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# **Biological Implications of Glycation in Breast Cancer**

by

**Katherine Rose Walter**

A thesis submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Masters of Biomedical Sciences in the College of Graduate Studies.

**Department of Pathology and Laboratory Medicine, 2015**

Approved by:

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(Chairman, David P. Turner)

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(Steven A. Rosenzweig)

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## **Abstract**

Breast cancer is a heterogeneous disease that affects close to two million women each year. Of these women, seventy percent express estrogen receptor alpha, a member of the nuclear receptor subfamily that is activated in response to estrogen. Our research has shown an alternate mechanism by which estrogen receptor alpha is activated and subsequently elicits its cancer promoting effects.

The process of glycation involves the non-enzymatic addition of sugar moieties to biological macromolecules which produce reactive metabolites known as advanced glycation end products (AGEs). These metabolites have been shown to be responsible for many of the complications associated with diabetes because of their ability to interact with the Receptor for Advanced Glycation End Products (RAGE) and produce a chronic inflammatory phenotype. Similarly, this same effect has been shown in several different cancers including prostate, melanoma, and colorectal.

Like other cancers, we observed greater levels of AGEs and RAGE within breast cancer tumor and serum samples and showed a correlation between tumor progression and intratumoral AGE concentration. Utilizing two ER positive breast cancer cell lines, T47D and MCF7, we have also identified a role for AGEs in the phosphorylation of the estrogen receptor at two different residues within the ligand independent activation domain: serine 118 and serine 167. By utilizing exogenous AGE treatment and inhibition with molecular inhibitors, we showed that, following exposure to AGEs, a signaling cascade occurs through Akt and ERK to phosphorylate

these two residues. Additionally, this signaling pathway produced a more proliferative phenotype in our cell lines. We were able to verify that AGE treatment was inducing this increase in proliferation through interaction with RAGE by using shRNA technology.

Activation of the estrogen receptor at these particular residues has been shown to be indicative of tamoxifen resistance. We identified a role for AGEs in tamoxifen resistance by performing a cell viability assay and found that AGE treated cells were indeed less sensitive to drug treatment. Elucidating a role for AGE-RAGE signaling in breast cancer creates potential for improved therapies and preventative interventions for patients with the disease.

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## **List of Abbreviations**

- AGEs—Advanced glycation end products
- Akt—Protein kinase B
- cDNA—Complimentary DNA
- DBD—DNA binding domain
- EGFR—Epidermal growth factor receptor
- ER—Estrogen receptor
- ERE—Estrogen response element
- ERK—Extracellular signal regulated kinase
- GLO--Glyoxalase
- HbA1C—Glycated hemoglobin
- HER2—Human epidermal growth factor receptor 2
- HMGB1—High mobility group box protein 1
- IDC—Invasive ductal carcinoma
- IGF-1—Insulin-like growth factor
- IGF-1R—Insulin-like growth factor receptor
- IHC—Immunohistochemistry
- IL-6—Interleukin 6
- JNK—c-jun N-terminal kinase
- LBD—ligand binding domain
- MAPK-mitogen activated protein kinase
- MOI—multiplicity of infection

mRNA—messenger RNA

NADPH—Nicotinamide adenine dinucleotide phosphate

NF- $\kappa$ B—nuclear factor kappa-light-chain-enhancer of activated B cells

PI3K—Phosphoinositide 3-kinase

PR—Progesterone receptor

RAGE—Receptor for advanced glycation end products

RT-PCR—Real time polymerase chain reaction

SERD—selective estrogen receptor downregulator

SERM—selective estrogen receptor modulator

SRB—Sulforhodamine B

SRC-3—Nuclear receptor coactivator 3

TMA—Tissue microarray

TNF $\alpha$ —Tumor necrosis factor alpha

UPL—Universal probe library

VEGF—Vascular endothelial growth factor

## **Acknowledgements**

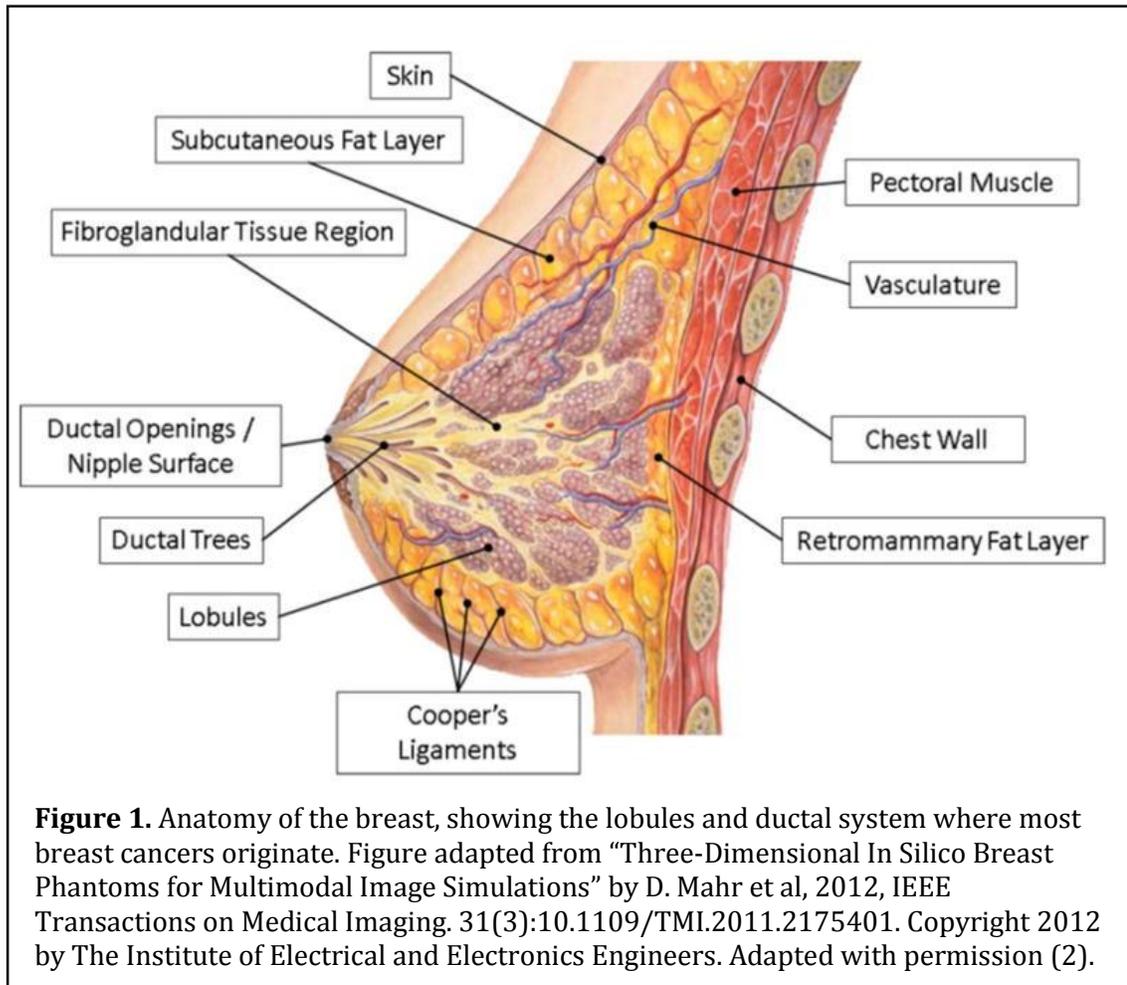
My passion for cancer research would have never been discovered if it weren't for the unfailing guidance of my mentor, Dr. David Turner. Thank you for welcoming me into your lab despite my lack of experience and forcing me to think independently, like a true scientist. Because of you, I feel as though I am more than ready for what lies ahead in my career and truly cannot thank you enough.

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## Introduction and Background

**Breast cancer:** Breast cancer is a carcinoma (or sarcoma, in extremely rare cases) that originates in the breast; which is the structure responsible for milk production and transport following childbirth. The breast is comprised of milk producing lobules that are grouped together into 15-20 grape-like clusters known as lobes. During lactation, milk is transported from the lobes and out the nipple through a network of thin, tube-shaped structures called ducts (Figure 1). Fibrous tissue as well as fat surround the lobes and ducts to make up the bulk of the breast.



Additionally, the breast contains blood and lymphatic vessels which play a key role in tumor metastasis in breast cancer patients. Around 70% of all breast cancers begin within the ducts, whereas only around 10-15% begin within the lobules (American Cancer Society). Mammary cells are undifferentiated until lactation following childbirth and regress to a less differentiated state after menopause. This lack of differentiation causes the breast to be more susceptible to cancer promoting stimuli and tumorigenic transformation (7)

There exist several different methods by which cancerous tissue is detected within the breast. Preventative screenings such as basic clinical breast exams and mammography are among the most widely used(8). These tests serve to examine and detect any abnormalities within the breast tissue. If a suspicious mass is found, a biopsy is then performed to determine malignancy status. Cancerous tumors are then further characterized using methods such as immunohistochemistry in order to classify the cancer into a variety of subtypes which will then warrant a specific treatment regimen(8).

A key issue in treating breast cancer is its vast heterogeneity. A particular tumor will respond to treatment differently than others, emphasizing the need for more personalized treatment. Relatively recently, genomic analysis has allowed for tumors to be classified into molecular subtypes based on the expression of a variety of genes (BRCA1/2, ERBB2, TP53, etc.). Many unique subtypes have been identified such as the luminal cancers (A and B), HER2 Enriched, and Basal-like. While our knowledge base continues to expand, genetic testing is expensive and not readily

available in the clinical setting(9). A key portion of the current diagnostic process in breast cancer is to determine a tumor's receptor status using immunohistochemistry (IHC). IHC is relatively inexpensive and aims to characterize the tumor based on the expression of three different receptors: estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2). Knowing a tumor's receptor status is crucial in the formation of a patient's treatment plan because receptor expression allows for targeted therapies that disrupt tumorigenic signaling, resulting in inhibition of cancer growth and survival. By characterizing these tumors, treatment is more personalized for the patient's particular cancer, leading to an improved prognosis.

**Breast Cancer Incidence/Risk Factors:** Breast cancer is a disease that affects post-pubescent women of all ages. Annually affecting 1.7 million worldwide, the incidence of breast cancer has steadily increased within the past decade and is only expected to continue to rise (9). In the U.S. alone, over 200,000 new cases are diagnosed each year with around 40,000 deaths attributed to the disease (American Cancer Society). Because of its breadth, breast cancer does indeed represent a substantial health burden and certain factors have been shown to modify a person's risk of incidence, reoccurrence, and mortality of the disease.

**Non-Modifiable Risk Factors:** The most influential risk factor associated with breast cancer development, other than gender, is age. Fewer than 10% of all invasive breast cancers are diagnosed in women under 40, with the

greatest risk present in post-menopausal women, peaking around age 70 (8). In addition to age, heritable genetic mutations have been shown to cause around 5-10% of all breast cancers (10). Research looking into the effects of gene mutations such as BRCA1, BRCA2, and TP53 (among many others) has allowed for preventative intervention and patient specific care. Another studied risk factor is race and ethnicity. Post-menopausal Caucasian women have the highest risk of breast cancer development. While the overall incidence is lower in the African American population, women of this race have an increased risk of developing a more aggressive disease at a younger age, resulting in a higher mortality rate (11).

**Lifestyle Risk Factors:** Certain factors that are modifiable also influence the risk of disease development. There is evidence that suggests weight gain and obesity, particularly following menopause, increases the risk of incidence, reoccurrence, and mortality of breast cancer (12-14). Relatedly, diet has been associated with increased incidence as well, which will be discussed in further detail later. Other risk factors are attributed to either lack of differentiation (i.e. nulliparous, non-breastfeeding, etc.) which leaves the mammary tissue more susceptible to transformation, or to exogenous hormones found in oral contraceptives or hormone replacement therapy (15, 16).

**Estrogen Receptor Positive Breast Cancer:** As stated previously, subtyping of breast cancers is imperative in developing the most effective treatment plan for patients. Genomic testing is not widely used in the clinic so IHC classification is a staple in the diagnostic process. Of all invasive breast cancers, around 70% express the estrogen receptor (17). These ER+ breast cancers are included in the Luminal molecular subtypes. Around 50-60% of breast cancers are Luminal A and have the best prognosis of all the subtypes. These tumors are generally ER+, PR+/-, and HER2- and have a low proliferation score, measured by Ki-67 staining. Luminal B tumors are generally ER+, PR+/- as well, but can also express the HER2 growth factor receptor. Additionally, these tumors usually have a high proliferation score, resulting in a worsened prognosis. While the correlation between pathological and molecular subtypes is not infallible, the overlap between the two diagnostic procedures allows for better insight into the characteristics of a patient's specific tumor. ER+ breast cancers tend to have an improved prognosis, not only because these tumors generally present with lower histological grades and little-to-no lymph node involvement at diagnosis, but also because expression of the estrogen receptor makes a patient a candidate for targeted therapies (9, 18). In ER+ positive breast cancer, there are two main categories of treatment: anti-estrogens and aromatase inhibitors. Anti-estrogens, which block the functionality of the receptor, can be further subdivided into selective estrogen receptor downregulators (SERD) and selective estrogen receptor modulators (SERM). SERDs act as full antagonists toward the receptor by causing ER degradation, whereas SERMs can function in an

antagonistic or agonistic manner, depending upon the targeted tissue location.

Aromatase inhibitors, which are most often prescribed secondary to anti-estrogens in post-menopausal women, block the functionality of the enzyme aromatase which is responsible for converting testosterone to estrogen. Blocking the production of the ligand therefore hinders ER mediated signaling. These aromatase inhibitors, while effective, do exhibit greater toxicity when compared to anti-estrogens, especially in premenopausal women (19).

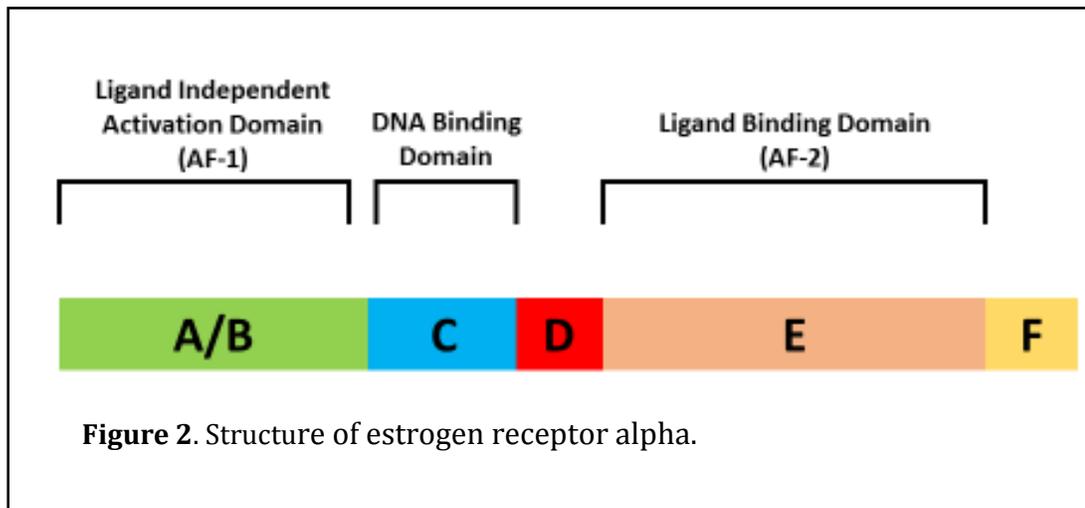
**Estrogen Receptor Alpha:** The estrogen receptors are a group of nuclear receptors that are included in the subfamily of ligand activated transcription factors (20).

Currently, two receptors are known: ER $\alpha$  and ER $\beta$ . These receptors are the product of unique genes and differ in their functionality and structure. The ligand and DNA binding domains of these receptors have been evolutionarily conserved, resulting in a great amount of homology between the two receptors in these regions. The ligand independent region located at the N-terminus of each receptor, however, varies markedly (21, 22). Despite it being the subject of research for over a decade, the exact functionality of ER $\beta$  remains unknown (23). Because of this, ER $\alpha$  (Figure 2) is the isoform detected during immunohistochemical analysis and subsequently used for diagnostic and therapeutic purposes in breast cancer.

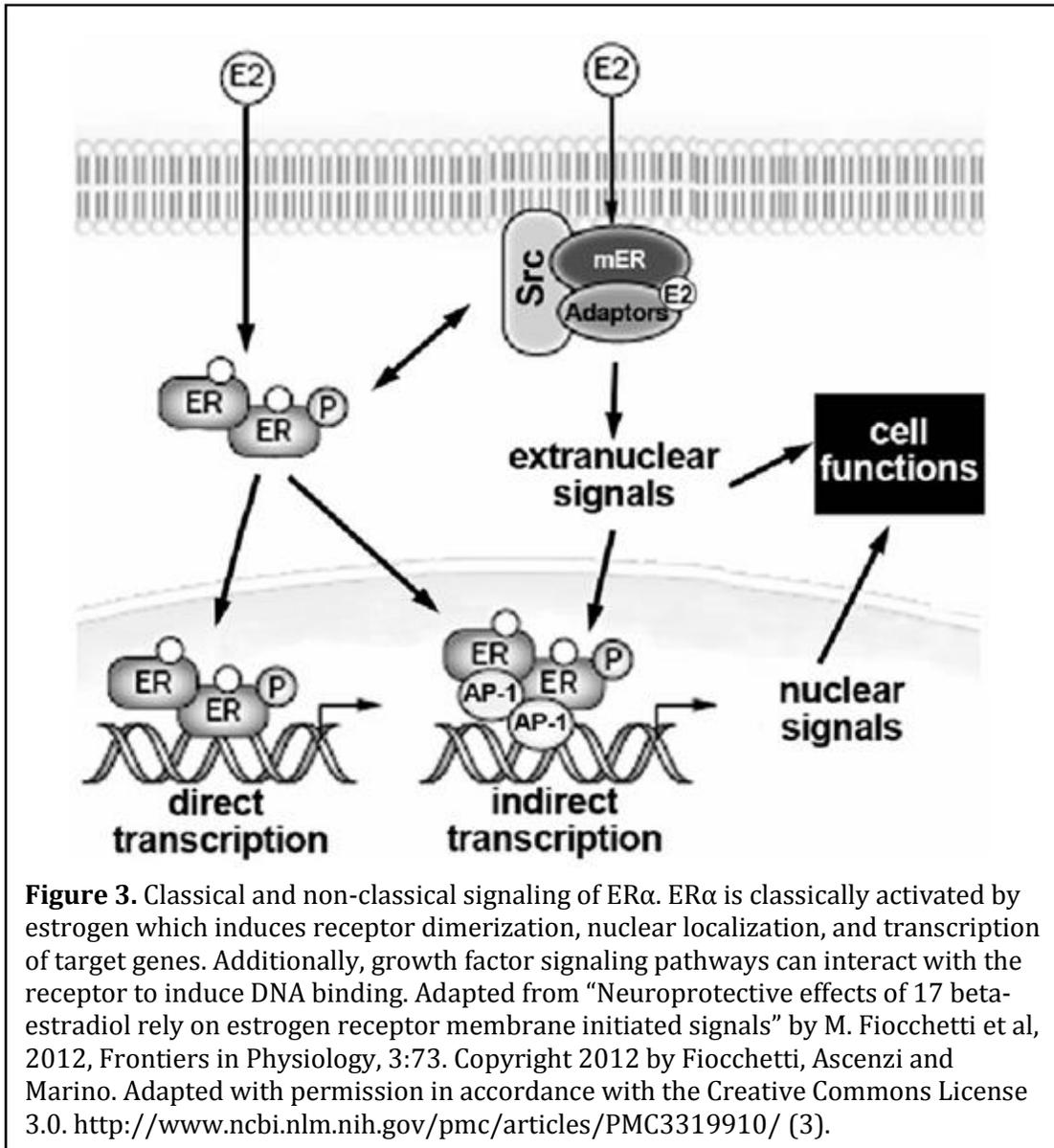
Structurally, ER $\alpha$  consists of three main functional domains (Figure 2).

