancy. After the mice gave birth the litters of pups (Experimental) were weighed daily starting from 1 day old until 21 days old, or time of weaning. The average of these weights was compared to average weights of mice born from a mom fed a regular diet as a control group. (Figure 18) A students t-test was utilized to compare the average weight per day of the control group (n=4) to the experimental group (n=3) where statistical significance was found at 11 days of age until 21 days of age with a resulting ~20% difference in body weight at weaning. These data allude to potential nutrient deficiency in the pups and/or possible lactation defect in the moms due to the high AGE diet. Upon weaning, the female pups were fed a regular diet until 7 weeks of age in order to assess multigenerational effects on mammary development.

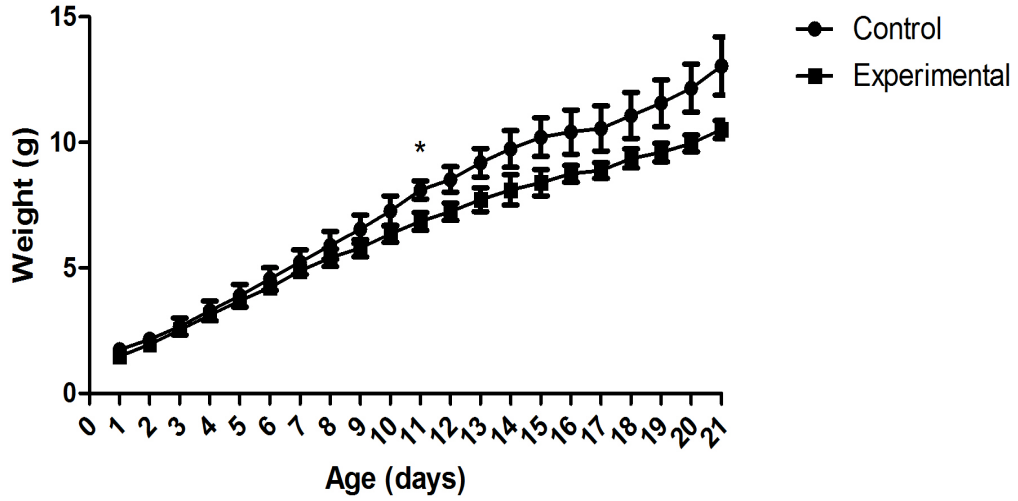


Figure 18. Pup Weight. Pups born from a mom fed a high AGE diet have significant weight differences compared to pups born from a mom fed a regular diet. *p<0.05 starting at 11 days of age to 21 days of age

Pubertal offspring from a mom fed a high AGE diet do not show changes in TEB number. To assess generational effects in mice nursing from a mom fed a high AGE diet compared to mice nursing from a mom fed a regular diet, we examined changes in mammary gland development at a pubertal time point. The female pups (n=3) discussed in the previous experiment were weaned and fed a regular diet until 7 weeks of age (puberty). At the experimental endpoint at 7 weeks of age, the mice were collected for mammary gland extraction. The prepared inguinal glands were used for whole mount analysis as previously described. TEB number per field of view was quantified and the pups from the mom fed a high AGE diet (Regular-Pups) showed a trend towards an increased TEB number similar to that observed in mice fed a high AGE diet (n=5) starting at weaning, with a p-value of 0.09, compared to a regular diet (n=4) (Figure 19).

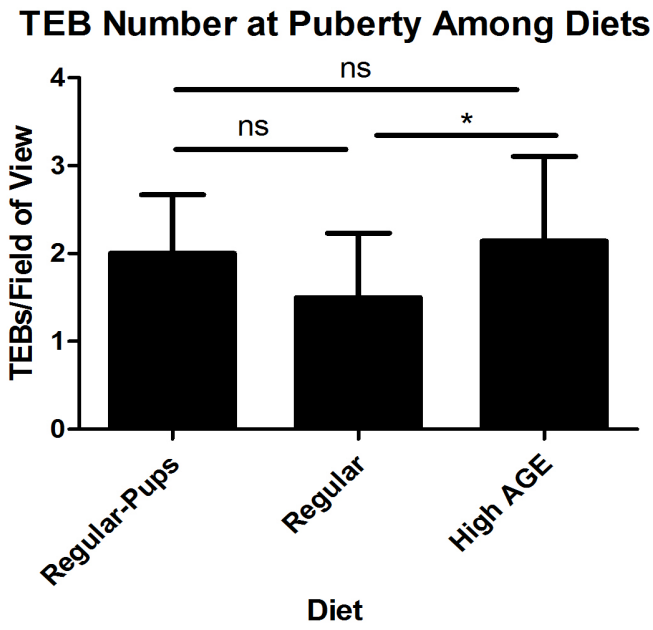


Figure 19. TEB Number at Puberty Among Diets. Carmine stained whole mount analysis revealed that the number of TEBS per field of view in the mice from a high AGE fed mom did not significantly differ from 7-week-old mice fed a regular control or high AGE diet. This trend did not reach statistical significance. *p<0.05

TEB area does not significantly differ between 7-week-old mice from a high AGE diet fed mom and mice fed a regular diet. As the number of TEBs present in the mice born from a mom fed a high AGE diet trend towards the phenotype observed in the mice fed a high AGE diet, we also wanted to assess whether the TEBs present differed in size. At the pubertal end point TEB size was measured, and we found that the TEB size did not significantly differ between mice that were exposed to a maternal high AGE diet (n=3) and mice fed a regular diet (n=4), while both groups of these mice did have significantly reduced TEB areas compared to mice fed a high AGE diet (n=5) (Figure 20).

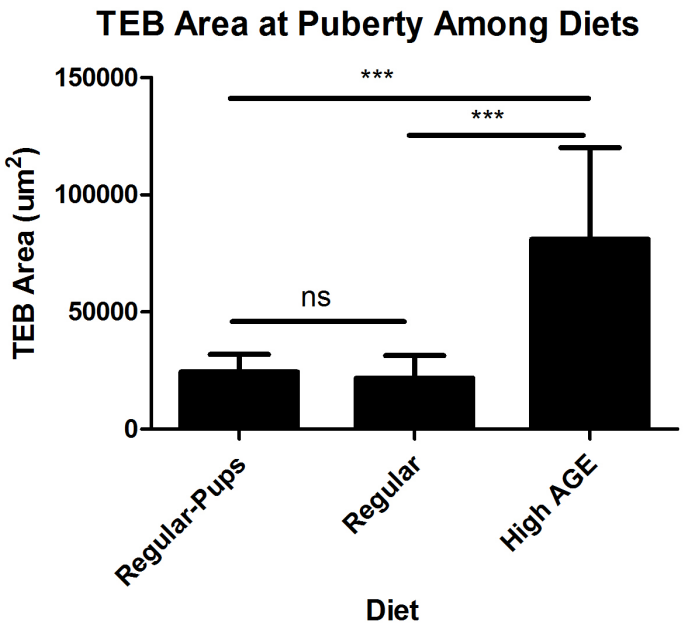


Figure 20. TEB Area at Puberty Among Diets. Carmine stained whole mount analysis revealed that TEB area between the 7-week-old mice from a high AGE diet fed mom and regular control diet did not significantly differ. However, TEB area from the 7-week-old mice from a high AGE fed mom and mice fed a high AGE diet were significantly different. ***p<0.0001, ns – not significant

Mice from a high AGE diet fed mom showed total ductal branching similar to mice fed a high AGE diet. Assessment of ductal branching between the mice from a high AGE diet fed mom (n=3) shows a similar trend in ductal branching of mice fed a high AGE diet (n=5), with a p-value of 0.10, compared to pubertal mice fed a regular diet (n=4) (Figure 21). Although the results are showing these trends, future experiments with more mice in each group should show a more definite relationship. While TEB number and size may not be affected by the exposure to a high AGE diet during nursing, the changes seen in ductal branching may be a result of multigenerational inheritance.