Because of its transcription factor functionality, ER $\alpha$  contains a DNA binding domain (DBD). The DBD is responsible for binding to the estrogen response element (ERE)

within the promoter sequence on the DNA of its target genes to enhance transcription. ER $\alpha$  also contains a ligand binding domain (LBD) where estrogens can interact and enhance DNA binding (21). This ligand dependent activation of ER $\alpha$  has been widely studied. Relatively recently, however, activation of ER $\alpha$  has been shown to occur independently of ligand via second messengers (20). These second messengers interact with the N-terminus of the receptor within the ligand independent domain to induce ER $\alpha$  DNA binding.



## Estrogen Receptor Signaling:



**Genomic (classical) Action of ER $\alpha$ :** Because estrogen is a steroid hormone, it is able to diffuse freely through the plasma membrane and interact with cytoplasmic estrogen receptors (Figure 3). Once estrogen binds, the receptor undergoes several conformational changes that allow it to dissociate from chaperone proteins and

dimerize(19). Following translocation to the nucleus, the dimerized receptor can act in one of two ways. The receptor can bind directly to the estrogen response element (ERE) located on the promoter region of its target gene and recruit regulatory proteins such as AIB1 or SRC3, or it can bind to transcription factor complexes such as AP-1 or NF- $\kappa$ B, thereby enabling it to enhance transcription of genes without an ERE(24). Target genes of ER $\alpha$  include several proliferative, pro-survival, and angiogenic factors such as c-myc, VEGF, IGF-1R, among several others(19). Estrogen mediated signaling is crucially important in the progression and survival of tumors in ER+ breast cancer.

**Estrogen Independent Activation:** ER $\alpha$  contains two transcription activation functional domains: AF1 and AF2. Following estrogen binding to the ligand binding domain, AF2 (estrogen dependent) synergizes with AF1, allowing the receptor to change conformation and form a hydrophobic groove to promote coactivator binding (17). While the AF2 domain is only able to enhance transcription in the presence of estrogen, the AF1 domain can be phosphorylated via second messenger pathways which can then induce an agonistic or antagonistic conformational change. Phosphorylation of the receptor by either estrogen binding or through secondary kinases then induces dimerization and subsequent transcriptional activity as described in the previous section. Phosphorylation is necessary in order for ER $\alpha$  to influence transcription, regardless of whether or not estrogen is present (19). Because of the variance in mechanisms behind the different

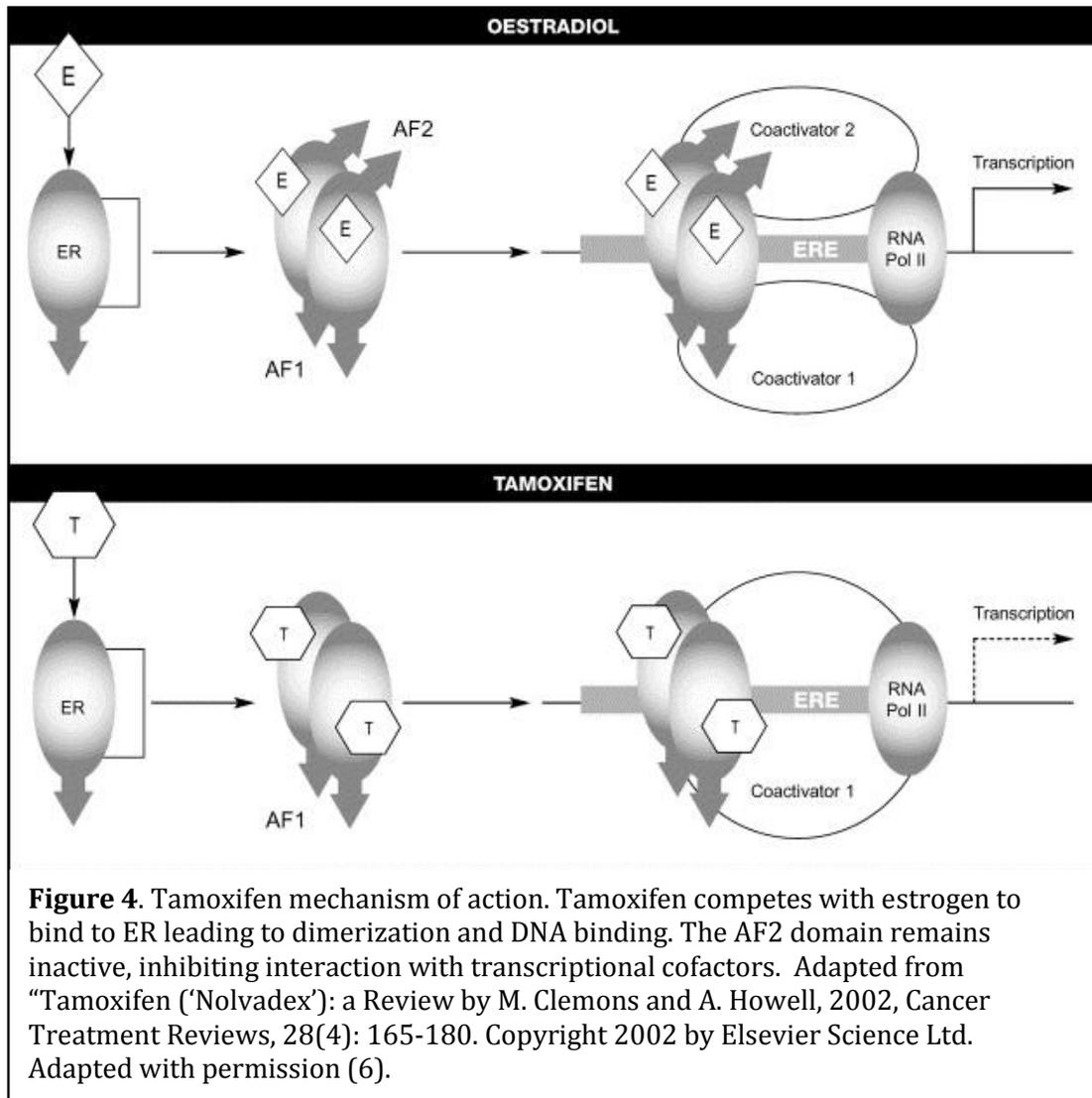
phosphorylation sites, this particular post-translational modification has been studied to determine if there is an influence on clinical outcomes in patients (25).

**Non-Genomic Action of ER $\alpha$ :** The nuclear action of ER $\alpha$  has been well established. Relatively recently, however, it has been discovered that ER $\alpha$  influences several signaling pathways throughout the cell. Membrane associated ER $\alpha$  has been shown to interact and directly phosphorylate membrane bound receptor tyrosine kinases such as the epidermal growth factor receptor (EGFR) and the insulin-like growth factor receptor (IGF-1R) following the binding of estrogen(26). In addition, ER $\alpha$  is able to directly interact with the p85 subunit of phosphoinositide-3 kinase (PI3K) leading to the activation of the pro-survival Protein kinase B (Akt)(21). While the mechanisms by which these interactions occur is still controversial, it is evident that there exists a bi-directional cross-talk between ER $\alpha$  and growth factor signaling leading to alterations in proliferation and cell survival, especially in breast cancer.

**Tamoxifen:** As stated previously, treatment for ER+ breast cancer falls under two categories: anti-estrogens and aromatase inhibitors. Anti-estrogens are most often the first line of defense following resection of the primary tumor. Among the most effective anti-estrogens given to patients with ER+ breast cancer is tamoxifen.

Tamoxifen is a selective estrogen receptor modulator that, when metabolized by the liver, is converted to 4-hydroxy tamoxifen and N-desmethyl 4-hydroxy tamoxifen which have up to 100 times greater affinity for the estrogen receptor than tamoxifen

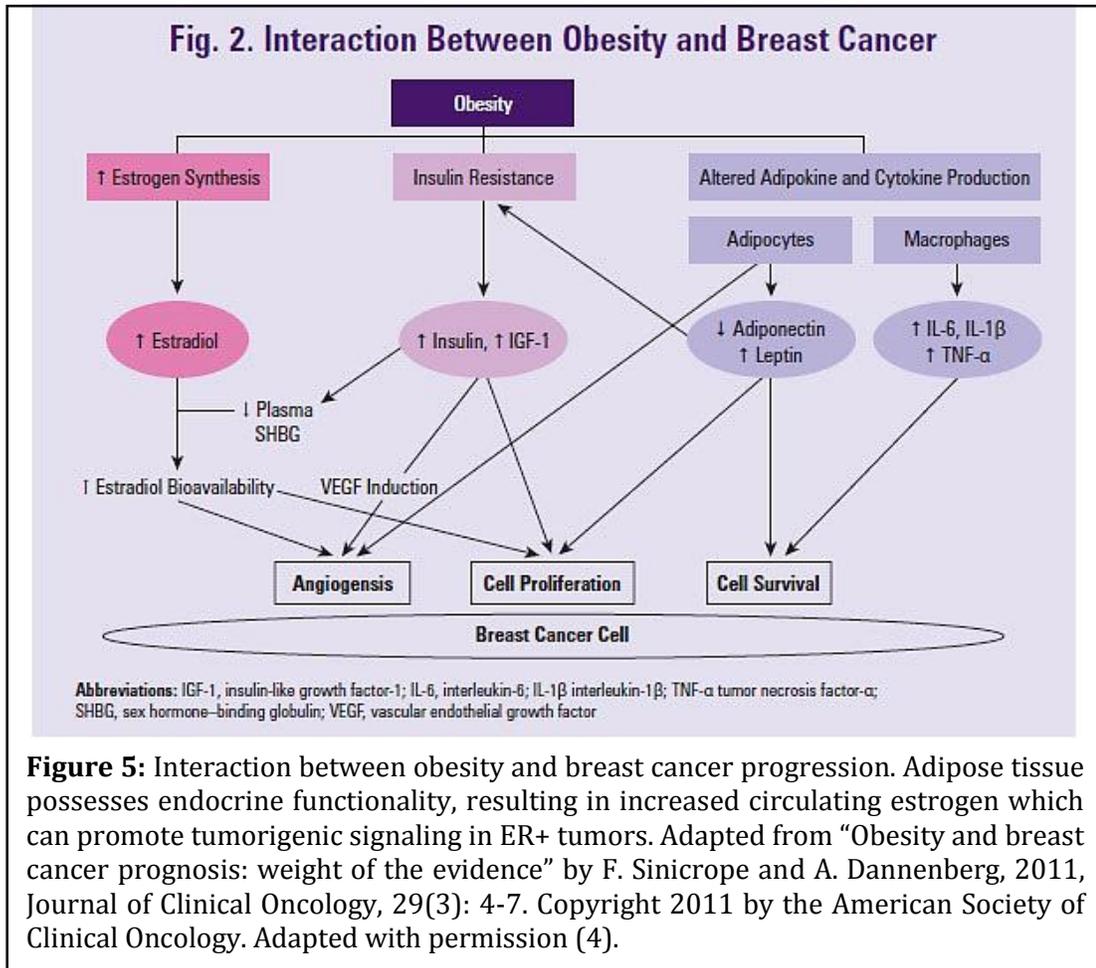
itself (19). These reactive species then compete with estrogens to bind to the receptor. When tamoxifen binds to the receptor, dimerization and DNA binding still occur, but the AF2 domain remains inactive and the conformational change required for the binding of cofactors does not occur (Figure 4). Transcription of estrogen receptor target genes is inhibited, resulting in decreased estrogen-mediated tumor growth. Because tamoxifen is considered a SERM, it possesses a dual functionality(17). It can function as an agonist in the endometrium and bone while acting as an antagonist in the mammary tissue of the breast. The efficacy of tamoxifen in the treatment of ER+ breast cancer has been well established, and because it has relatively low toxicity and is able to be given to both pre- and post-menopausal women, it has become the drug of choice for most patients(6).



**Tamoxifen resistance:** Despite encouraging results in many patients, around 30% of women taking tamoxifen will not receive benefit from treatment despite still expressing the estrogen receptor and are considered de novo resistant. In addition to de novo resistance, almost all patients with metastatic disease will eventually acquire a resistance to tamoxifen, usually relapsing within 1-2 years (25-27). These patients are then given second- and third-line treatments in the form of SERDs

and/or aromatase inhibitors, but even then, these tumors will most often still acquire a resistance to these drugs (28). Because of the prevalence of ER+ breast cancer, it is imperative to identify the mechanism behind the development of tamoxifen resistance in order to better improve first-line therapies for these patients. Identification of this mechanism is widely studied but very controversial and currently there are four theories as to how this resistance occurs: 1) resistance to specific anti-estrogens (i.e. inability to convert tamoxifen to reactive metabolites), 2) loss of ER $\alpha$  expression, 3) altered ER $\alpha$  signaling, and 4) ligand independent activation of ER $\alpha$  (28, 29). Phosphorylation within the AF1 domain of ER $\alpha$  independent of estrogen is thought to contribute to tamoxifen resistance because transcription is still able to occur despite the absence of ligand. Among the most widely studied phosphorylation sites within the ligand independent activation domain are serine 167 and serine 118. These two residues are emerging as potential predictive markers for patient response to tamoxifen because of their influence on the structure and functionality of ER $\alpha$ . Ser118 has been shown to be phosphorylated in response to the activation of several kinase pathways including the mitogen activated protein kinases (MAPK) ERK 1/2 and p38 (30). Functionally, phosphorylation of ER $\alpha$  at ser118 by MAPK serves to not only enhance binding of cofactor SRC-3, but also causes the receptor to become hypersensitive to estradiol, therefore lowering its affinity for tamoxifen(17). Furthermore, one study utilized a tamoxifen resistant breast cancer cell line and found that MAPK activity was significantly increased (31). This activation of MAPK (and subsequent ER $\alpha$

phosphorylation) can either be estrogen dependent or estrogen independent (25). Ser167 is also phosphorylated by a variety of kinases, most notably by Protein Kinase B (Akt) within the PI3K signaling pathway (30). Like ser118, phosphorylation at this residue can occur with or without the estrogen ligand making it a potential contributor to tamoxifen resistance. While the specific mechanism by which ser167 influences tamoxifen resistance is currently understudied, investigators have shown that phosphorylation of this residue by Akt results in the increased recruitment of cofactors to ER $\alpha$  resulting in enhanced transcription (30, 32). The MAPK and PI3K signaling cascades are frequently upregulated in several cancers because of their proliferative and pro-survival effects. This could potentially be leading to an upregulation of ER $\alpha$  mediated signaling, independent of estrogen. In an effort to combat tamoxifen resistance, inhibitors of these pathways in combination with endocrine therapies have been tested and some have proven to be relatively effective (33). The sheer complexity of ER $\alpha$  signaling, however, allows the tumor the ability to adapt to even the most effective drugs (34). Because of this, understanding the complete mechanism by which tumors become resistant is important in identifying an appropriate and effective drug target.



**Breast Cancer, Obesity, and Diet:** As stated previously, poor diet and obesity are modifiable risk factors contributing to the incidence, reoccurrence, and mortality of breast cancer in women. Several epidemiological studies have shown an association between increased adiposity and poorer prognoses, increased incidence, and a greater mortality rate, especially among post-menopausal women (15). This is potentially caused by the dual functionality of adipose tissue in both metabolism and hormone production (Figure 5). Whereas pre-menopausal women produce

estrogen primarily in the ovaries, postmenopausal women are restricted to peripheral sources of estrogen production. Adipose tissue contains aromatase, which is a complex of enzymes that is responsible for converting androgens produced in the adrenal gland into estrogen(35). Increased adipose tissue leads to greater amounts of circulating estrogen, which is a major fuel for ER+ breast cancers. In addition to production of estrogen, obesity can also cause insulin resistance leading to glucose intolerance and increased production and secretion of pro-inflammatory markers such as IL-6 and TNF $\alpha$  (36).

It is no secret that one of the primary causes of obesity is a poor diet. Because of this, several types of diets (i.e. high fat, high carbohydrate, Mediterranean, etc.) have been studied to determine what diet in particular has the greatest effect on breast cancer development and prognosis. Of particular interest for this proposal is a study utilizing the Italian portion of the European Prospective Investigation into Cancer and nutrition (EPIC) cohort that looked at the effects of diets with a high glycemic load on breast cancer incidence in over 26,000 women over eleven years. After controlling for several external factors such as menopausal status and total energy intake, the investigators found that women consuming diets high in carbohydrates, measured by glycemic load, had a significantly increased incidence of breast cancer development(37). Glycemic load measures both the quality and quantity of a particular food (unlike glycemic index which just measures quality) making it a more accurate estimation of post-prandial circulating glucose and insulin demand. This is especially relevant for cancer because of insulin's ability to

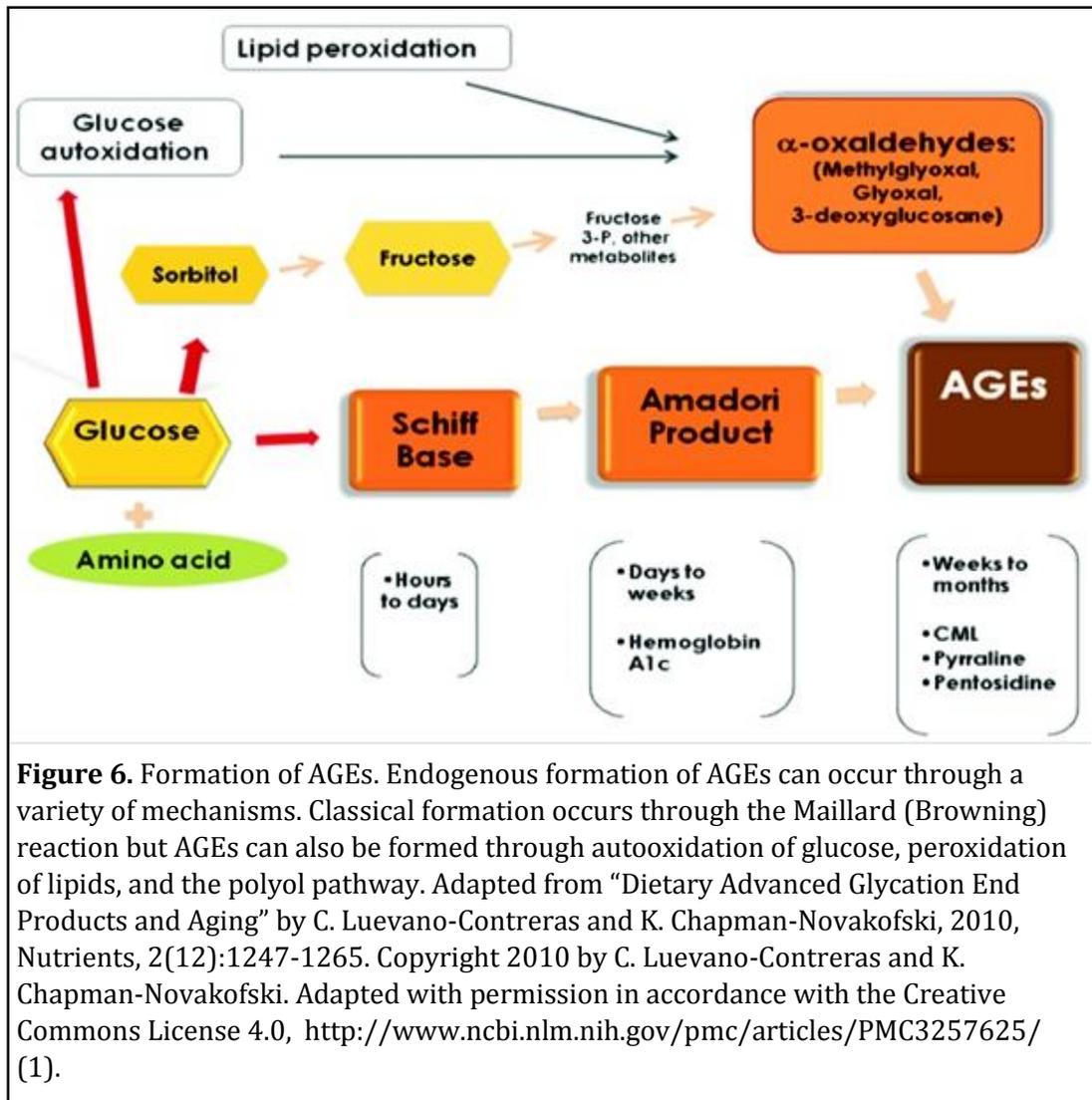
interact with transmembrane receptors and increase proliferation. Furthermore, as stated previously, activated receptor tyrosine kinases (including IGF1Rs) can result in estrogen-independent activation of ER $\alpha$  (15). In addition to the EPIC cohort study, other epidemiological studies have also looked at glucose metabolism in breast cancer incidence, including one study investigating fasting blood glucose in a cohort of over 10,000 women. After a five year period, 144 breast cancer cases were matched with 4 control participants who did not develop breast cancer over the examination period. The investigators found that in post-menopausal women, glucose, insulin, and IGF-1 were all associated with increased breast cancer incidence, leading the investigators to conclude that altered glucose metabolism is a risk factor for breast cancer development (38).