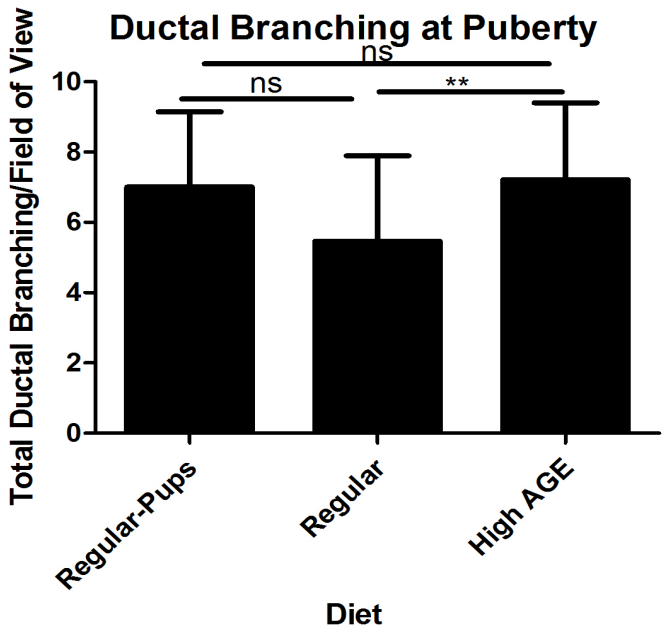


Figure 21. Ductal Branching at Puberty Among Diets. Carmine stained whole mount analysis revealed that ductal branching between pubertal mice from a high AGE diet fed mother does not significantly differ between mice fed a regular diet or high AGE diet. **p<0.005, ns – not significant

Ductal extension of pubertal mice is slightly affected by a maternal high AGE diet. Assessment of ductal extension in mice born from a high AGE diet fed mom (n=3) shows a significant difference compared to ductal extension in mice fed a regular diet (n=4) (Figure 22). Additionally, there was a significant difference observed in ductal extension between the mice from a high AGE diet fed mom and mice fed a high AGE diet (n=5).

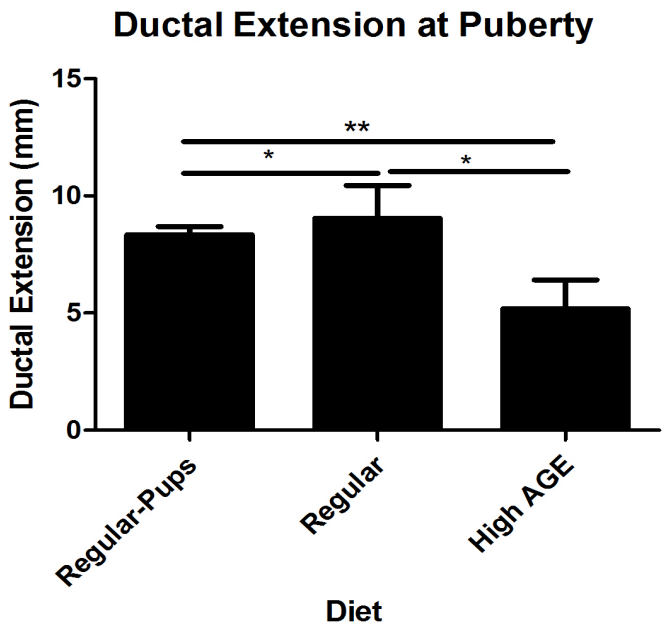


Figure 22. Ductal Extension at Puberty Among Diets. Carmine stained whole mount analysis revealed that ductal extension in mice born from a mom fed a high AGE diet are significantly different than mice fed a regular control diet. *$p<0.05$, **$p\leq0.005$

A high AGE diet affects lobular-alveoli development in pregnant mice. The data first described in Specific Aim 3 demonstrated that a lack of weight gain in pups fed from mice fed a high AGE diet may suggest a potential lactation defect in pregnant moms. To examine these findings further, female mice were fed a high AGE diet for 2 weeks during mating (n=6). Once pregnant, the female mice were put on a regular diet during gestation. Mice fed a regular diet from mating until pregnancy were used as controls (n=4). Pregnant mice were collected at various pregnancy day (Pd) time points (Pd 7.5, Pd 9.5, Pd 11.5, Pd 13.5). These time points were confirmed upon collection where the embryo was microscopically examined for developmental features that hallmark the various time points. During pregnancy, the mammary fat pad is encompassed by developing lobular-alveolar structures that develop from the mature ductal branches throughout the gland. These structures allow for the synthesis and secretion of milk for feeding the pups during lactation. Once the pups are born, the entire fat pad is filled with epithelial lobular-alveolar units. [23]