**Advanced Glycation End Products:** Persistently high levels of glucose within the blood, often resulting from a carbohydrate-rich diet, not only alters insulin mediated signaling, but also leads to the increased formation of reactive metabolites known as advanced glycation end products (AGEs)(39).

**AGE Formation:** This heterogeneous, complex group of molecules is formed during normal metabolism, classically through what's known as the Maillard, or browning, reaction (40) (Figure 6). This AGE-forming reaction occurs in a series of three phases. The first step is glycation, which is the non-enzymatic addition of reducing sugars to the amino groups on biological

macromolecules such as proteins, lipids, or nucleic acids to form Schiff bases. This first step is reversible and its initiation is heavily dependent upon sugar concentration. The second step of the Maillard reaction involves the Schiff base undergoing a series of rearrangements over the course of a few days to form Amadori products, or early glycation products. Glycated hemoglobin, or HbA1C, is an example of an Amadori product that is a widely used measurement of long-term blood glucose concentration in diabetic patients. These products are more stable than Schiff bases, but the total reaction is still reversible at this point. Accumulation of Amadori products, however, causes an even further series of rearrangements that can take several weeks and irreversibly forms the reactive, cross-linking proteins known as AGEs (1, 41).

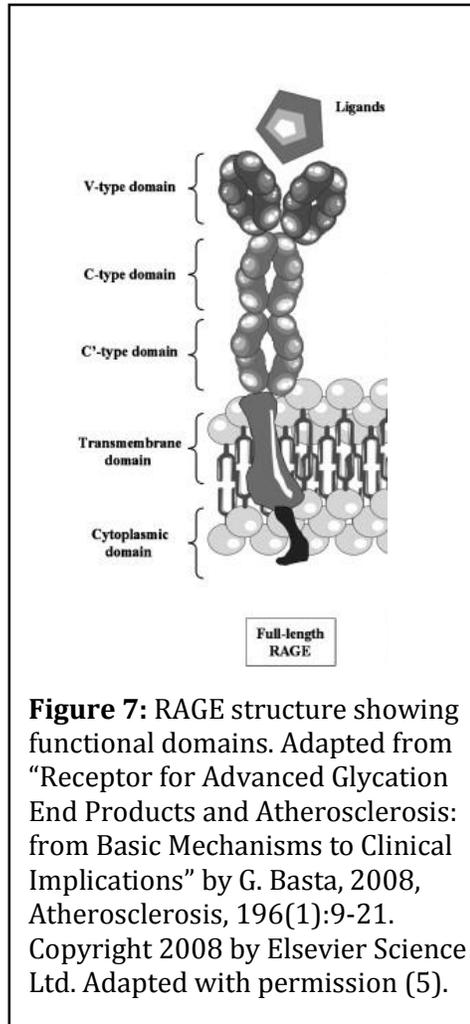
While the Maillard reaction is the classical pathway for AGE formation within the body, there exist other mechanisms capable of producing these reactive metabolites.  $\alpha$ -oxoaldehydes (glyoxal, methylglyoxal, etc.) formed from the autooxidation of monosaccharides or the peroxidation of lipids can interact with monoacids to form AGEs in high oxidative stress environments (1). Additionally, these  $\alpha$ -oxoaldehydes can be formed through the polyol pathway in which glucose is converted to fructose intermediates (Figure 6). The variety of precursors and reactions responsible for the formation of AGEs are what give this group of reactive metabolites their heterogeneity in both structure and pathogenic functionality(42).



One of the key internal mechanisms inhibiting AGE formation is the glyoxalase system (GLO). GLO is responsible for detoxifying methylglyoxal, a reactive carbonyl and AGE precursor (43). This is accomplished by catalyzing the conversion of methylglyoxal to lactate and is a process that has been shown to be very active in cancer because of the high rate of glycolysis and glucose consumption that is characteristic of the tumor microenvironment (44).

**Exogenous Sources of AGEs:** In addition to normal metabolism, AGEs are naturally present in uncooked, animal derived foods. Furthermore, particular food preparations can increase the AGE content of a food by driving AGE forming reactions. Cooking methods involving dry heat such as grilling, broiling, and searing induce the greatest AGE forming effect (40). Thermal processing for food safety and/or preservation purposes can also increase the AGE content of a particular food. AGEs themselves can even be added to food in order to enhance taste, appearance, and color (45, 46).

The western diet generally refers to a diet high in fat, sugar, and processed foods and is a key contributor in the dramatic increase in rates of both obesity and type 2 diabetes. These foods, coincidentally, are also high in AGE content. The exact fraction of exogenous AGEs being successfully absorbed remains controversial, but several studies have found a post-prandial increase in serum AGE levels several days following consumption of an AGE rich meal, suggesting that these reactive molecules can indeed contribute to overall AGE accumulation with the body (47). Because cellular and renal clearance of AGEs is inefficient, increased consumption of these foods leads to an increase in the deleterious effects associated with this reactive species that can potentially worsen overtime (40, 42).



**Figure 7:** RAGE structure showing functional domains. Adapted from “Receptor for Advanced Glycation End Products and Atherosclerosis: from Basic Mechanisms to Clinical Implications” by G. Basta, 2008, *Atherosclerosis*, 196(1):9-21. Copyright 2008 by Elsevier Science Ltd. Adapted with permission (5).

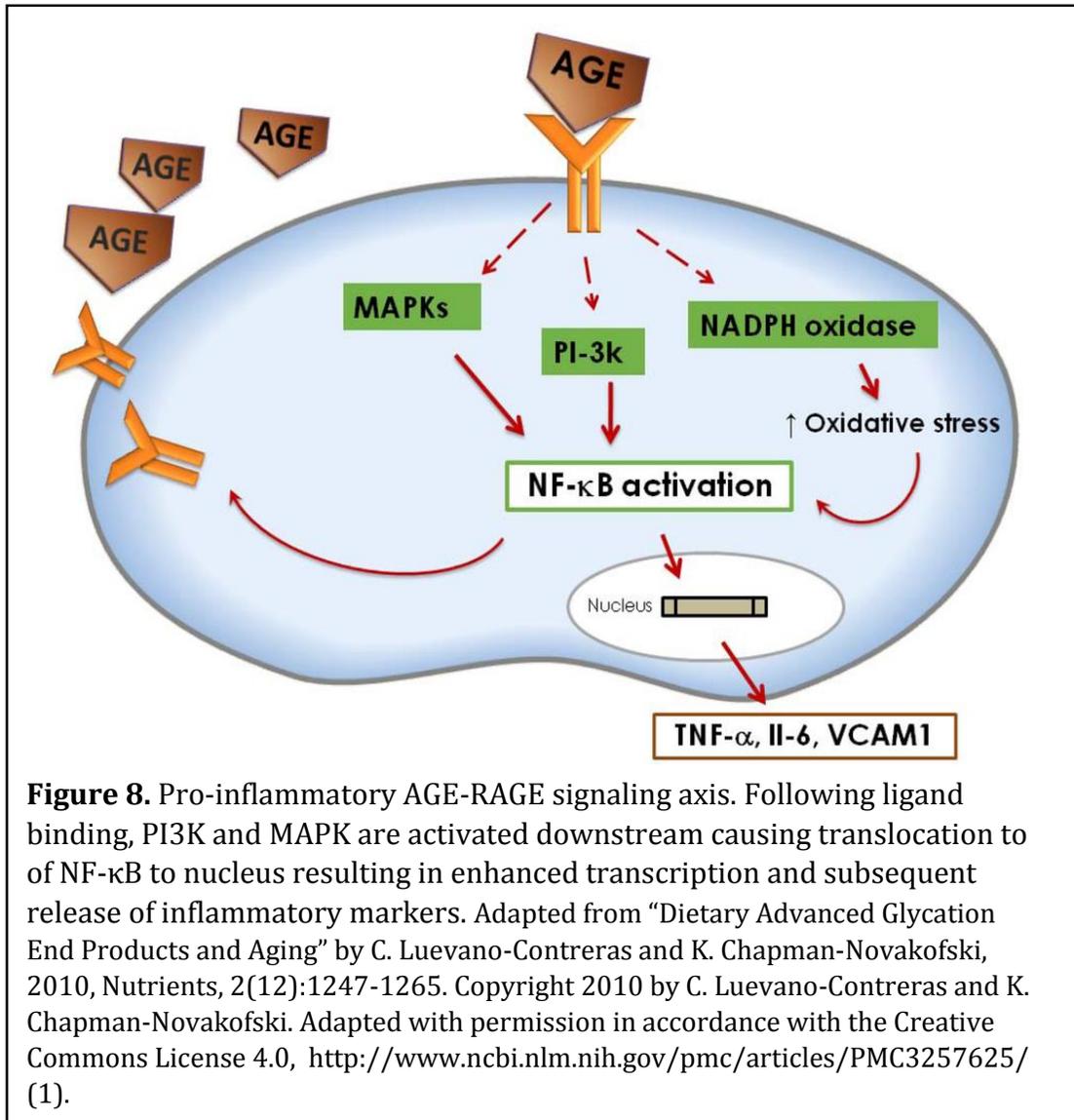
**Receptor for Advanced Glycation End Products (and pathogenic signaling):**

AGEs can contribute to pathogenesis in one of two ways. They can act independently of a receptor and cause damage to protein structure and function, particularly within the extracellular matrix by causing collagen crosslinking, or by acting as a ligand for the receptor for advanced glycation end products (RAGE) (1). RAGE is a transmembrane, pattern recognition receptor that is part of the immunoglobulin superfamily (48) (Figure 7). It is expressed in relatively low levels in all tissues on multiple cell types throughout the body, but

is found to be greatly increased in pathophysiological settings (i.e. hypoxic and inflammatory environments) (49). Structurally, RAGE contains a variable domain that is responsible for ligand binding, two constant domains, a transmembrane domain, and a cytoplasmic domain responsible for carrying out RAGE intracellular signaling. In addition to binding AGEs, RAGE also interacts with a variety of other ligands including the S100 family of proteins and High Mobility Group Box Protein 1

(HMGB1). Because of the variety of its ligands, RAGE signaling encompasses several different pathways involved in the inflammatory and immune responses (42).

A key effector in the propagation of the inflammatory response following injury is the activation of the transcription factor NF- $\kappa$ B (Figure 8). After AGE binds RAGE, several downstream signaling pathways are activated including the phosphatidylinositol-3-kinases (PI3K) and mitogen activated protein kinases (MAPK) which lead to translocation of NF- $\kappa$ B to the nucleus (1). The transcription factor then enhances production and subsequent release of inflammatory cytokines, growth factors, and adhesive molecules which serve to recruit more leukocytes to the site of injury or infection (Figure 8). Additionally, NF- $\kappa$ B also enhances transcription of RAGE itself, causing a positive feedback loop with enhanced AGE-RAGE signaling, resulting in a chronic inflammatory phenotype (1). Because of its role in inflammation, AGE signaling has been implicated in the progression and complications of a variety of diseases including diabetes, cardiovascular disease, and Alzheimer's (41).



**AGE-RAGE Signaling in Cancer:** It has been suggested that a pro-inflammatory environment can serve to promote tumorigenesis (49-51). This functionality is partly due to the ability of cytokines released during the inflammatory response to promote un-controlled proliferation(52). Relatively recently, several studies have shown that AGEs are increased and RAGE is upregulated in a variety of cancers including prostate, colorectal, and gastric (53). In

addition, AGE-RAGE signaling has been implicated in several cancer-associated signaling pathways that are pro-survival and contribute to increased proliferation, migration, and invasion, leading to a more aggressive phenotype with greater metastatic potential (54). As stated previously, AGE binding RAGE leads to the activation of the PI3K and MAPK pathways. Interestingly, these two pathways are also known to be upregulated or altered in a variety of cancers. Protein kinase B (Akt), within the PI3K pathway, promotes survival by inhibiting apoptosis and cell cycle arrest within cancerous cells (55). RAGE binding AGEs can also activate several different proteins within the MAPK pathway including ERK1/2, JNK, and p38 which are heavily involved in the growth potential of malignant tumors (48). The activation of PI3K, MAPK, as well as NADPH oxidase by AGE-RAGE signaling then activates NF- $\kappa$ B which, as stated previously, causes the enhanced transcription of not only several inflammatory cytokines and growth factors, but RAGE as well. This causes a feed-forward, pro-tumorigenic loop, leading to a more aggressive phenotype in cancer (1).

**Clinical Implications of AGEs** Implicating AGEs in the progression of several diseases has led to attempts at identifying a way to utilize these reactive metabolites in the clinic.

**Inhibition of AGEs:** It has been widely studied that AGEs are poor prognostic markers for complications in inflammatory diseases, especially in diabetes. Relatively recently, however, it has been suggested that AGEs could

potentially be successful drug targets by either blocking their formation or inhibiting intracellular signaling. (56). Drugs such as Metformin, which inhibits formation of AGEs are already being clinically utilized for treatment of other chronic diseases and could be potentially helpful as a dual therapy in AGE-associated cancer (57).

**AGEs as a biomarker:** In diabetes and cardiovascular disease, assessing AGE content in patient serum and tissue has proved valuable in predicting complications associated with these diseases. In diabetes, high levels of AGEs are shown to be predictive of microvascular complication and nephropathy (58). Interestingly, AGEs seem to have greater predictive capacity for these complications when compared to glycated hemoglobin, an AGE precursor and already established risk factor for diabetic complications. This may be in part due to AGEs' ability to assess overall metabolic stress, rather than just glucose metabolism (58).

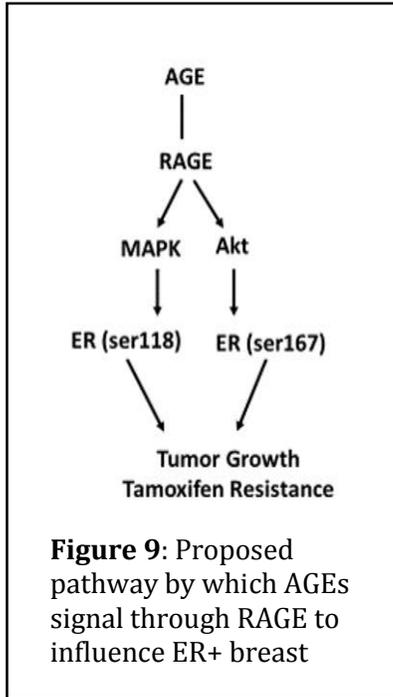
**Disease Prevention:** The pathogenic functionality of AGEs is increased in obese individuals. This could be the result of a combination of factors: hyperglycemia, hyperlipidemia, increased consumption of AGE-rich foods, etc (43). Obesity is, of course, a major risk factor for a variety of chronic diseases, including breast cancer. Therefore, reduction of AGEs either through decreased consumption of AGE-rich foods or through AGE/RAGE inhibitors could have potential preventative implications in the development and prognosis of several diseases.

## **RESEARCH PLAN**

**Overall Rationale:** Because of its pro-inflammatory nature, the AGE-RAGE signaling axis has been previously implicated in the complication and progression of a variety of chronic diseases including diabetes, cardiovascular disease, rheumatoid arthritis, along with many others (54, 59). Only recently, however, has RAGE signaling been shown to have tumorigenic effects in several different types of cancer. RAGE signaling is able to activate a variety of kinase pathways including PI3K and MAPK which are known to enhance proliferation and cell survival in cancer. Our lab has previously shown an association between AGEs, RAGE, and disease progression in prostate cancer (60). Because of this, we believe that AGE-RAGE signaling may be serving this same functionality in breast cancer. Identifying a role for AGEs in breast cancer may have potential therapeutic and preventative implications for patients because of our ability to consume exogenous AGEs in the form of high fat, high sugar, and thermally processed foods. Additionally, because a high AGE diet promotes weight gain and obesity, implicating AGEs in breast cancer could potentially explain the increase in incidence, reoccurrence and mortality that is already known to be associated with obesity (43).

Around 70% of all invasive breast cancers express the estrogen receptor (17). Activation of this receptor is the driving force behind the progression of these types of cancer. Tamoxifen, which is often the first line of adjuvant therapy for these patients, is an effective drug that combats the ability of the estrogen receptor to enhance transcription of its target genes. A third of patients taking tamoxifen,

however, will not respond to the drug and most who initially respond will develop resistance. Several studies have attempted to find a molecular cause for this acquired resistance by investigating specific, estrogen-independent



phosphorylation sites on the receptor itself (17, 25, 32, 61). Two sites in particular, ser167 and ser118 within the ligand independent activation domain, have been suggested to influence tamoxifen resistance. Ser167 and ser118 are phosphorylated by the second messenger kinase pathways, PI3K and MAPK, respectively. Because RAGE signaling has already been shown to activate these signaling cascades in other diseases, we believe that AGEs could be a potential factor contributing to tamoxifen

resistant tumors by causing phosphorylation of the estrogen receptor at these two residues through the PI3K and MAPK pathways. We are therefore proposing AGE-RAGE signaling (Figure 9) as a potential mechanism of progression and tamoxifen resistance in estrogen receptor positive breast cancer.

**Hypothesis:** We hypothesize that AGE levels are increased in breast cancer and cause activation of cancer associated signaling pathways and contributes to the development of tamoxifen resistance in estrogen receptor positive breast cancer.

**Specific Aim #1: Determine whether or not presence of AGEs and RAGE are indicative of disease progression in breast cancer.** The RAGE signaling axis has been implicated in a variety of diseases including diabetes, Alzheimer's, and cardiovascular disease. Additionally, RAGE signaling has been previously shown to activate pro-survival and proliferative pathways in several types of cancer causing its presence (along with AGEs) to be associated with increased grade and disease progression in prostate, colorectal, and gastric cancers. Because of its pro-tumorigenic ability, we believe identifying the RAGE signaling cascade as a marker of increased breast cancer aggression will help elucidate its functional role in tumor development and survival.

**Task #1:** Quantify AGE levels in breast cancer tissue and serum samples to define:

- 1) Differences between normal, benign, and cancerous samples
- 2) Correlation between AGEs and level of differentiation as an indicator of disease progression.

**Task #2:** Quantify RAGE levels in breast cancer tumor samples to define:

- 1) Differences between normal, benign, and cancerous samples

2) Correlation between RAGE protein level and tumor differentiation as an indicator of disease progression.

### **Experimental Design**

**Biological Samples.** Human serum samples (n=39) were obtained from MUSC's Tissue Biorepository. Tissue microarray was obtained from US BioMax (Rockville, MD) and contained 96 samples of normal, benign, and cancerous tissue from 48 patients. Demographic data such as age and race were included. Pathological data available from each patient included: tumor grade and receptor status (some).

**AGE ELISA.** In order to quantify AGE levels in serum samples from breast cancer patients, a competitive ELISA assay was utilized to determine concentration of circulating AGEs and comparisons were made based on level of tumor differentiation as well as ER status. The OxiSelect™ Advanced Glycation End Product Competitive ELISA kit was purchased from Cell Biolabs, Inc. (San Diego, CA). The wells of the provided 96 well plate were coated with 5 ug/mL BSA-AGE conjugate and incubated at 4°C overnight. Wells were then washed twice (250uL each) with phosphate buffered saline (PBS) using an automatic plate washer (Bio-Tek Instruments, Inc. Winooski VT). Plate was then blocked to prevent non-specific binding using 200uL of assay diluent and incubated on an orbital shaker at room temperature for one hour. After removal of diluent, 50uL of BSA-AGE standards or serum samples were added to the wells in triplicate. Ten standards were prepared

according to the manufacturer's instructions to produce serial dilutions ranging from 1.56 ug/mL to 100 ug/mL. After addition of the sample, the plate was then incubated at room temperature with light agitation for 10 minutes. 50uL of diluted anti-AGE antibody (Cell Biolabs, Inc. San Diego, CA) was then added to each well and allowed to incubate at room temperature with light agitation for 1 hour. Wells were washed with 250uL of 1X wash buffer three times (Cell Biolabs, Inc. San Diego, CA) using an automatic plate washer and thoroughly dried using absorbent strips. 100uL of diluted secondary antibody-horseradish peroxidase (HRP) conjugate (Cell Biolabs, Inc. San Diego, CA) was added to each well and again allowed to incubate for one hour at room temperature with light agitation. Plate was then washed in the same manner described previously. 100uL of the substrate solution (Cell Biolabs, Inc. San Diego, CA), after being warmed to room temperature, was added to each well and incubated in the dark for fifteen minutes on an orbital shaker. To stop enzymatic reaction, 100uL of stop solution (Cell Biolabs, Inc. San Diego, CA) was added to each well and absorbance was read immediately on microplate reader (Bio-Tek Instruments, Inc. Winooski, VT) using 450nm as the primary wavelength.

**AGE/RAGE IHC.** Quantification of AGEs within the tumors themselves was accomplished using immunohistochemical (IHC) staining of the TMA. Because RAGE is a transmembrane protein, expression levels are only quantifiable using IHC in tumor samples. Tissue microarrays contained 96 total punched, paraffin embedded samples, each 1mm in diameter mounted on glass slides 2mm apart. Removal of

paraffin was accomplished by incubating slides at 60°C for 30-45 minutes. A series of washes were then utilized in order to rehydrate the tissue samples: 2-xylene for 20 minutes each; 2-100% ethanol, 2-95% ethanol, 1-70% ethanol, and 1-50% ethanol for ten minutes each; 3-distilled water for five minutes each. Vector unmasking solution (Vector Labs, Burlingame, CA) was then used at a 1:100 dilution for antigen retrieval. Slides were placed in a pap jar containing the diluted unmasking solution and placed in a vegetable steamer, also containing diluted unmasking solution, at 90°C for 30 minutes. The slides were then allowed to cool to room temperature and placed in pap jar containing 0.3% hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ) for 30 minutes to remove any endogenous peroxidase activity. The slides were then washed in 1x tris buffered saline containing 0.01% Tween-20 (TBST) (Fisher Scientific, Fair Lawn, NJ) for 5 minutes on an orbital shaker. In order to prevent solution run-off in the following steps, tissue samples were outlined using a hydrophobic pen. Slides were then incubated in VECTASTAIN® horse serum (Vector Labs, Burlingame, CA) to block non-specific binding for 30 minutes at room temperature in a humidified chamber. Primary antibodies for AGE and RAGE (Abcam, Cambridge, MA) were both diluted 1:50 using horse blocking serum diluted with TBST and applied to respective slides and allowed to incubate overnight at 4°C in a humidified chamber. Primary antibody was then removed by washing 3 times in TBST for five minutes on an orbital shaker. Slides were incubated with VECTASTAIN® secondary antibody solution (Vector Labs, Burlingame, CA) for thirty minutes at room temperature in a humidified

chamber. Slides were then washed as described previously. Next, slides were incubated with immPACT™ NovaRED™ substrate solution (Vector labs, Burlingame, CA) for 8 minutes and then placed in distilled water to stop enzymatic reaction. Following a five minute wash in distilled water, slides were dehydrated in: 1-50% ethanol for five minutes; 1-70% ethanol for five minutes; 2-95% ethanol for 5 minutes each; 2-100% ethanol for five minutes each; 2-xylene for 10 minutes each. Glass cover slips were then mounted on top of slides using Permount® (Fisher Scientific, Fair Lawn, NJ). Imaging was performed using an Olympus BH-2 microscope and DP 70 digital camera (Olympus American, Inc. Center Valley, PA). Quantification of the staining of advanced glycation end products (AGEs) was completed using a 0 to 4+ scoring method by a single pathologist. Each separate tissue on the microarray was scored individually based on staining intensity where 0 was no staining of the cells of interest and 4+ was intense cytoplasmic staining. The lowest score any tissue received in this series was 1+. To gain a score of 4+ the staining had to be diffuse across the cells of interest. Each tissue was given a score for both the epithelial component and the stromal component, when a stromal component was present. Visual scoring for RAGE was used to quantify the staining intensity in each IHC core sample. Reference samples were chosen and given a score of 1 through 4 with 1 being the lowest and 4 being the highest staining intensity. Scores were then assigned based upon the four reference samples.

Analyses outlined in the tasks above were then performed in order to investigate whether or not AGEs and RAGE are associated with a more aggressive disease in breast cancer.

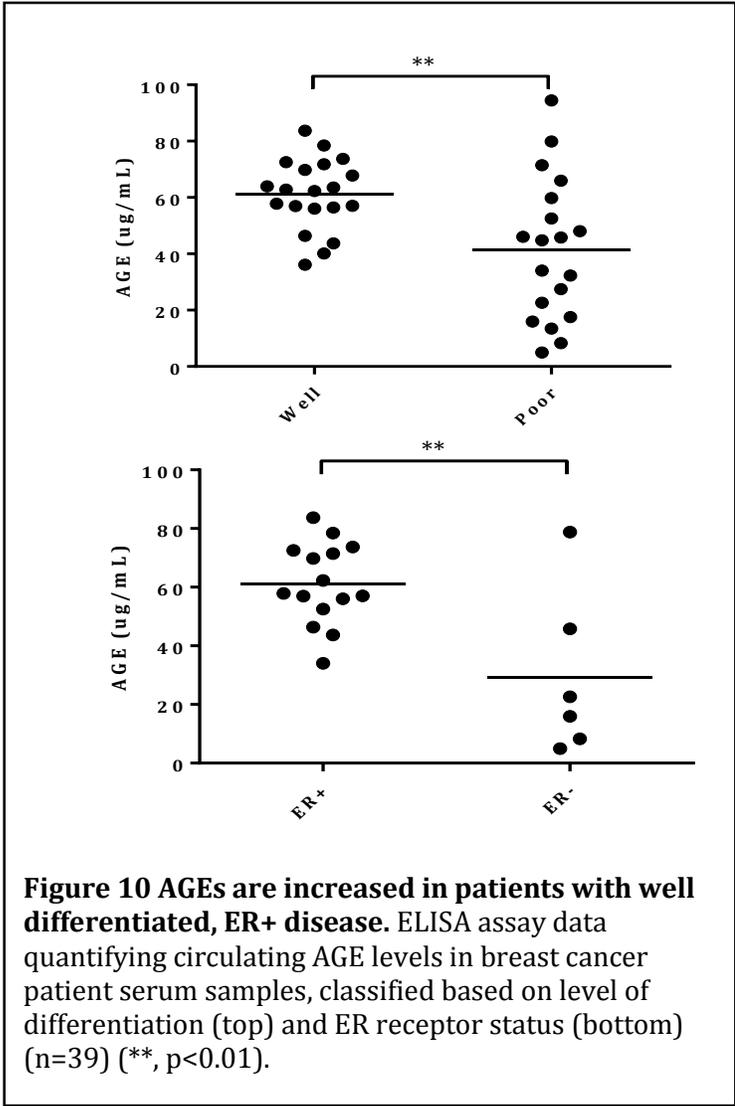
**RNA Extraction and GLO1 mRNA Analysis.** In order to quantify glyoxalase mRNA in a panel of breast cancer cell lines and to gain insight into tumor detoxification of AGE precursors, quantitative real time PCR was used. Cells were cultured on 10cm plates and once 75% confluency was reached, media was aspirated and cells were washed with 5mL of PBS. 1mL of Trypsin was then added to the plate and allowed to incubate for five minutes to disrupt cell adhesion. 4mL of media was added in order to neutralize the trypsin and then collected into a 15mL tube. The tube was centrifuged at 1,000 RPM for 5 minutes to pellet the cells. The supernatant was then aspirated and the cells were again washed with PBS. The tube was centrifuged once more and again the supernatant was removed leaving only the pellet of cells. Total RNA was extracted from the cell pellet using RNeasy® Plus Mini Kit (Qiagen, Valencia, CA). 1ug of RNA was then reverse transcribed to produce cDNA using iScript (BioRad, Hercules, CA) according to manufacturer's instructions. The cDNA was then diluted 1/20 using molecular grade water (Roche, Nutley, NJ). The cDNA was used as a template for probe based, quantitative real-time PCR (RT-PCR) to measure the total quantity of GLO1 mRNA (and GAPDH mRNA as control). Primers were diluted 1/5 using molecular grade water (primer sequences shown in Table 1). Probes for each mRNA transcript were obtained from the universal probe library

(UPL) and RT-PCR was ran in a LightCycler480 (Roche, Nutley, NJ) with the following conditions: after pre-incubation to 95°C, amplification occurred in a series of 50 cycles of 5 seconds at 95°C then 30 seconds at 60°C. After 50 cycles, the plate was cooled to 40°C. Each sample was run in triplicate and with corresponding negatives (no cDNA in reaction mixture). The relative mRNA levels were quantified using the Ct value measured against an internal standard curve using the software provided by the instrument manufacturer (Roche, Nutley, NJ). Glyoxalase 1 mRNA data obtained was then normalized to GAPDH mRNA data and analyzed.

<b>Table 1: Primer Sequences for probe-based qRT-PCR</b>			
<b>Gene</b>	<b>5' Primer</b>	<b>3' Primer</b>	<b>Probe #</b>
<b>RAGE</b>	ggtcacatctggcaaag	cctcctcttctcctctgggt	23
<b>Glyoxalase 1</b>	cccagtagcaaggattttct	tgggaaaatcacatttttggga	84
<b>GAPDH</b>	agccacatcgctcagacac	gcccaatacgaccaaattcc	60

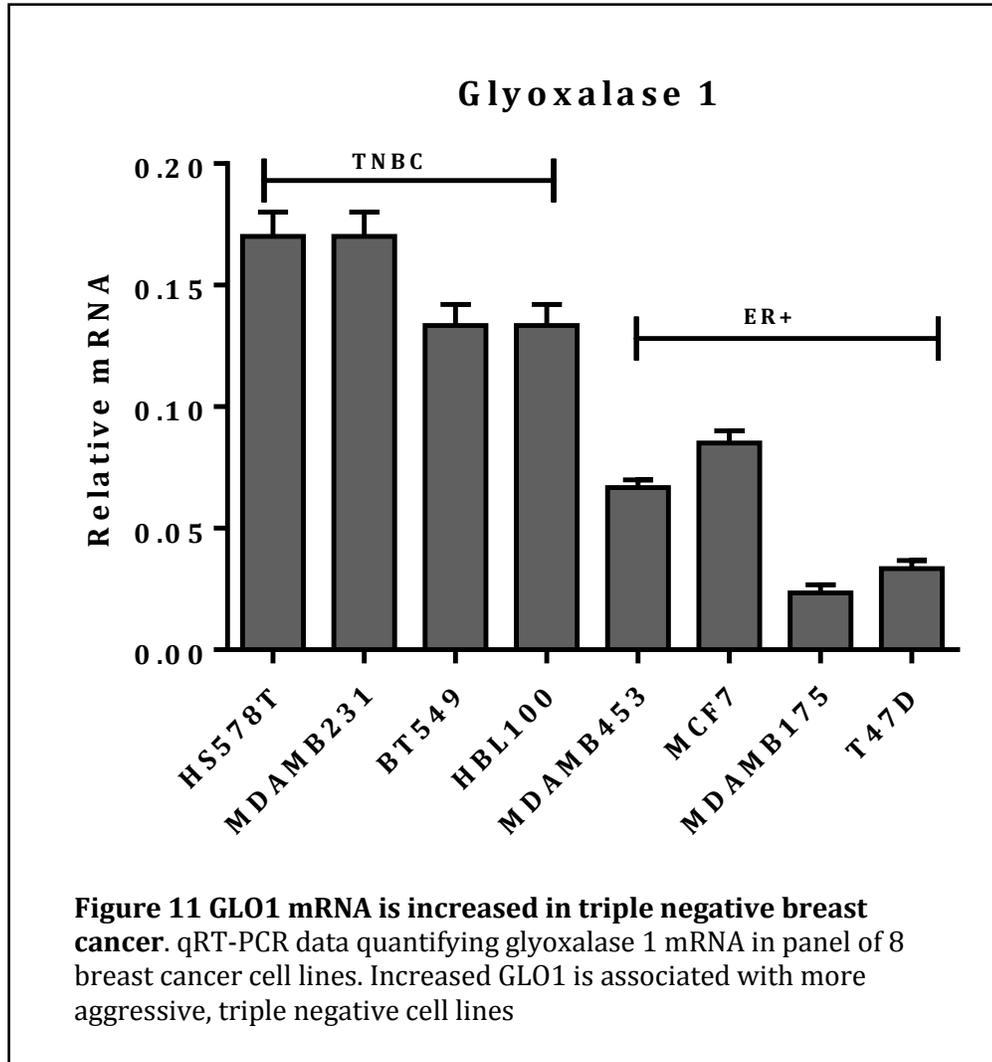
## **Results and Discussion**

**AGEs exist in greater quantities in well differentiated serum samples:** ELISA assay data (Figure 9) suggests that well differentiated breast cancer patients have higher circulating AGE levels when compared to the patients with a more progressive, less differentiated disease. While this is not as we expected, a potential explanation could be a difference in glyoxalase 1 activity in more aggressive diseases. A recent paper cited that more aggressive, triple negative tumors exhibited lower concentrations of AGE precursors, but greater glyoxalase 1 activity in both tumor and tissue(62). Glyoxalase 1, as discussed previously, is an internal detoxification mechanism that serves to lower concentrations of reactive carbonyls within the cell. To attempt to verify this cited correlation between GLO1 and cancer aggression, we quantified GLO1 mRNA in a panel of 8 breast cancer cell lines (Figure 11). On average, triple negative tumors had a greater level of GLO1 mRNA. Because triple negative tumors are more aggressive than other subtypes, this data suggests that more aggressive tumors are compensating rising AGE levels by increasing GLO1 activity. As can be seen in Table 2, the majority of the well differentiated serum samples, as expected, were ER+/PR+/HER2- (Luminal). The poorly differentiated samples however contained more samples with higher expression of HER2 (poor prognostic factor) and more triple negative subtypes. As can be seen in the bottom of Figure 10, when classified based on ER status, expression of the receptor is indicative of a greater concentration of circulating AGEs in our patient samples.



**Table 2.** Receptor status of serum samples used for ELISA assay AGE quantification. Well differentiated were mostly ER+ whereas poorly differentiated samples were more heterogeneous in subtype.

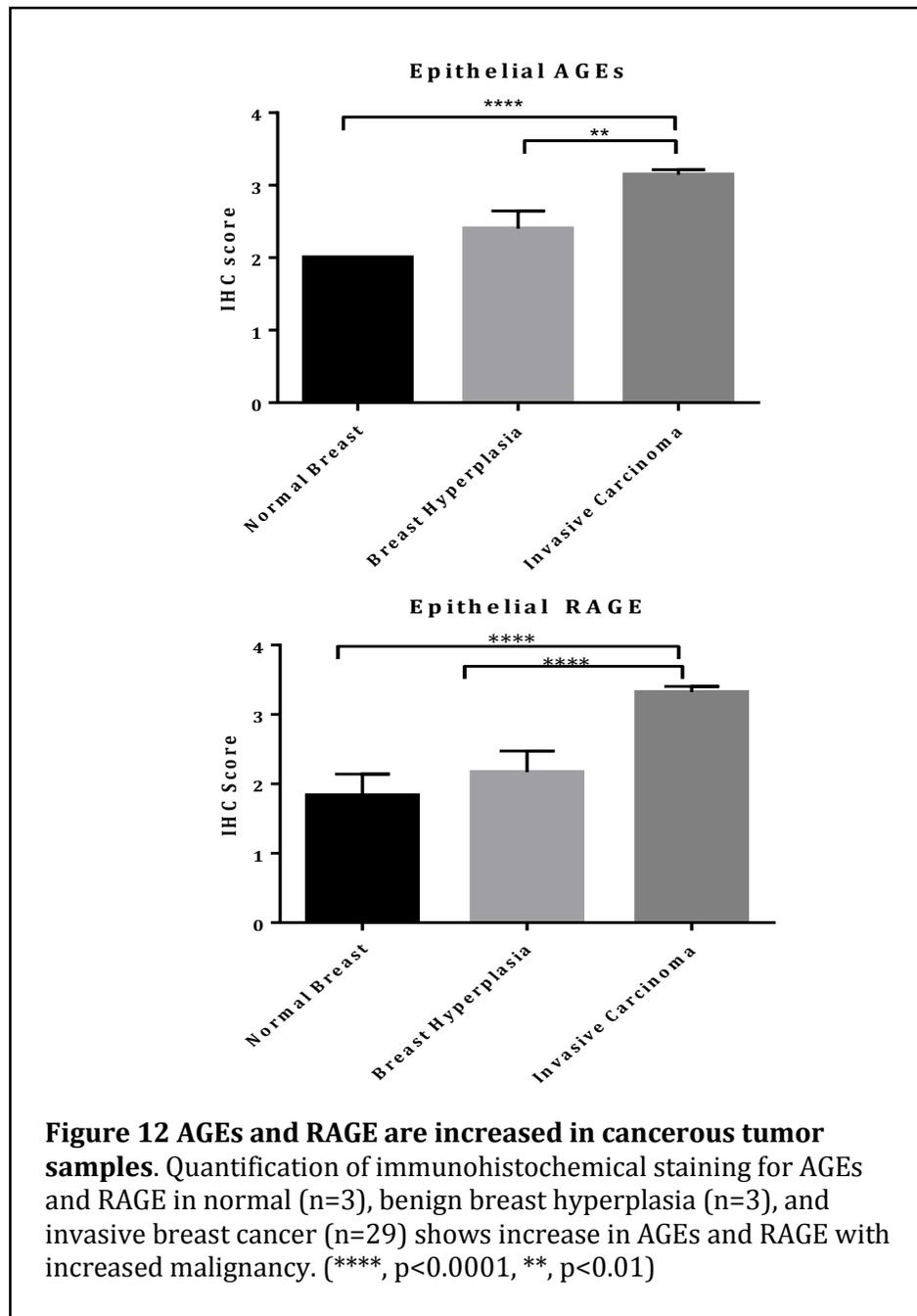
	Well Differentiated (n=20)	Poorly Differentiated (n=19)
ER+/PR+, HER2 -	12	4
ER+/PR+/HER2+	0	3
ER-/PR-/HER2+	0	3
Triple Negative	1	2
Unknown	7	7