Mammary glands were extracted to be used for paraffin embedding and H&E staining as previously described. The H&E stained inguinal mammary gland tissues were imaged and used to determine the average area of the gland covered by lobular-alveoli compared to the total area of the gland (Figure 23). The percent epithelial density was compared to the total area of the gland reported as a percentage and reported in Figure 24. Overall, mice fed a high AGE diet before pregnancy showed a statistically significant decrease in percentage of area of

lobular-alveoli epithelial density compared to mice fed a regular diet. A student's t-test reveals that at each stage of pregnancy assessed, a significant reduction in epithelial density was observed. From this, we can postulate that the high AGE diet inhibits the proliferation and/or differentiation of the lobular-alveolar structures during pregnancy. These data suggest that the short-term exposure of the high AGE diet in mice had a lasting effect during pregnancy as they were only fed the high AGE diet for the initial two weeks of the experiment (while mating).

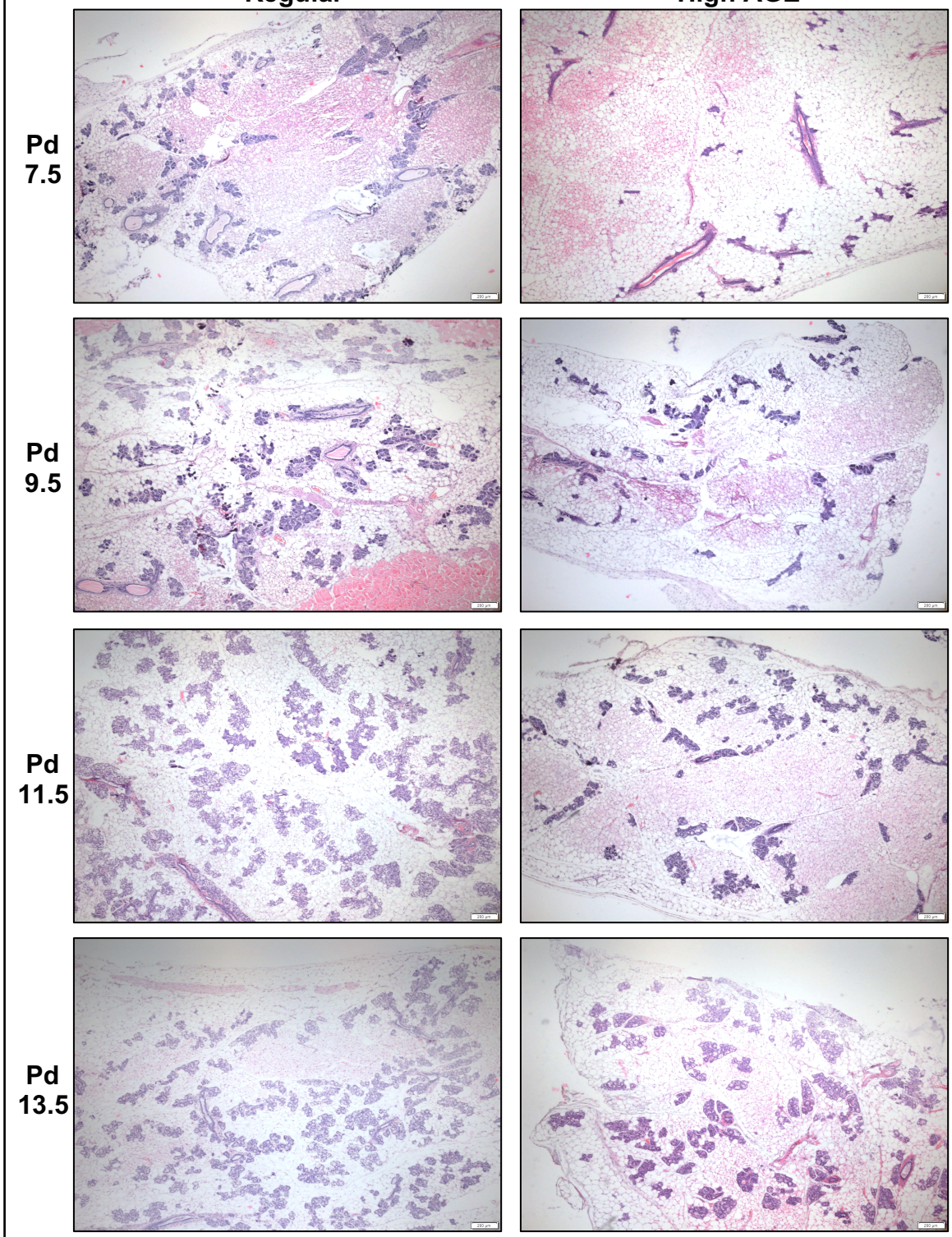


Figure 23. Morphological Changes in Pregnant Mice. Representative images of H&E stained mammary glands from pregnant mice at various stages fed a regular and high AGE diet before pregnancy shown to compare epithelial density.

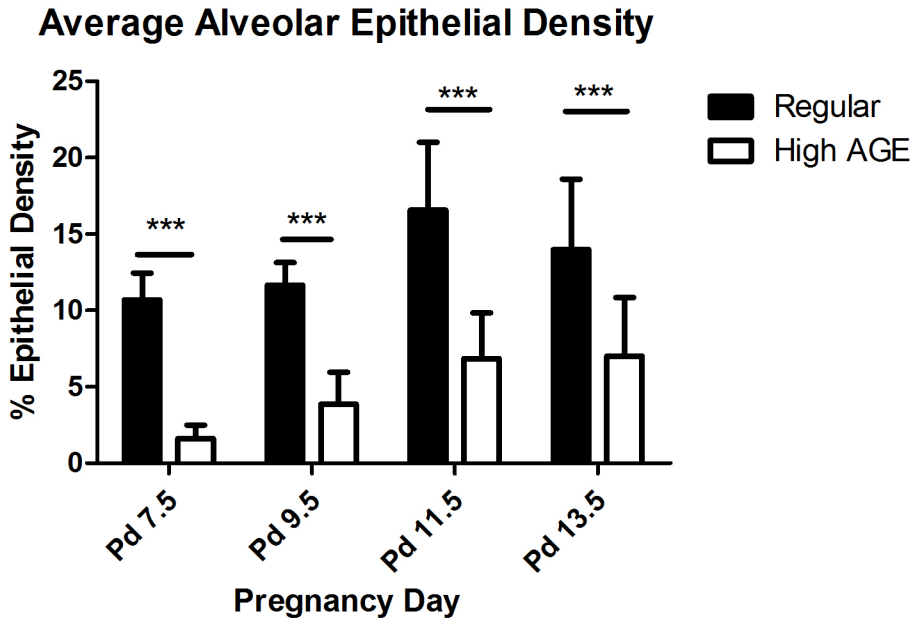


Figure 24. Average Alveolar Epithelial Density of Pregnant Mice. H&E stained inguinal mammary gland tissue sections were used to quantitatively assess the area of the mammary gland covered by epithelial alveolar structures compared to the total area of the gland in mice exposed to a high AGE diet (n=6) compared to a regular diet (n=4). ***p<0.0001

Discussion

Specific Aim 3 showed that as a result of consuming a high AGE diet before and during pregnancy, the alveoli in the mammary gland did not fully develop which may have affected offspring development. Offspring were exposed to a high AGE diet via the milk of the lactating mom, and weights of offspring were monitored from birth until 3 weeks of age. A significant reduction in the weight of pups was observed starting at 11 days of age and persisted through 21 days of age. To investigate the effects of a high AGE diet on the developing pregnant mammary gland, pregnant mice were fed a high AGE diet and taken at various time points defined by how long the mouse had been pregnant for (Pd 7.5, Pd 9.5, Pd 11.5, Pd 13.5). Mice fed a regular diet were used as controls. H&E stained inguinal mammary glands showed overall less epithelial density in mice fed a high AGE diet compared to mice fed a regular diet. Quantitation of these results confirmed the initial qualitative assessment showing that at all time points, the epithelial density was significantly lower in mice that consumed a high AGE diet.