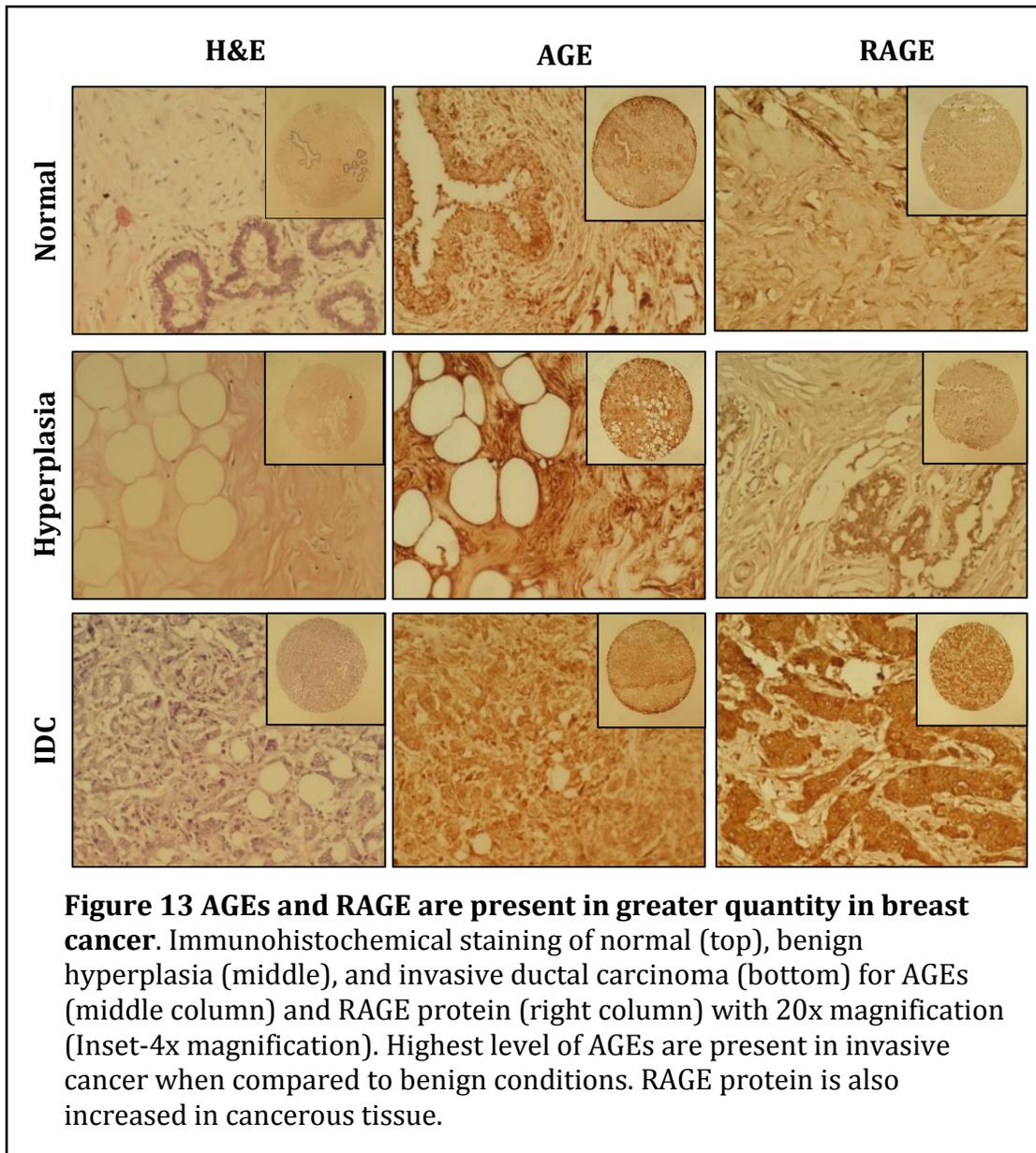


**AGEs and RAGE are increased in cancerous tumor samples:**

Immunohistochemical staining of the tumor microarray for AGEs and RAGE revealed that, on average, invasive carcinomas exhibited greater levels of AGEs and RAGE protein within the tumor epithelium (Figure 12). Representative images are shown in Figure 13. More intense staining was found in the tumor epithelium with only slight staining within the tumor stroma for both AGE metabolites and RAGE

protein. These data indicate that AGEs and RAGE are present and may potentially be associated with increased malignancy in breast cancer.

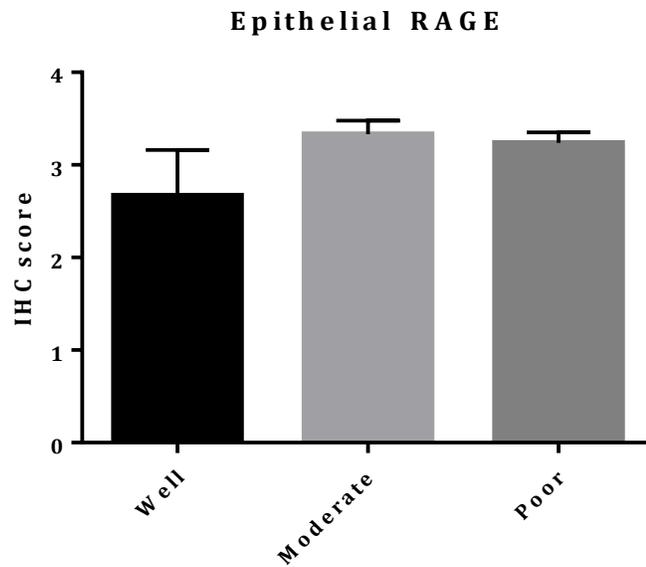
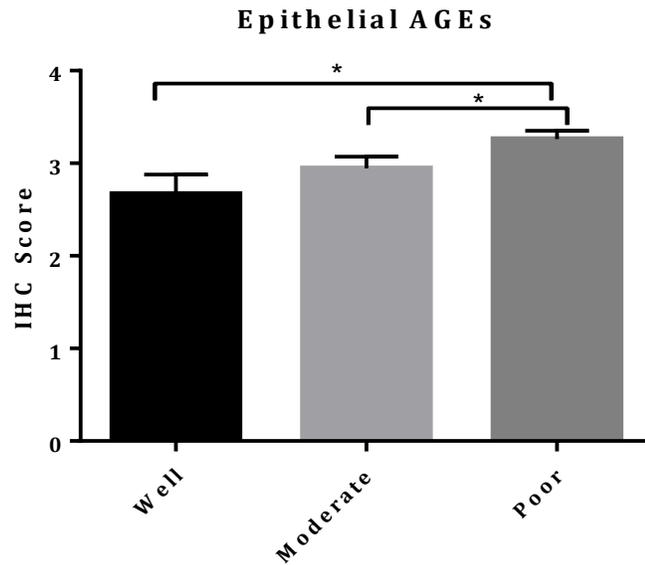




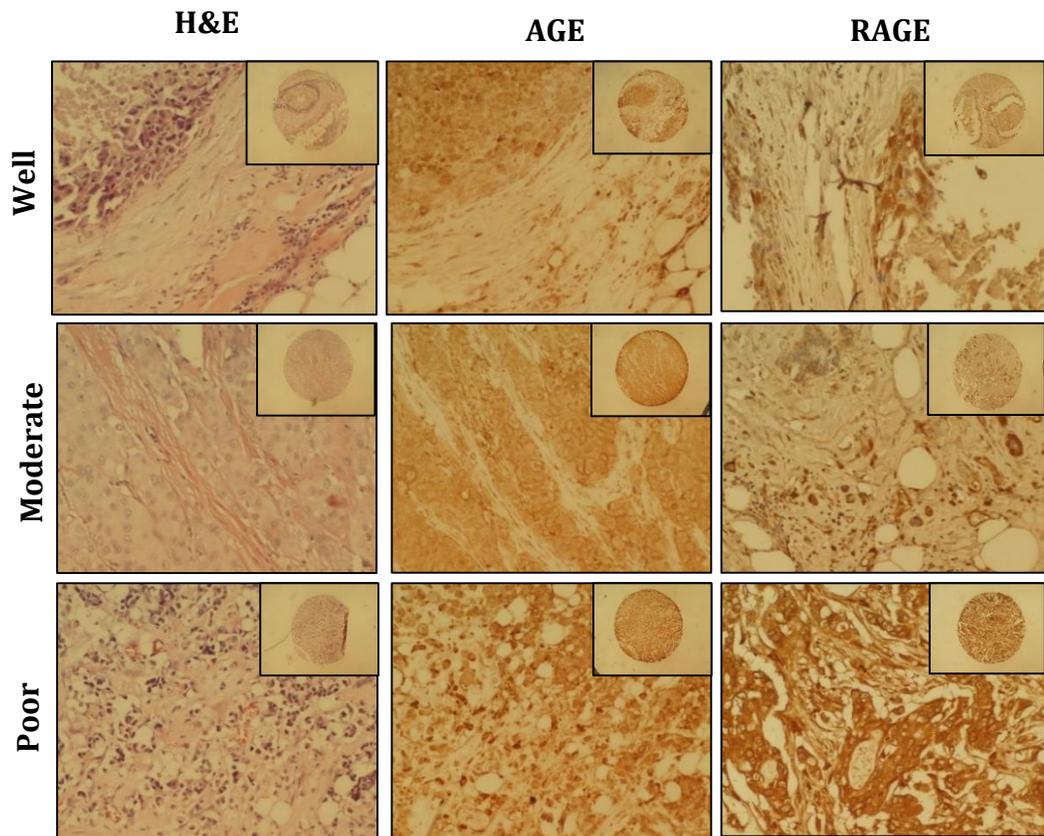
**AGEs and RAGE are increased in poorly differentiated tumors:**

Immunohistochemical staining of the tumor microarray for AGEs and RAGE revealed that, on average, AGEs were indicative of less differentiation within the tumor. Moderate and poorly differentiated tumors exhibited greater levels of AGEs

within the tumor epithelium (Figure 14). Representative images are shown in Figure 15. More intense staining was found in the tumor epithelium with only slight staining within the tumor stroma for AGE metabolites. RAGE protein seemed to trend towards being increased in more aggressive tumors but values were not statistically significant. Again, these data indicate that AGEs (and potentially RAGE) are indicative markers of a more advanced disease in breast cancer.



**Figure 14 AGEs and RAGE are associated with decreasing tumor differentiation.** Quantification of immunohistochemical staining for AGEs and RAGE in malignant tumors separated by level of differentiation: well differentiated (n=3), moderately differentiated (n=12), and poorly differentiated (n=16). Data shows increase in AGEs with more advanced disease (\*,  $p < 0.05$ ). RAGE seems to trend toward same conclusion despite being statistically insignificant.



**Figure 15 AGEs and RAGE increase with lack of differentiation.** Immunohistochemical staining of breast carcinomas with varying levels of differentiation for AGEs (middle column) and RAGE protein (right column) with 20x magnification (Inset-4x magnification). Highest level of AGEs are present in poorly differentiated tissue when compared to better differentiated tissues. RAGE protein is also increased (to lesser extent) in poorly differentiated cancerous tissue indicating both are potentially associated with a more advanced disease.

## **Discussion and alternative approaches**

In this aim, we showed that AGEs are present in greater levels in serum samples from patients with a less aggressive disease. Additionally, by looking at tumor samples from breast cancer patients, we were able to show an increase intratumoral AGEs and RAGE in cancerous tissue when compared to normal breast and benign hyperplasia. Level of differentiation was also investigated and AGEs were associated with more advanced tumors. RAGE showed a similar trend, but was not statistically significant despite RAGE levels already having been shown to correlate with a worsened prognosis in other cancers such as gastric and prostate (63, 64). There are numerous possible explanations for the differences observed between AGE serum and tumor correlations. One potential explanation is that the tumors themselves are producing AGEs. This is plausible because of the high glycolytic rate that is characteristic of malignant tumors and, as stated previously, glycolytic intermediates are often precursors to AGE formation. This would explain why we do not observe high AGE levels in more aggressive serum, but we see the expected increase in the tumors themselves.

Observing AGEs and RAGE in greater levels within the malignant tumor when compared to benign and normal breast tissue suggests that AGE-RAGE signaling may be potentially contributing disease progression. This is reinforced in our data showing increasing AGE levels in the moderate and poorly differentiated tumors compared to the less progressed, well differentiated tumors. All of this data together

suggests a possible role in breast cancer disease progression, but many more tasks must be accomplished before reaching a final conclusion.

Identifying a role for AGEs in breast cancer could have potential preventative implications. As can be seen in Table 2, the majority of the samples from patients with well differentiated tumors were ER+ and exhibited higher AGE levels when compared to the poorly differentiated tumors. As stated previously, ER+ positive patients are often given the drug tamoxifen and many develop resistance. Specific Aim 2 investigates AGEs in tamoxifen resistance and, by showing AGEs are increased in serum for these patients, a low AGE diet and/or dual treatment with AGE inhibitors may be beneficial for these patients.

A caveat with the experimental design in this specific aim is our small sample size. While a tissue microarray allows for the testing of several tumor samples at once, each sample is only a small representation of the tumor as a whole. The tumor microenvironment is incredibly diverse and it is absolutely possible that AGEs and RAGE are present and functioning in one part of the tumor to a greater extent than in the remaining portion. A future direction for this aim would be to perform these same tests in a much larger and more diverse sample size in order to gain a better insight into the role of AGEs and RAGE in breast cancer tumor progression. Matched serum and tumor samples would also be helpful in identifying a correlation between circulating AGEs and intratumoral AGEs in a patient-specific manner. Additionally, quantification of IHC staining is undoubtedly imperfect and it is important to keep in mind that correlation does not necessarily imply causation. That is to say, even

though we may see higher levels of RAGE within the higher grade tumors, RAGE signaling may not be causing this progression. Because of this, it is necessary to investigate the signaling pathway by which AGEs and RAGE are inducing this effect in ER+ breast cancer.

**Specific Aim #2: Determine if AGE-RAGE signaling through Akt and ERK induces estrogen receptor phosphorylation leading to increased proliferation and tamoxifen resistance.** Previous studies have shown that RAGE signaling can activate the specific kinase signaling cascades, PI3K and MAPK, which contribute to tumor survival and growth in many cancers. Additionally and separately, previous studies investigating a molecular cause for tamoxifen resistance in estrogen receptor positive cancer have identified two residues on the receptor itself that, when phosphorylated, are indicative of this resistance. Interestingly, these two residues, ser167 and ser118, are phosphorylated by kinases within the PI3K and MAPK pathways. This specific aim proposes that AGE signaling is connected to these phosphorylation events and resistance to tamoxifen in ER+ breast cancer through the mechanism shown in Figure 9. By characterizing this pathway, we can elucidate a functional role of AGEs and RAGE in the development of this resistance.

**Task #1:** Determine AGE and RAGE levels in ER+ breast cancer cell lines in order to:

- 1) Show that AGEs and RAGE are present in ER+ breast cancer
- 2) Create base line reading of AGE and RAGE protein levels before manipulating pathway in following tasks

**Task #2:** Perform proliferation experiments following AGE treatment to demonstrate:

- 1) AGEs role in cancer progression

- 2) Gain a more functional perspective of AGEs and RAGE in breast cancer in vitro

**Task #3:** Demonstrate that AGE-RAGE signaling induces phosphorylation of the estrogen receptor on ser167 and ser118 through phosphorylation of Akt and ERK 1/2 in order to:

- 1) Demonstrate AGEs signaling through RAGE activates pro-tumorigenic signaling pathways
- 2) Create a connection between AGE-RAGE signaling and ER mediated signaling in ER+ breast cancer

**Task #4:** Identify a role of AGEs in tamoxifen resistance by exposing ER+ cells to AGEs and monitoring cell viability during tamoxifen treatment to determine if:

- 1) AGE-mediated activation of ER at ser167 and ser118 results in a more resistant phenotype

## **Experimental Design**

**Cell Culture.** ER+ MCF7 and T47D cell lines were a gift from Dr. Dennis Watson at the Medical University of South Carolina. Cells were incubated at 37°C, 5% CO<sub>2</sub> in their respective media. T47D cells were incubated in RPMI (Fisher Scientific, Fair Lawn, NJ) with 10% fetal bovine serum (Fisher Scientific, Fair Lawn, NJ), and 1% Penicillin/Streptomycin (Fisher Scientific, Fair Lawn, NJ). MCF7 cells were incubated in DMEM/High Glucose media (Fisher Scientific, Fair Lawn, NJ) also containing 10% FBS and 1% Penicillin/Streptomycin with the following additives: 1% MEM Non-essential Amino Acids (Mediatech, Manassas, VA), 1% Sodium Pyruvate (100mM) (Life Technologies, Grand Island, NY), 1% Sodium Bicarbonate (7.5% stock) (Invitrogen, Grand Island, NY), and 1% insulin (Life Technologies, Grand Island, NY). Media on each cell line was changed every 3 days and were passaged once 75% confluency was reached using 0.05% HyClone Trypsin (Fisher Scientific, Fair Lawn, NJ).

**AGE and Protein Assessment.** In order to assess endogenous AGE content in our ER+ in vitro model, dot blot analysis was performed. RAGE, p-Akt (and total Akt), p-ERK1/2 (and total ERK), and p-ER $\alpha$  at both ser167 and ser118 (and total ER $\alpha$ ) expression were examined using Western Blot analysis. For each, cells were cultured on 10cm plates. After reaching 75% confluency, the old media was removed and plates were washed with 7mL of PBS. After removal of the PBS, cells were lysed using 100uL RIPA lysis buffer +Halt phosphatase inhibitors (Pierce,

Rockford, IL) and manually removed from the surface of the plate using a cell scraper and placed into eppendorfs. Tubes were then centrifuged for 20 minutes at 13,000 RPM at 4°C. After centrifugation, the supernatant was collected and placed into new eppendorfs. Protein concentration of each sample was then assessed using the BCA protein assay (Pierce, Rockford, IL). A 2mg/mL standard of BSA was diluted to 16ug/mL in 1% SDS and then serial diluted to produce the remaining samples for the standard curve: 8ug/mL, 4ug/mL, 2ug/mL, 1mg/mL, and a blank of only 1% SDS. 25uL of each standard was added into their respective wells on a 96 well plate in duplicate. 5uL of the unknown samples were diluted into 20uL 1% SDS and then added into their respective wells on the same plate. 200uL of the BCA protein assay reagent (mixed according to manufacturer's instructions) was added to each well. The plate was incubated at 37° for thirty minutes, allowed to cool, and immediately read on a spectrophotometer plate reader at a primary wavelength of 540nm (Bio-Tek Instruments, Inc., Winooski, VT) to determine protein concentration of each unknown sample. Samples were diluted to produce 50ug of protein using distilled water.

For the western blot analysis, the diluted protein sample was added to a tube containing Laemmli Sample Buffer (BioRad, Hercules, CA) plus 0.1%  $\beta$ -mercaptoethanol and boiled for five minutes. The proteins in the samples were separated using 10% Mini-PROTEAN® TGX (BioRad, Hercules, CA) pre-cast gels in running buffer containing 25mM Tris-base, 192mM Glycine, and 1% SDS run at 200 volts for 45 minutes for blots investigating RAGE, Total Akt, ERK1/2, and ER $\alpha$ . For

phosphorylated proteins (p-Akt, p-ERK1/2, and p-ER $\alpha$ ), gels were run at 100 volts for approximately two hours with the gel-containing chamber on ice. The protein ladder, PageRuler Plus (BioRad, Hercules, CA), was used to estimate protein size. Gels were removed from their casts and prepped for transfer to a PVDF (Millipore, Billerica, MA) membrane that had been charged in methanol and rinsed in distilled water. The blotting sandwich containing the gel and membrane was placed in a chamber containing chilled transfer buffer (25mM Tris-base, 192mM Glycine, 20% methanol). Transfer was performed at 100 volts for one hour for RAGE, Total Akt, ERK1/2, and ER $\alpha$ . For phosphorylated proteins, transfer was performed at 40 volts, on ice, for 90 minutes. Membranes were then removed and placed in 1x TBST overnight at 4°C. To reduce non-specific binding, the membrane was blocked in 10% milk diluted in TBST for one hour. For phosphorylated proteins, the membrane was blocked in 5% BSA diluted using TBST for one hour. After blocking, blots were incubated with primary antibody at 4°C overnight with gentle agitation. Antibodies were diluted as follows using either 5% milk or 2.5% BSA: RAGE (rabbit) (Abcam, Cambridge, MA) 1:1500; p-Akt (rabbit), p-ERK1/2 (rabbit), p-ER $\alpha$  ser167 (rabbit), p-ER $\alpha$  ser118 (mouse) (Cell Signaling, Danvers, MA) 1:1000; Total Akt, Total ERK1/2, Total ER $\alpha$  (Cell Signaling, Danvers, MA) 1:1000. Membranes were washed three times in TBST for ten minutes each. Anti-rabbit and anti-mouse HRP linked secondary antibodies (Cell Signaling, Danvers, MA) were then diluted in 5% milk or 2.5% BSA (1:4000 for all). Blots were incubated in appropriate secondary antibody at room temperature for one hour on an orbital shaker. After washing excess

secondary from the membrane (3 x 10 minutes each in TBST), Blots were covered with Super Signal West Pico Chemoluminescence substrate (Fisher Scientific, Fair Lawn, NJ) for 5 minutes in order to activate the horseradish peroxidase. Finally, using autoradiography film (Delville Scientific, Metuchen, NJ) various time exposures were taken of each blot and then developed on an automatic developer (Kodak, Rochester, NY). After achieving ideal exposure, membranes were stripped by washing with 200mM NaOH for five minutes, followed by two five minute washes with distilled water in order to prepare membrane for loading control. Membranes were blocked in 10% milk for thirty minutes and then incubated with GAPDH primary antibody (Cell Signaling, Danvers, MA) at a 1:5000 dilution for two hours at room temperature on an orbital shaker. Blots were then washed as before and incubated with an anti-rabbit HRP linked secondary antibody (Cell Signaling, Danvers, MA) diluted at 1:5000 for one hour at room temperature on an orbital shaker. The blots were then washed and developed as described previously.