There are many potential explanations for these observations. The high AGE diet may not be providing adequate nutrition for the nursing mom and therefore cannot produce milk rich in nutrients for her offspring. Signaling pathways that drive ductal and alveolar development and differentiation which could be dependent or independent of RAGE may be affected by the maternal high AGE diet. These may include, but are not limited to: STAT5 (signal transducer and activator of transcription), RANKL or RANK, Cyclin D1, p27, Id2 and C/EBPβ. [67]

Further work must be done to determine a potential cause of the decrease in pup weight and characterizing the molecular causes of decreased epithelial density.

At three weeks old, the offspring were weaned and fed a regular diet until 7 weeks of age. Since the offspring were nursed from a mom fed a high AGE diet and exposed to AGEs via the milk, several concepts can potentially explain the effects that we observed. First, we could apply the concept of metabolic memory to this study. As mentioned in Specific Aim 2, the concept of metabolic memory postulates that an early glycemic environment is remembered or imprinted leading to disease complications and disease progression. [40-43] We proposed that AGEs are a potential mechanism in the development of metabolic memory, as AGEs have regulatory effects on oxidative and immune stress pathways. Second, the observed effects seen in the mice from a high AGE diet fed mom may be due to multigenerational inheritance. As previously mentioned, multigenerational inheritance is seen in F1 and F2 generations. [65] This work showed that the effects of a high AGE diet were observed in the F1 generation. However, in order to fully assess whether the observations are due to multigenerational inheritance, this study would have to be continued in order to assess the F2 generation. Lastly, we postulate that the observed effects are due to the pups being exposed to a high AGE diet via the mom's milk. Although the offspring were not fed a high AGE diet directly, the AGE mediated effects that we observed in pubertal mice fed a high AGE diet could have similar effects on newborn mice, especially since most of mammary gland development occurs postnatally.

Overall, whole mount analysis of TEB number, ductal branching and ductal extension show pubertal mammary glands from a high AGE diet fed mom to be more characteristic of mammary glands from 7-week-old mice fed a high AGE diet. Although this data may not be significant, the data does trend towards those phenotypes. By repeating the experiment with continuation into the F2 generation with more mice in each group, we could gain a better understanding of the observed phenotypes and what could be causing them.

Conclusion and Significance of Study

Previous research supporting the effect of diet on mammary gland development and breast cancer risk has been mostly limited to high- and low- fat diets. Limited studies have examined the effects of a diet high in sugar and polyunsaturated fats on mammary gland development. While sugar and fat content values are readily accessible on food packaging labels, the content of AGEs in food is not. Therefore, exploring the effects of a high AGE diet on mammary gland development is crucial to educating others to make healthy, AGE-conscious food choices.

The current work in our lab focuses on a diet high in AGEs, which is associated with the Western diet. Consumption of a Western diet is associated with fostering a sedentary lifestyle, two risk factors that increase breast cancer risk, particularly in susceptible populations such as pubertal and pregnant groups. Puberty and pregnancy are developmental times hallmarked by tightly regulated proliferative processes. Disruption of these processes could lead to an increased risk of breast cancer. Previous work in our lab as well as work discussed in this thesis have established a novel link between increased AGE consumption and disruption of mammary gland development. We observed hyperproliferation in the mammary gland as a result of a high AGE diet despite introducing an intervention diet, dysregulation of development in mammary glands in pregnant mice, and effects on pup weight due to a high AGE diet.

Raising awareness of the effects of a high AGE diet on mammary gland development is crucial particularly in susceptible populations. Individuals are often unaware of the potential long-lasting effects that food can have on their development and future risk of disease. Educating prepubescent and young populations of females on these effects could mitigate the chance of increasing the risk of breast cancer later in life. Leading an active lifestyle and making conscious, healthy food choices contribute to this cause.

Future Directions

Previous and current work in our lab has shown that chronic consumption of a pubertal high AGE diet leads to dysregulation of mammary gland development, including the formation of hyperproliferative ducts, resulting in a permanent change in mammary gland function via metabolic imprinting. [57] These disruptions in the mammary gland microenvironment allow for a greater risk of breast cancer susceptibility, especially during pubertal and pregnancy development. However, the mechanism of AGE-RAGE mediated actions and their implications on cancer susceptibility and development has yet to be elucidated and must be further explored (Figure 25).