For the dot blot analysis, the diluted samples were boiled for five minutes. 10uL (50ug) of each sample was then carefully applied on a nitrocellulose membrane in duplicate (one for AGE content and one for loading control) and allowed to dry at room temperature for 90 minutes. The membrane was blocked in 5% BSA for thirty minutes. The blot was then incubated overnight at 4°C in primary AGE antibody (Abcam, Cambridge, MA) diluted 1:4000 in 2.5% BSA. The membrane was washed, incubated in secondary anti-body (1:10,000), and exposed to ECL in the same manner as was detailed for the western blot analysis.

**RNA Extraction and RAGE mRNA Analysis.** Endogenous RAGE mRNA as well as change in RAGE mRNA following AGE treatment was performed using quantitative real time PCR as previously described in Specific Aim #.1

**Formation of Exogenous AGEs and AGE Treatments.** In order to examine the effects of AGEs on proliferation and estrogen receptor mediated signaling, we needed to first produce exogenous AGEs that we could expose our ER+ cell lines to. We chose to produce glyceraldehyde-derived AGEs because this type has been shown to be biologically relevant (prevalent in total circulating AGEs) and also enhances disease malignancy more so than other types of AGEs (i.e. glucose derived AGEs) (65-67). Our protocol for production of these types of AGEs is as follows: 125mg BSA (Fisher Scientific, Fair Lawn, NJ) suspended in 3mL of distilled water, 1mL of 1M NaP (pH=7.4), 250uL of 2M glyceraldehyde (Fisher Scientific, Fair Lawn, NJ), 31uL of 800mM diethylene triamine penta acetic acid (DENPA) (Fisher Scientific, Fair Lawn, NJ), and then filled with distilled water to a total volume of 5mL. The solution was then incubated for seven days at 37°C with gentle agitation. Following incubation, excess glyceraldehyde not conjugated to BSA was removed using a dextran desalting column (Pierce, Rockford, IL) according to the manufacturer's instructions. Finally, the samples were concentrated to remove excess PBS (used in previous step as exchange buffer) in 0.5mL centrifugal filter units (Millipore, Billerica, MA) according to the manufacturer's instructions. Final AGE concentration was determined to be approximately 10mg/mL using an AGE

ELISA assay (described previously in specific aim #1). A control AGE solution was made using the same protocol, but without glyceraldehyde in order to prevent any glycation (and therefore no AGE formation) to ensure that the AGEs themselves are causing an effect and not any of the other ingredient used in our AGE formation process.

We then proceeded to treat our ER+ breast cancer cell lines with exogenous AGEs. First, approximately 24 hours before cell collection was to occur, the cells' media was changed to serum-free. After 24 hours of serum-starvation, Glyceraldehyde AGEs were pipetted directly into the media for a final concentration of 50ug/mL. The cells were then placed in a 37°C cell culture incubator for the time specified for each experiment. The media containing the AGEs was then aspirated, cells were washed with PBS, and cell collection proceeded as described previously. Cells treated with the control AGE solution underwent the same treatment protocol.

**Proliferation Assays.** To look at the effects of AGE treatment on cell growth, a proliferation assay was performed. Cells were plated in triplicate in a 96 well format with 2,000 cells per well. Cells were allowed 24 hours to fully adhere to the bottom of the plate. The zero time point was fixed using 5% cold trichloroacetic acid incubated at 4°C for one hour. After incubation, the plate was washed four times with water and stored until conclusion of experiment. The remaining cells were either treated with 50ug/mL AGEs or left untreated and allowed to grow. The final time point was then fixed at 48 hours using 10% cold TCA as described previously.

Fixed plates were then stained with 0.4% sulforhodamine B diluted in 1% acetic acid for 30 minutes at room temperature and washed four times with 1% acetic acid. After allowing the plates to air dry, SRB staining was solubilized using 10mM Tris Base for five minutes with gentle agitation and absorbance was immediately read at 560nm using microplate reader (Bio-Tek Instruments Inc., Winooski, VT). Percentage growth was then quantified by normalizing 48 hour time point with corresponding zero time point.

**shRNA Mediated Knockdown of RAGE Function.** In order to verify that the proliferative effect observed was dependent upon the presence of RAGE, shRNA was used to stably knockdown RAGE in MCF7 cells. 150,000 MCF7 cells were plated into the wells of a 12 well plate and allowed to adhere to the plate for 24 hours. Various shRNA lentiviral vectors (Table 3) (Sigma-Aldrich CO. LLC, St. Louis, MO) were then added to the appropriate well with an anticipated multiplicity of infection (MOI) of 2 along with polybrene (8ug/mL). 24 hours after infection, the media was changed back to normal media. After 24 hours, media containing 2ug/mL puromycin was added to each well in order to select only the cells that were infected with the shRNA vector. After original selection, cells were cultured in media containing 200ng/mL puromycin. Knockdown of RAGE was verified using western blot analysis.

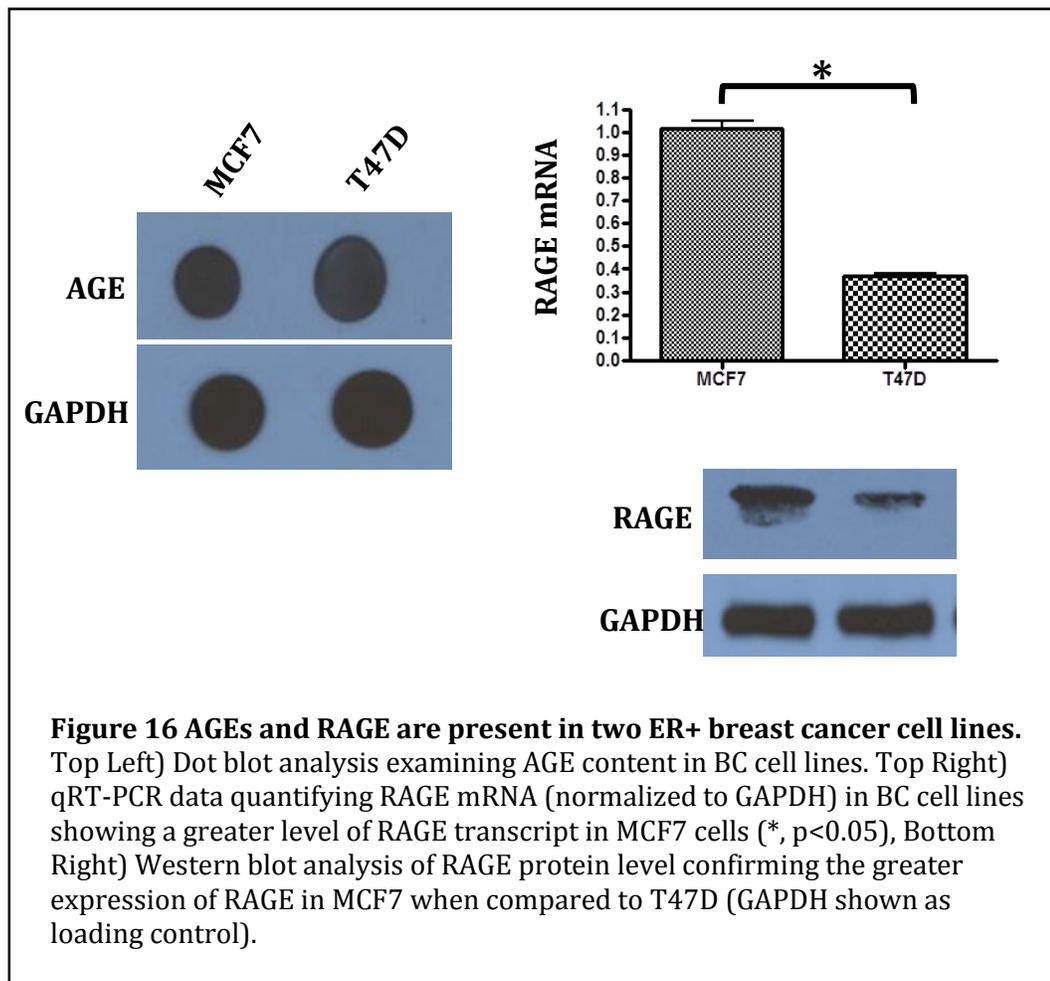
<b>Clone #</b>	<b>shRNA Sequence</b>
<b>165</b>	CCGGGCGGCTGGAATGGAACTGAACTCGAGTTCAGTTTCCATTCCAGCCGCTTTTTG
<b>878</b>	CCGGTGCTGATCCTCCCTGAGATAGCTCGAGCTATCTCAGGGAGGATCAGCATTTTTG
<b>582</b>	CCGGCAGACTGCAGTCGGAGCTAATCTCGAGATTAGCTCCGACTGCAGTGTGTTTTG
<b>963</b>	CCGGCCGTGCTGTCAGCATCAGCATCTCGAGATGCTGATGCTGACAGCACGGTTTTG

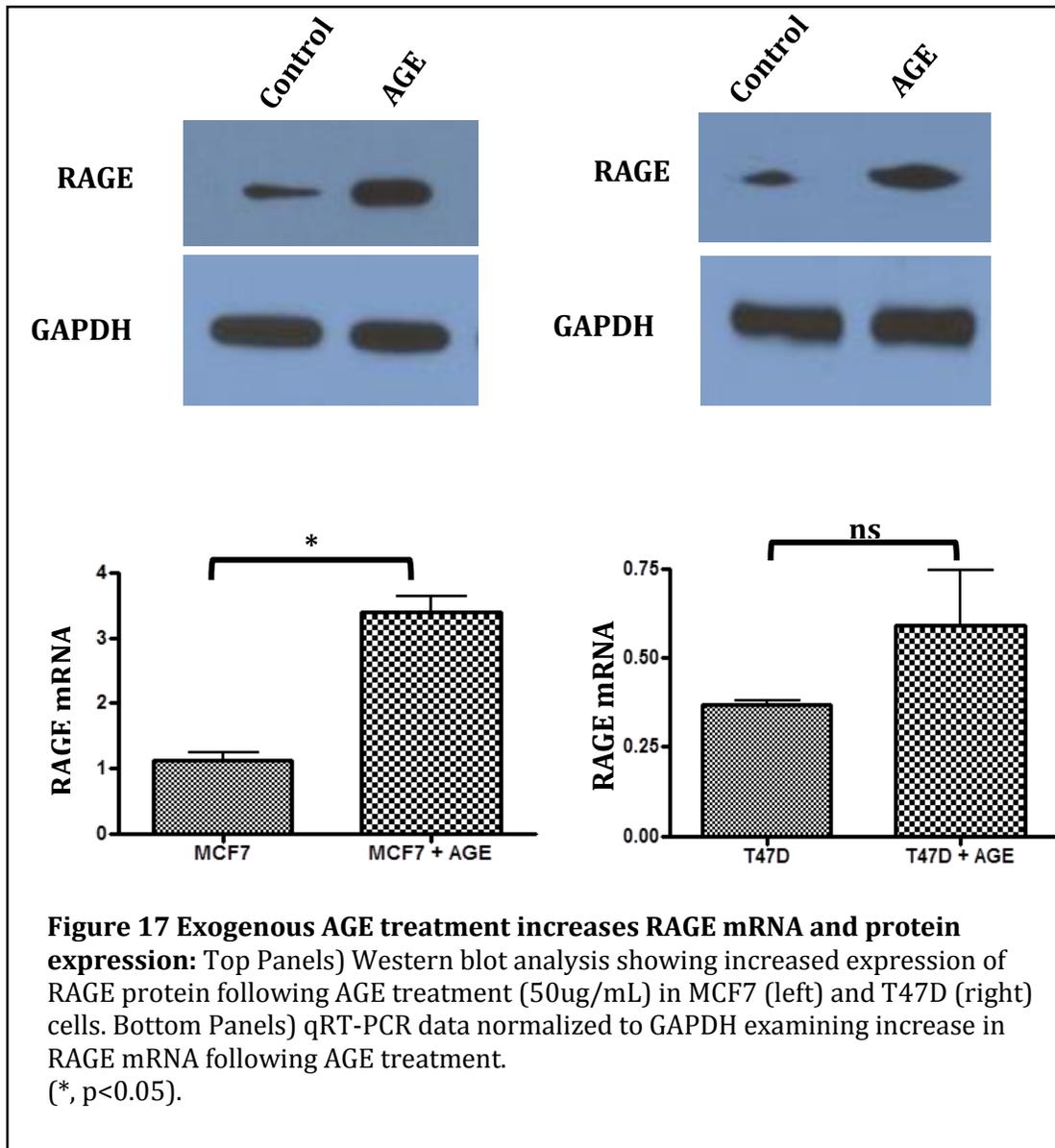
**Inhibition of Akt and ERK1/2.** So that we may confirm AGE mediated activation of the estrogen receptor is occurring through Akt and ERK1/2, we inhibited the functionality of both kinases using the molecular inhibitors LY294002 and U0126, respectively. MCF7 and T47D cells were cultured as described previously. Before AGE treatment and subsequent cell collection, cells were treated with 15uM of the appropriate inhibitor for 12 hours before being exposed to AGE treatment. Cells were collected and subjected to western blot analysis for phosphorylated ER $\alpha$  as described previously.

**AGE Treatment and Tamoxifen Resistance.** In order to investigate the effects of AGE signaling on tamoxifen resistance, we performed a cell viability assay by plating 3,000 cells per well in triplicate on a 96 well plate. The cells were allowed 24 hours to adhere to the plate and were then treated with varying doses of tamoxifen (0, 5, 10, 20uM) (Sigma Aldrich, St. Louis, MO) in combination with varying doses of AGEs (5ug/mL, 10ug/mL, 50ug/mL). After 24, 48, and 72 hours, SRB staining was used to quantify cell growth as described previously.

## Results and Discussion

**AGEs and RAGE are present in two ER+ breast cancer cell lines:** Dot blot analysis shows baseline levels of AGEs present in MCF7 and T47D cell lines (Left, Figure 16). Additionally, RAGE expression seems to be higher in the MCF7 cells when compared to the T47D cells on both the protein and transcript level measured by western blot and qRT-PCR analysis, respectively (Right, Figure 16). This also happens to correlate with GLO1 mRNA levels observed in specific aim #1 (Figure 11). These data suggest that AGEs and RAGE are present in ER+ breast cancer.





**RAGE mRNA and protein are upregulated following AGE treatment:** Following AGE treatment (50ug/mL for 5 minutes) in both cell lines, RAGE mRNA (bottom panels, Figure 17) and RAGE protein (top panels, Figure 17) were increased when compared to untreated controls. Increase in RAGE mRNA was statistically significant in MCF7 cells. The ability of our exogenous AGEs to upregulate the RAGE receptor is

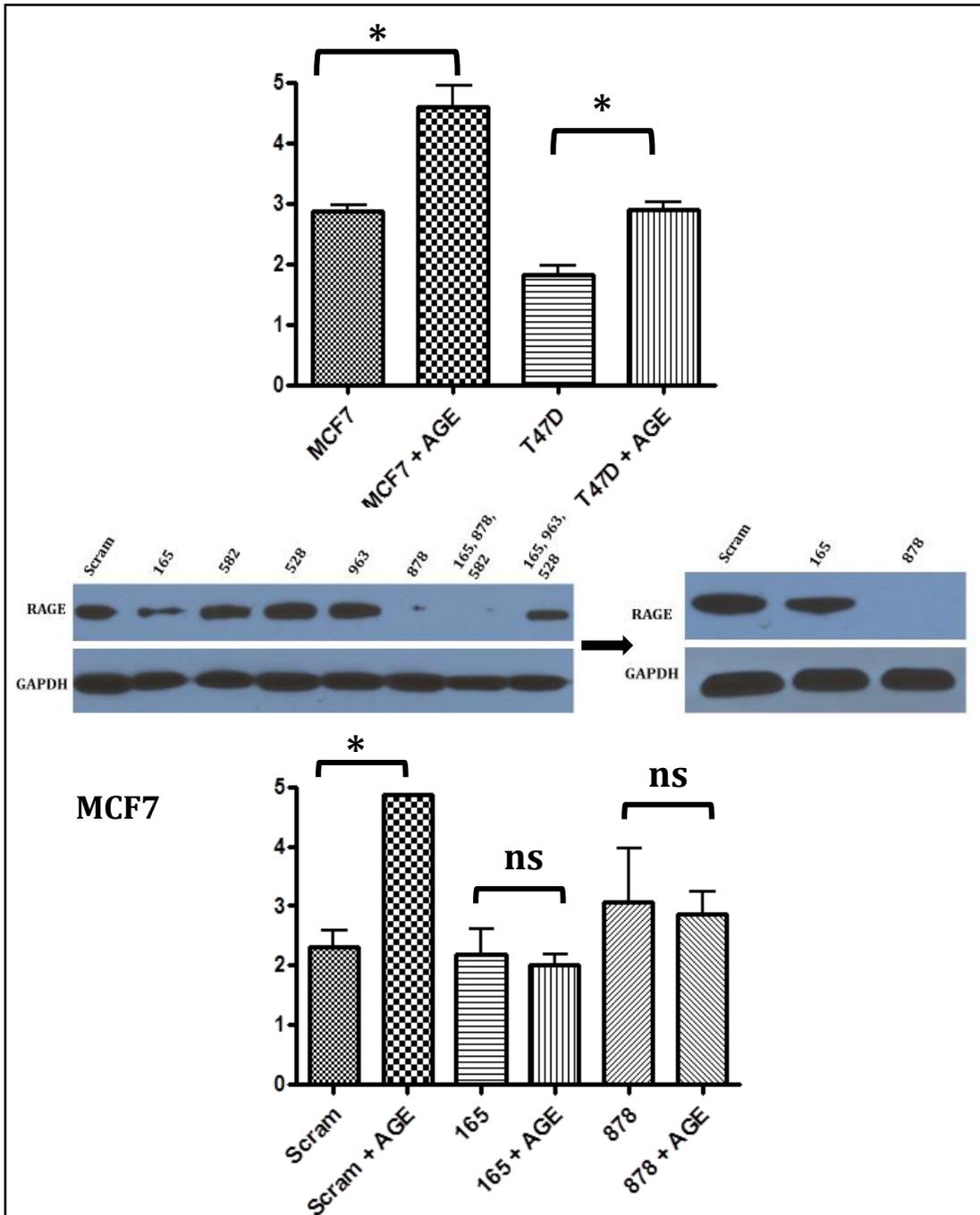
most likely a result of the positive feedback loop (discussed in background, Figure 8) and seeing these results indicate that we are able to manipulate the RAGE signaling pathway for the following experiments.

**AGEs signaling through RAGE result in more proliferative phenotype:** To gain a more functional perspective of the effects AGE-RAGE signaling has in ER+ breast cancer, we performed proliferation assays because uncontrolled proliferation is considered to be a hallmark of cancer. We utilized MCF7 and T47D cells and examined proliferation after 48 hours with or without AGEs (50ug/mL). In both cell lines, we found a statistically significant increase in growth in the presence of AGEs when compared to the untreated control (Top, Figure18).

We then needed to determine if AGEs were signaling through RAGE to produce this more proliferative phenotype. To do this, we utilized shRNA to stably knockdown RAGE. We began with a variety of different clones including two infections using a combination of shRNA. After successful infection and subsequent puromycin selection, western blot analysis was used to verify knockdown (Middle, Figure 18). We chose clone #165 as a partial knockdown model and #878 as an almost total knockdown. A repeat western blot in our chosen clones verified our results.