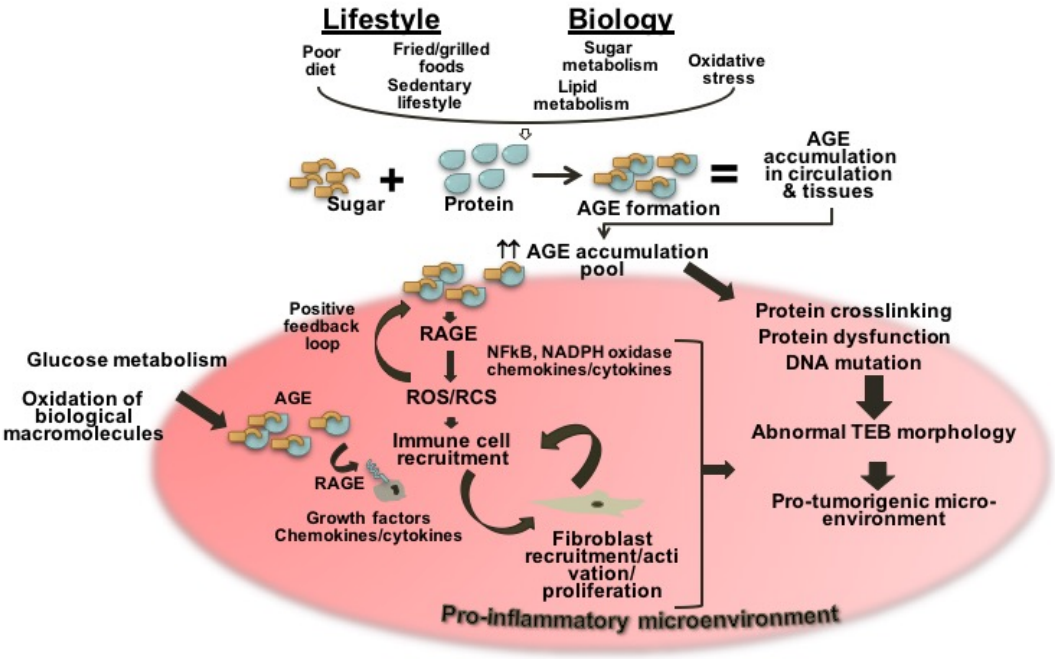


Figure 25. Working Hypothesis of AGE Mediated Effects. Overall hypothesis of AGE-RAGE signaling and its physiological effects contributing to an increased risk of cancer susceptibility.

Test the hypothesis that a high AGE diet induces tumor formation and growth in vivo

Breast cancer risk can be partly attributed to inheritance of breast cancer susceptibility genes. Environmental influences, such as carcinogen exposure, also contribute to breast cancer risk. The use of DMBA (7,12-dimethylenz[a]anthracene) has been associated with mammary tumorigenesis by epidemiological and laboratory studies. [68, 69] Administration of DMBA via oral gavage can produce mammary tumors in rodents. Initial steps in tumorigenesis involve DMBA metabolism into a mutagenic epoxide intermediate that readily forms DNA adducts. These adducts are associated with DNA mutations and the malignant transformation that is proposed to be involved in DMBA-mediated carcinogenesis. A study conducted with FVB mice given 6 weekly 1mg doses of DMBA resulted in mammary tumors in 75% of the mice. Oncogenes in human breast cancer, cyclin D1 and c-myc, were found to be increased in many of the DMBA-induced mammary tumors. [69]

Because a high AGE diet alone has produced hyperproliferative ductal structures, use of DMBA in conjunction with a high AGE diet will test induction of tumor formation and growth. Furthermore, this model of DMBA-induced tumors will allow us to examine the effects of a high AGE diet on circulating and tissue AGE and tissue RAGE levels. We anticipate that mice fed a high AGE diet with the addition of DMBA will have decreased tumor latency compared to the mice fed a regular diet with weekly DMBA treatments. This contention is supported through

previous studies examining a high fat diet effect on tumor latency with DMBA with tumor occurrence beginning around 15 weeks of age and all mice having tumors at 34 weeks of age. [36-38, 69, 70] Tumor formation may occur due to off target effects of the DMBA and not due to AGE-RAGE signaling. To address this caveat, it would be beneficial to use knockout RAGE mice in this experiment.