We performed the same proliferation assay as before to see if the increase in proliferation that we observed was attenuated when AGEs were unable to interact with the RAGE receptor (Bottom, Figure 18). In our scramble control, we observed

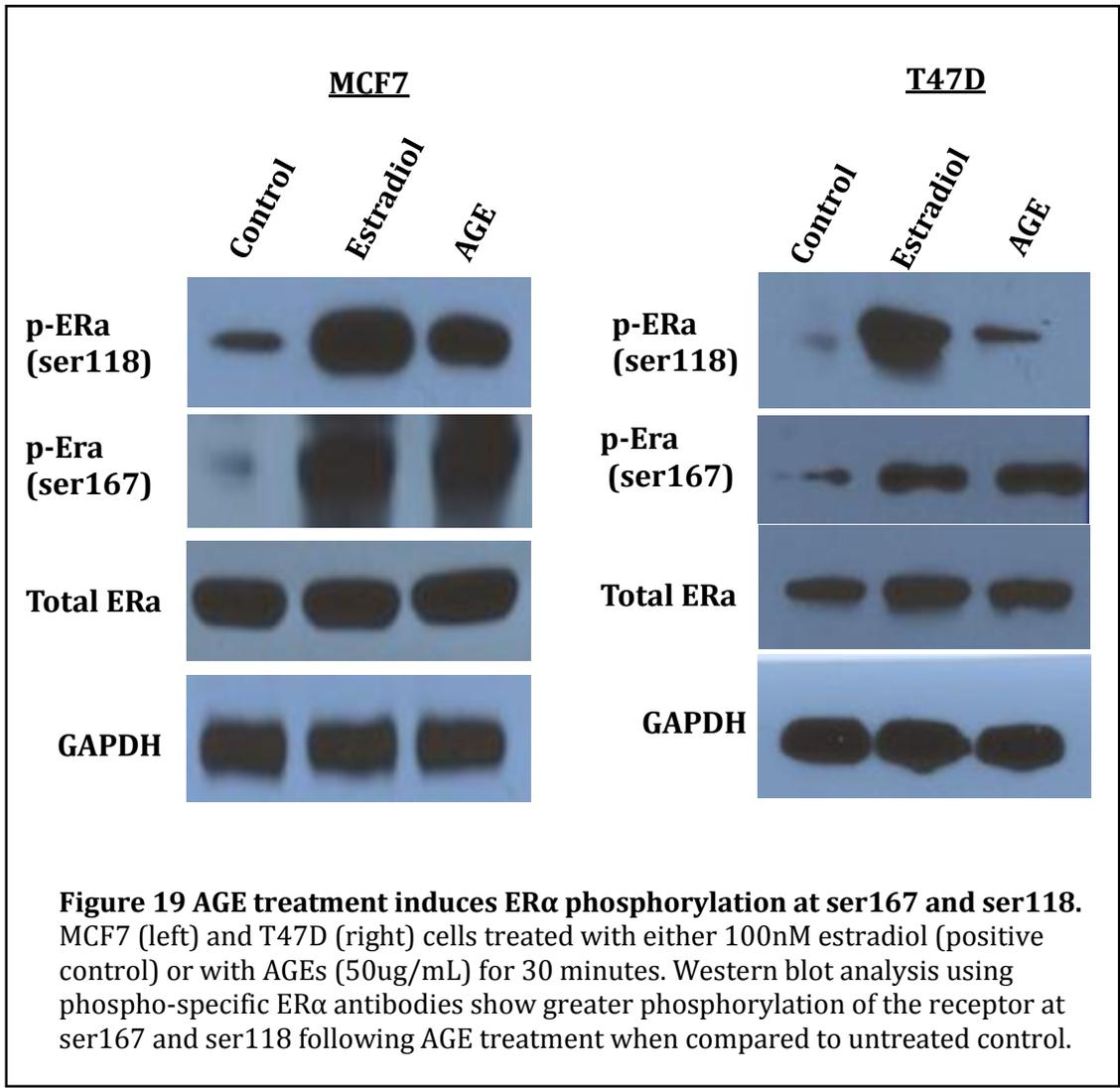
the same increase in proliferation that we saw in our wild type MCF7 and T47D cells. The RAGE knockdown clones, however, showed no difference between the AGE treated and untreated cells indicating that RAGE is necessary for the AGE-mediated increase in proliferation observed previously.

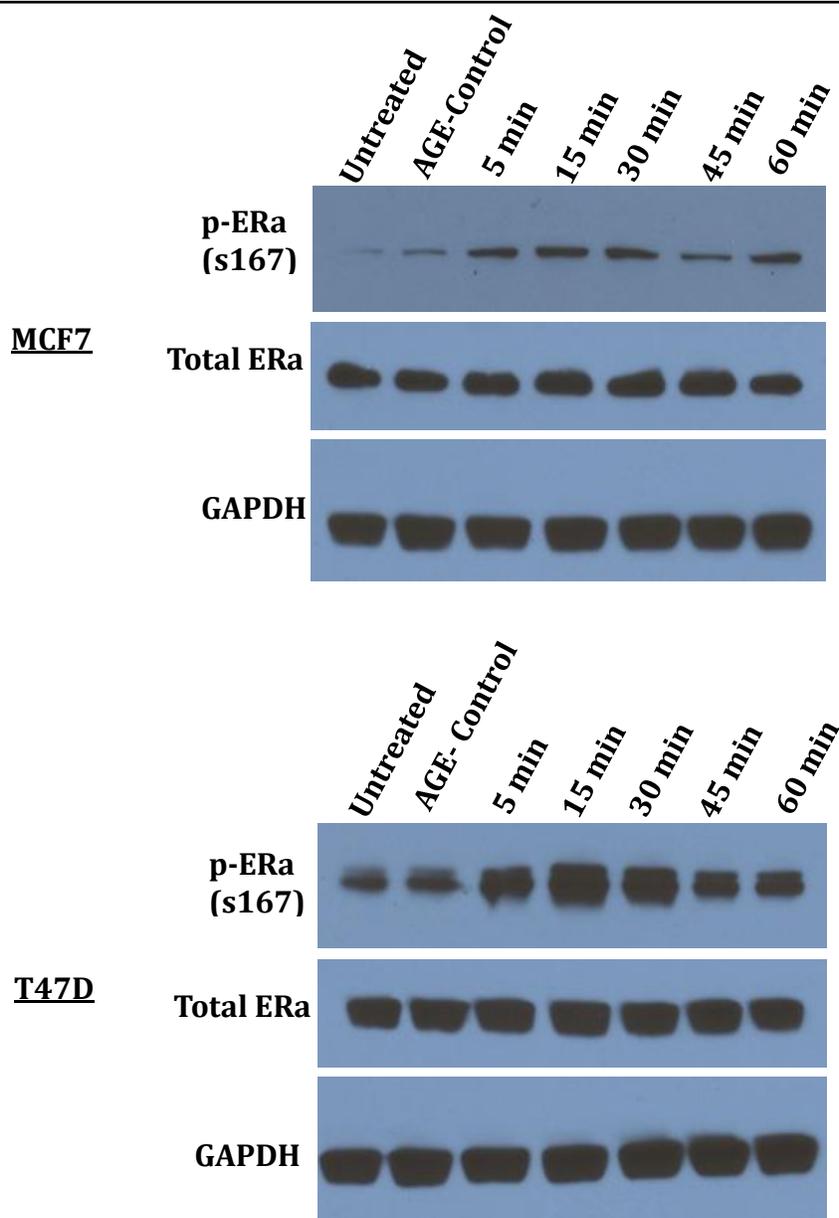


**Figure 18 AGE increases proliferation through interaction with RAGE receptor.** Top) Proliferation assay quantifying percentage growth after 48 hours in the presence of AGEs (50ug/mL). Increased proliferation occurs with AGEs when compared to untreated control (\*,  $p < 0.05$ ). Middle) Western blot analysis verifying shRNA mediated RAGE knockdown in MCF7 cells. Bottom) Proliferation assay in stable RAGE knockdown cell line. Proliferation was unaffected despite presence of AGEs (50ug/mL) in RAGE stable knockdowns (\*,  $p < 0.05$ ).

**AGE treatment induces ER $\alpha$  phosphorylation:** To begin examining AGEs influence on estrogen receptor-mediated pathways, we first needed to look into the effects on the estrogen receptor following AGE treatment. MCF7 and T47D cells were treated with 50ug/mL of AGEs for 30 minutes and were then examined for ser167 and ser118 phosphorylation. 100nM estradiol, one of the key estrogenic ligands for the receptor was used as a positive control to ensure optimization of phospho-specific antibodies. In both cell lines and at both serine residues, we found a significant increase in phosphorylation following AGE treatment when compared to untreated control (Figure 19). The estrogen independent phosphorylation of ER $\alpha$  at these residues has been shown to be indicative of tamoxifen resistance. Our data suggests that AGEs are capable of influencing these phosphorylation events, and therefore potentially contributing to tamoxifen resistance.

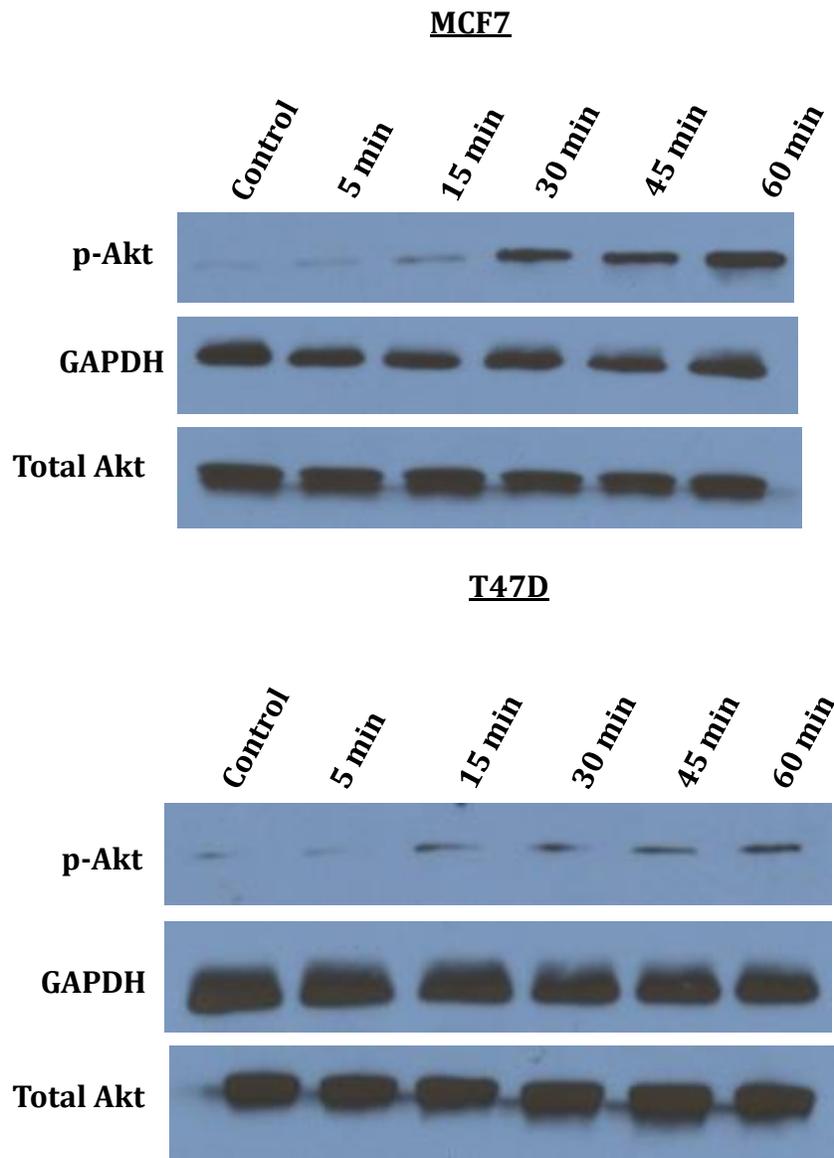
Additionally, we examined the influence of different time durations of AGE treatments on the phosphorylation of ER $\alpha$  at ser167 because of the transient nature of this post-translational modification. We did indeed find a time dependent activation of the receptor with a peak around 15-30 minutes following initial treatment (Figure 20).



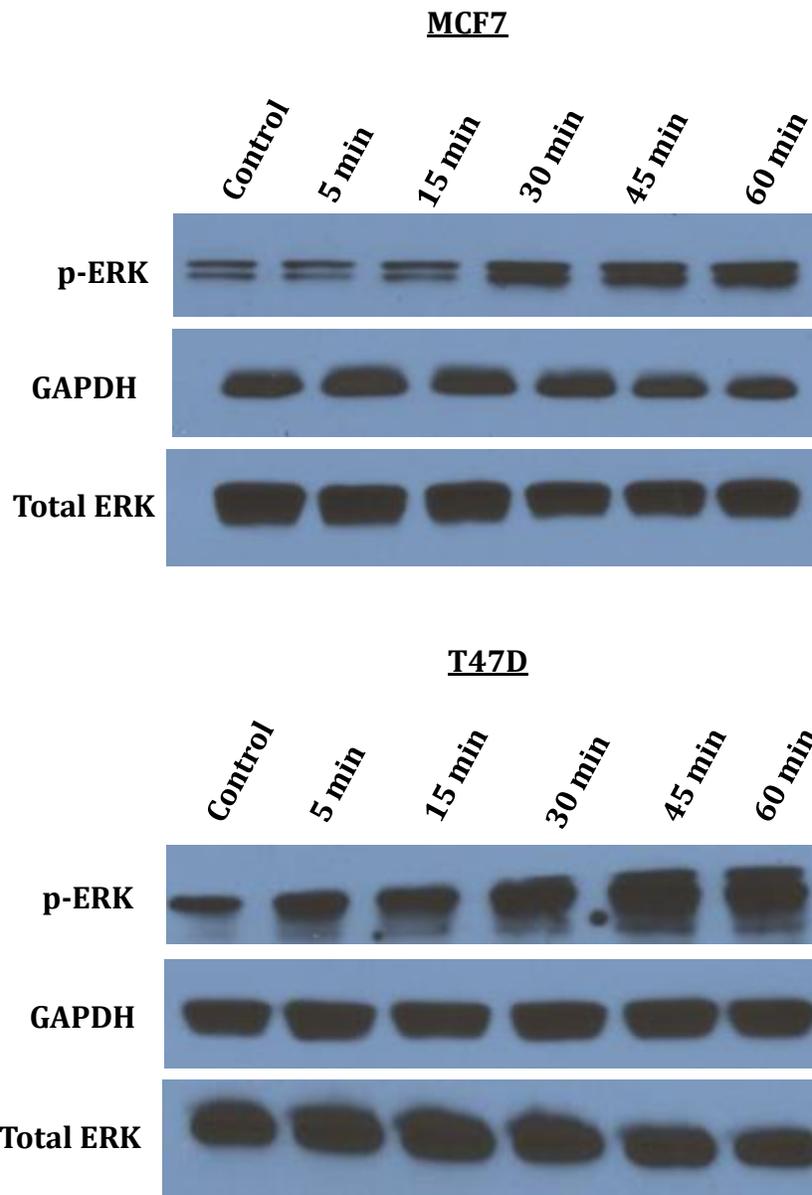


**Figure 20 Phosphorylation of ER $\alpha$  at ser167 occurs following AGE treatment in a time-dependent manner.** MCF7 (left) and T47D (right) cells were treated with AGEs (50ug/mL) or with AGE control for varying durations of time. Western blot analysis shows peak of phosphorylation around 15-30 minutes following AGE treatment.

**AGE treatment induces Akt and ERK phosphorylation:** Because we hypothesized that RAGE was signaling through Akt and ERK to mediate estrogen receptor phosphorylation, we needed to examine the effects of AGEs on activation of both these kinases. To investigate this, we again treated MCF7 and T47D cells with AGEs (50ug/mL) for varying time durations. In both cell lines, we found a correlation between Akt (Figure 21) and ERK (Figure 22) phosphorylation and duration of AGE treatment. The time-dependent manner in which these phosphorylation events occur indicate that sustained exposure to AGEs may induce pro-tumorigenic signaling pathways and influence estrogen-receptor mediated signaling in ER+ breast cancer.

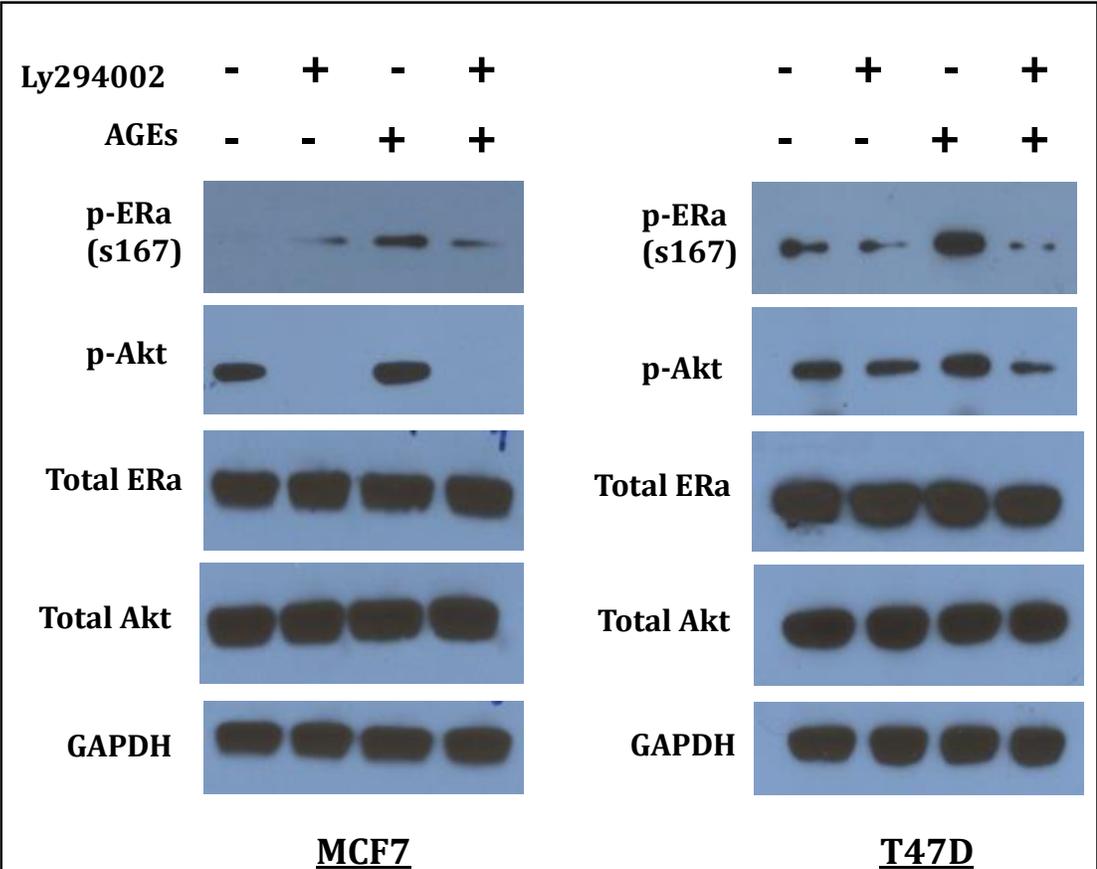


**Figure 21 AGE treatment induces phosphorylation of Protein Kinase B (Akt) in a time-dependent manner.** MCF7 (left) and T47D (right) cells were treated with AGEs (50ug/mL) for varying time durations. Western blot analysis shows correlation between Akt phosphorylation and duration of treatment suggesting AGEs influence Akt activation in ER+ breast cancer.