Determine whether RAGE is necessary in AGE-mediated mammary gland dysregulation

To further explore the mechanism of AGE-RAGE mediated effects observed in the mammary gland, it would be necessary to perform all experiments discussed in this thesis and previous thesis with FVB/n RAGE -/- mice. [57] The time of introducing the high AGE diet and regular diet (3 weeks of age) and the time points of collecting the mice (7 weeks and 12 weeks old) for mammary gland extraction would remain the same. All data points would be compared to data previously collected from FVB/n RAGE +/+ mice. If the presence of RAGE is necessary for the dysregulation of mammary gland development due to a high AGE diet, we expect to see little-to-no change in the morphology of mammary glands collected from RAGE -/- mice. However, if comparable results are noted in mammary glands from RAGE -/- mice as was observed in RAGE +/+ mice, another signaling pathway must be at least partially responsible for the AGE-mediated effects in the mammary glands. As previously mentioned, independent of RAGE, AGEs can damage protein structure and alter extracellular matrix metabolism.

Therefore, we potentially would expect to see some deleterious effects that AGEs have on mammary gland development.

Exploration of metabolic memory and diet intervention

To further investigate the time necessary to observe the effects of a high AGE diet on mammary gland development, it would be crucial to feed the high AGE diet to pubertal mice for shorter durations of time. We observed that consumption of a high AGE diet for 5 weeks resulted in mammary gland dysregulation, particularly hyperproliferation in ductal structures. By feeding the mice a high AGE diet for 1, 2, 3 and 4 weeks, we could narrow down the time needed for the high AGE diet to “imprint” the mammary gland. The mammary glands collected from these mice will be used in the same analyses as used in this project such as ductal extension, ductal branching, TEB number and size, as well as morphological changes with H&E staining. Furthermore, Ki-67 IHC can be used to compare the newly generated time points to previously established time points generated by work completed towards this thesis. Overall, data collected from this experiment will strengthen our hypothesis that consumption of a high AGE diet results in a permanent change in mammary gland function via metabolic imprinting. Previous studies that observed the effects of diet intervention on mammary gland development measured metabolic parameters, such as glucose and insulin levels, as well as body weight. [36, 37] By assessing potential hyperglycemic and hyperinsulinemic conditions over time in mice fed an intervention diet, our findings may further support the theory of metabolic memory that early glycemic conditions

are imprinted leading to disease complications later on. Measuring body weight over time will complement the metabolic parameter measurements, thus allowing us to have a more complete assessment of metabolic memory in our diet and mouse model.

Localization of immune cell populations in high AGE diet fed mice

The AGE-RAGE signaling pathway includes an inflammatory response through the NF-κB pathway. To determine the mediators of this response, flow cytometry methodology can be used to sort immune cell populations and examine the localization of RAGE within the populations. This can be performed by isolating cells from extracted mammary glands in female FVB/n pubertal (7-week-old) and adult (12-week-old) mice fed a high AGE diet compared to a regular diet. Detection of fibroblasts will be completed by labeling cells with vimentin and sorting the cells. Furthermore, M1 and M2 polarized macrophages can be differentiated by sorting co-labeled cells with CD86, a marker of pro-inflammatory (M1) macrophages, and CD206, a marker of tumorigenic (M2) macrophages. Additionally, by labelling the cells with a marker of RAGE, localization of RAGE among macrophage cell populations can be deduced thus narrowing down potential mechanisms of AGE-RAGE signaling amongst fibroblast and macrophage populations. We expect to see an increased fibroblast cell population in mice fed a high AGE diet compared to a regular diet. Additionally, we expect to see an increased macrophage presence in the high AGE diet cell population. More specifically, we expect more M1 polarized macrophages as a result of a high AGE diet. The presence of M2

macrophages may be less populous since we do not expect the formation of tumors at 12 weeks of age in mice fed a high AGE diet.

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