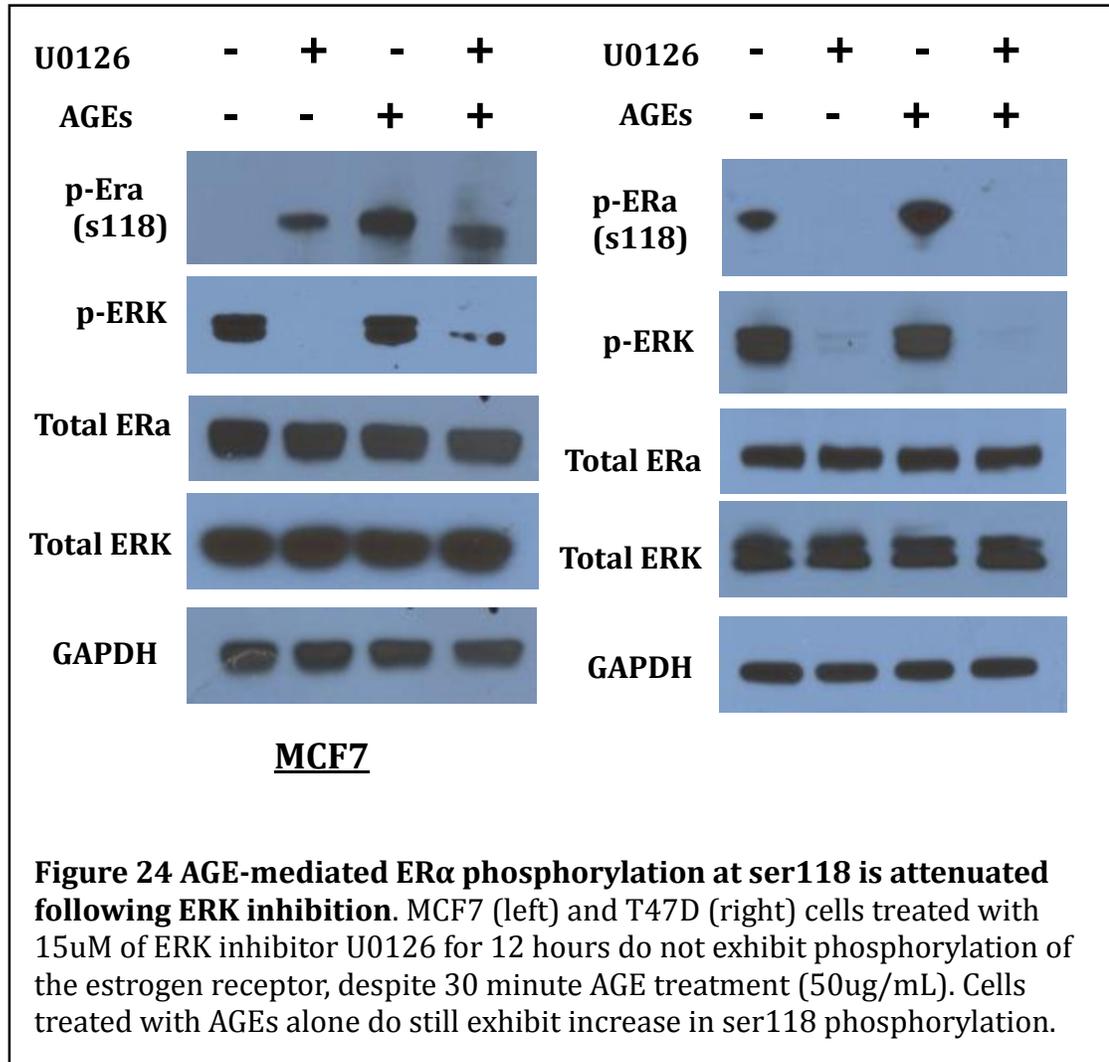


**Figure 22 AGE treatment induces phosphorylation of ERK1/2 in a time-dependent manner.** MCF7 (left) and T47D (right) cells were treated with AGEs (50ug/mL) for varying time durations. Western blot analysis shows correlation between ERK phosphorylation and duration of treatment suggesting AGEs influence ERK activation in ER+ breast cancer.

**Inhibition of Akt and ERK results in decreased ER $\alpha$  phosphorylation following AGE treatment:** To ensure that Akt and ERK were responsible for estrogen receptor phosphorylation, we needed to inhibit these two kinases and examine downstream effects. Using Ly294002, we successfully inhibited Akt phosphorylation (Figure 23) in both MCF7 and T47D cell lines. Western blot analysis probing for p-ER $\alpha$  at ser167 revealed that, despite being exposed to AGEs (50ug/mL) for thirty minutes before cell collection, little to no phosphorylation occurs when Akt is inhibited. We saw a similar result when using U0126 to suppress ERK activation (Figure 24). When ERK phosphorylation was successfully inhibited, phosphorylation of ER $\alpha$  at ser118 was attenuated, even after AGE treatment. This suggests that AGEs, through interaction with RAGE, signal through Akt and ERK to induce estrogen-independent activation of the estrogen receptor.

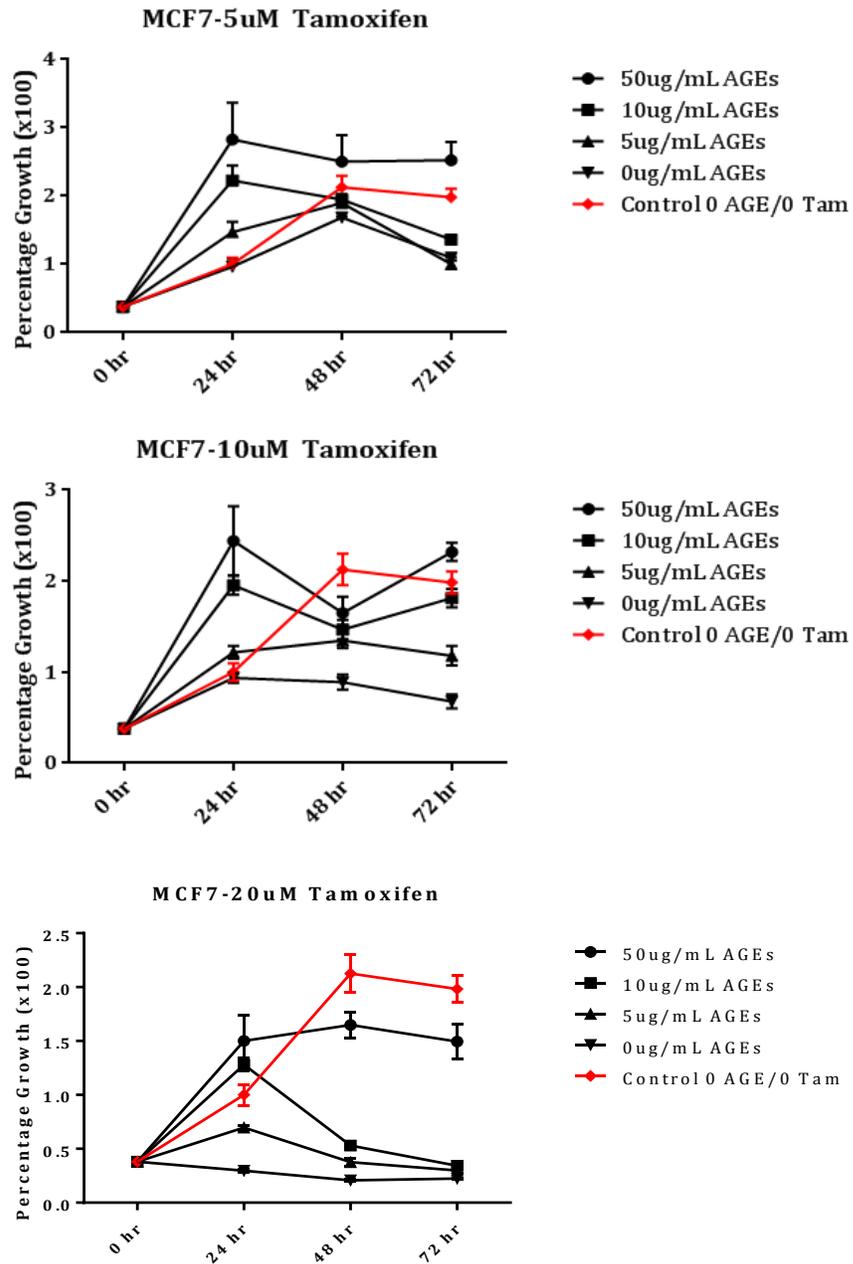


**Figure 23 AGE-mediated ER $\alpha$  phosphorylation at ser167 is attenuated following Akt inhibition.** MCF7 (left) and T47D (right) cells treated with 15 $\mu$ M of Akt inhibitor Ly294002 for 12 hours do not exhibit phosphorylation of the estrogen receptor, despite 30 minute AGE treatment (50 $\mu$ g/mL). Cells treated with AGEs alone do still exhibit increase in ser167 phosphorylation.

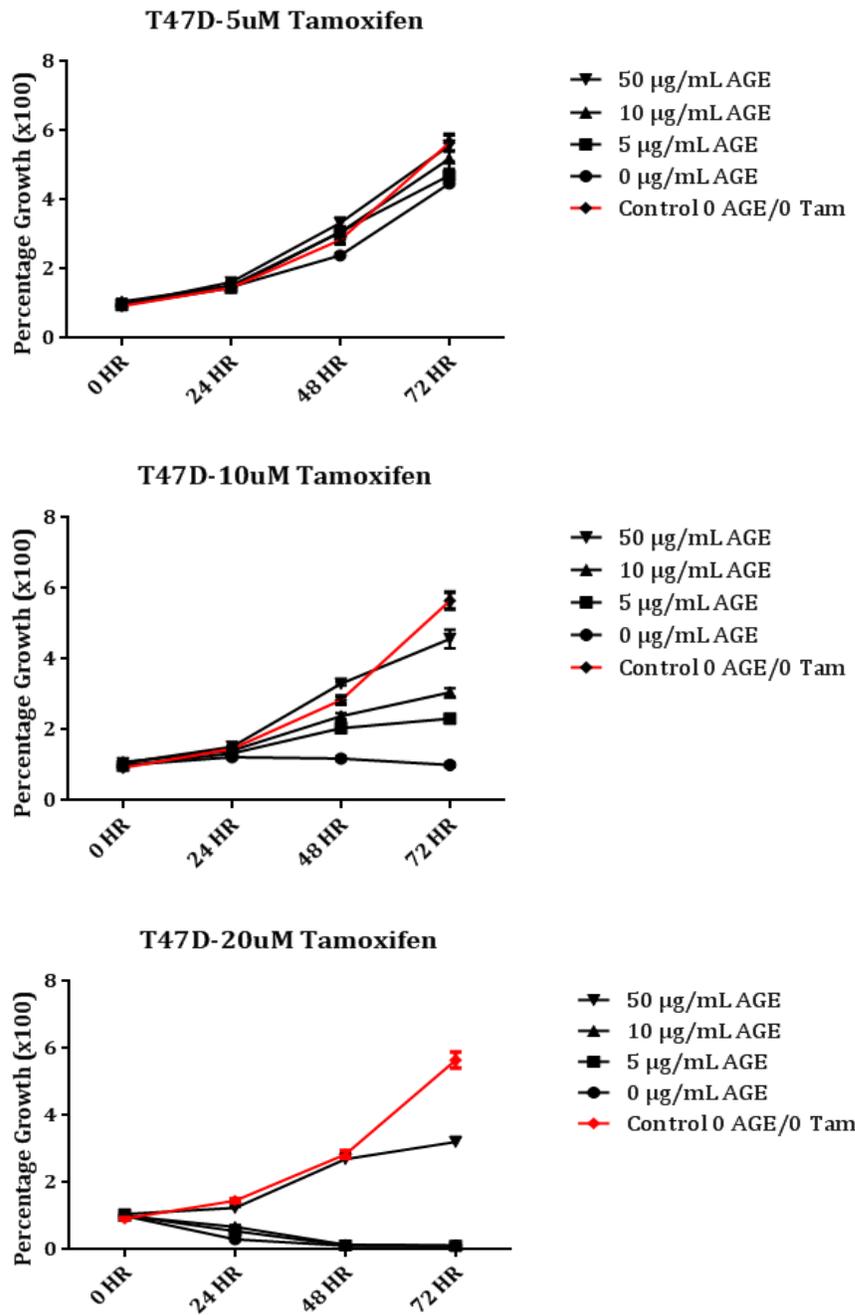


**AGE treated cells are more resistant to tamoxifen:** After identifying at least part of the pathway by which AGEs induce ER $\alpha$  phosphorylation, we wanted to investigate whether or not AGEs were, in fact, inducing tamoxifen resistance through this mechanism. We examined this by performing a proliferation assay. MCF7 and T47D cells were treated with varying levels of AGEs and varying levels of tamoxifen and were allowed to grow for 24, 48, and 72 hours (Figures 25 and 26). At the highest concentrations of tamoxifen (20uM), the 50ug/mL AGE treated cells

still remained viable in both cell lines. Additionally, resistance to the effects of 20uM tamoxifen occurred in an AGE dose-dependent manner in the MCF7 cells after 24 and 48 hours. Similar results occurred early on in the assay in the 5uM and 10uM tamoxifen treated MCF7 cells. Response in T47D cells, however seemed to be more dependent on AGE concentration later on in the assay in the 5uM and 10uM tamoxifen treated cells. All of this data suggests that AGEs do, in fact, contribute to tamoxifen resistance in vitro which may potentially occur by AGEs inducing ER $\alpha$  phosphorylation at ser167 and ser118 through Akt and ERK, respectively.



**Figure 25 AGE treated MCF7 cells exhibit more resistant phenotype following tamoxifen treatment.** Proliferation assay showing percentage growth in MCF7 cells treated with 5uM (top), 10uM (middle), and 20uM (bottom) tamoxifen and with varying concentration of AGEs. AGE treated cells, especially at higher concentrations, remained more viable when compared to AGE untreated cells despite treatment with tamoxifen.



**Figure 26 AGE treated T47D cells exhibit more resistant phenotype following tamoxifen treatment.** Proliferation assay showing percentage growth in T47D cells treated with 5uM (top), 10uM (middle), and 20uM (bottom) tamoxifen with varying concentration of AGEs. AGE treated cells, especially at higher concentrations, remained more viable when compared to AGE untreated cells despite treatment with tamoxifen.

## **Discussion and alternative approaches**

The goal of this aim is to identify a role for AGE-RAGE signaling in ER+ breast cancer progression and tamoxifen resistance. We have been able to determine that AGEs and RAGE are normally present in the ER+ breast cancer cell lines MCF7 and T47D. After exposing these two cell lines to AGEs, we have found that the expression of RAGE is increased on both the transcript and protein levels. Additionally, we showed that AGE treatment induces phosphorylation of ER $\alpha$  at ser167 and ser118 potentially through the activation of Akt and ERK, respectively. These two kinase cascades are frequently altered in cancer and influence cell growth and survival. Additionally, estrogen independent phosphorylation at these two residues are significant because they have been previously identified as indicators of poor clinical response to the SERM, tamoxifen. We have been able to show that AGEs do possess the capability to alter tamoxifen response in vitro which may have very important clinical implications in the future. In specific aim#1, we suggest that AGEs and RAGE are associated with increased progression in breast cancer. Using a more functional perspective with our breast cancer cell lines, we have shown AGE treatment increases proliferation in wild type MCF7 and T47D cell lines because of AGEs' ability to interact with their receptor, RAGE. This may explain why we observed an increased presence of AGEs and RAGE in the more progressed tumor samples.

While our in vitro work is an important foundation in investigating a role for AGEs in tamoxifen resistance, immortalized cell lines are not the ideal method for

examining drug sensitivity because it fails to address the complexity of the tumor microenvironment and intracellular signaling. We can only begin to scrape the surface of the numerous signaling pathways that influence a tumor's ability to grow and metastasize within the mammalian body. Because of this shortcoming of our model, *in vivo* mouse models in which tamoxifen treated mice are exposed to exogenous AGEs will be necessary in the future to reliably identify any of the AGE-mediated effects we observed in our cell lines.

In addition to the general failings of using an *in vitro* model to show clinically relevant data, it is also important to recognize the complexity of intracellular signaling itself. As stated previously, non-genomic action of ER $\alpha$  is a complex network of bi-directional cross-talk so it is not reasonable to identify a linear signaling pathway as we have suggested in this study. We can show this simple pathway is occurring, but it is incredibly plausible that several other proteins may be affecting the activation we are observing in our ER+ breast cancer cell lines. Additionally, the disparity in peak phosphorylation between Akt/ERK and ER $\alpha$  may indicate that ER $\alpha$  may be phosphorylating these kinases as well. Because of this, suggesting that AGE signaling is the cause of all these effects is indeed oversimplification.

Despite these shortcomings, we have shown data that suggests AGEs signaling through RAGE is causing activation of the estrogen receptor independently of estrogen, potentially leading to tamoxifen resistance through phosphorylation at ser167 and ser118 in ER+ breast cancer.

## **Future Experiments**

While the data attained in this study suggests a functional role for AGEs in breast cancer, many more studies need to be performed to reach a full conclusion. As stated previously, our tissue and serum samples need to be expanded into a wider set of patients with greater diversity. Doing so would allow us to investigate AGE differences between varying demographics. As discussed previously, African American women are more likely to die from their disease and it would be interesting to investigate a role for AGEs in this disparity. Additionally, since the focus of our study is on ER+ breast cancer for the most part, focusing in on this specific subtype would allow us to gain insight into the role of AGEs in specific ER+ disease progression.

Characterizing the specific signaling pathway by which AGEs influence tamoxifen sensitivity requires more gain of function and loss of function studies. By investigating knockdown and overexpression of RAGE protein in our tamoxifen treated cells, we can verify that the AGE mediated tamoxifen resistance is occurring because of RAGE signaling. It would also be important to investigate ER $\alpha$  signaling using immunofluorescence. It is known that the estrogen receptor can be located throughout the cell and its functionality can be dependent upon its location. It would be interesting to identify where the receptor is localizing following AGE treatment in order to gain more insight into exactly how AGEs are inducing these phosphorylation events. And, if we observe nuclear localization, identifying which target genes are being transcribed will allow us to gain a functional perspective as to

how increased proliferation and tamoxifen resistance are occurring. Additionally, several studies have documented development of tamoxifen resistant cell lines. Usage of such a cell line could allow us to examine basal levels of AGEs in these cells compared to tamoxifen sensitive cells as well as the effects of AGE inhibitors on rescuing tamoxifen sensitivity. Finally, breast cancer patients can also exhibit resistance to aromatase inhibitors, so it would be ideal to investigate whether or not AGEs are contributing to this phenotype as well.

A key goal to further the knowledge gained in this project is to investigate this signaling pathway in an in vivo model. It would be ideal to look at the effects of a high AGE diet on tamoxifen resistance in a breast cancer mouse model because of its preventative and clinical implications.

### **Significance of study**

Previously, several studies have implicated AGEs in the pathogenesis of a variety of different diseases including diabetes, cardiovascular disease, and Alzheimer's. AGE-RAGE signaling has only recently been shown to contribute to tumorigenic signaling in cancers such as colorectal, prostate, and pancreatic. Very little research, however, has investigated the effects of AGE and RAGE signaling in breast cancer. It is important to elucidate the role of these reactive metabolites in breast cancer disease progression because of our ability to consume exogenous AGEs. By showing that high levels of AGEs leads to a more progressed disease, our data could potentially possess preventative implications in breast cancer. It is

already known that obesity is a risk factor for development and reoccurrence in breast cancer and our data could possibly offer a mechanistic explanation for this.

In addition to simply showing this association between AGEs and breast cancer progression, identifying a mechanism for tamoxifen resistance is both a widely investigated and severely misunderstood field of study. About 70% of all invasive breast cancers express the estrogen receptor, making it of utmost importance to create more effective treatments for these patients. By characterizing a functional role of AGEs in the development of tamoxifen resistance, targeted therapies for this type of cancer can potentially become more effective, leading to an improved prognosis. This could occur through modification of diet or through AGE inhibition with drugs already on the market in tamoxifen treated patients. Many studies have shown an association between certain estrogen independent phosphorylation sites on ER $\alpha$ . Here, we have shown that two of these identified residues are activated in response to AGE exposure and that AGE treatment reduces cell sensitivity to tamoxifen treatment. By identifying an effect on sensitivity to this drug, we can add to the existing knowledge base that is reinforcing the need for more personalized medicine in cancer.

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