Chemopreventive Activity of Tocopherols in Mammary Tumorigenesis

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ABSTRACT OF THE DISSERTATION

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Vitamin E is a dietary micronutrient that is recognized as a lipid-soluble antioxidant and suggested to reduce cancer risk. Tocopherol, a member of the vitamin E family, consists of four forms designated as α, β, γ, and δ. Several large cancer prevention studies with α-tocopherol have reported no beneficial results. Recent laboratory studies have suggested that γ-enriched mixed tocopherols (γ-TmT), δ-tocopherol and γ-tocopherol inhibit cancer by inhibiting cell proliferation and inducing apoptosis. The purpose of this thesis is to characterize the cancer preventive activities of γ-TmT and individual (α-, γ-, δ-) tocopherols in the prevention of two subtypes of breast cancer: estrogen receptor (ER) positive and human epidermal growth factor 2 (HER2) positive.

As a complex and heterogeneous disease, breast cancer is divided into subtypes such as ER positive, HER2 positive, or basal-like. Animal models are utilized to define etiology of breast cancer and generate new prevention and treatment strategies.

In three different animal models of breast cancer, the chemopreventive activities of γ-TmT or individual tocopherols (α, δ or γ) were assessed. Due to their sensitivity to 17β-estradiol (E$_2$) to induce mammary hyperplasia, female August Copenhagen Irish (ACI) rats were utilized. Immunohistochemical analysis of the mammary glands revealed a decrease in estrogen receptor α (ERα) and proliferating cell nuclear antigen (PCNA), while there was an increase in cleaved-

caspase 3, peroxisome proliferator activated receptor γ (PPARγ), and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) in γ-TmT treated rats.

In a second animal model, individual δ- and γ-tocopherols inhibited hormone-dependent mammary tumorigenesis in N-methyl-N-nitrosourea (NMU)-treated female Sprague Dawley rats, whereas α-tocopherol did not decrease tumor burden. In mammary tumors, markers of cell proliferation (PCNA, PKCα), survival (PPARγ, PTEN, phospho-Akt) and cell cycle (p53, p21) were affected by δ- and γ-tocopherols.

However, in the third animal model, administration of individual tocopherols did not prevent HER2/neu-driven tumorigenesis. There were modest effects by γ-tocopherols on increased tumor latency, but the overall tumor burden was not significantly decreased.

In conclusion, δ-tocopherol, γ-tocopherol and γ-TmT, but not α-tocopherol, have exhibited chemopreventive properties in two estrogen-dependent animal models, but not in transgenic HER2-driven breast cancer.

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1,25(OH)2D3	1α,25-dihydroxyvitamin D3
15-S-HETE	15-S-hydroxyeicosatetraenoic acid
2-OHE2	2-hydroxyestradiol
3-MC	3-methylcholanthrene
4-OHE2	4-hydroxyestradiol
8-oxo-dG	8-hydroxy-2'-deoxyguanosine
9cRA	9-cis-retinoic acid
α-TTP	α-tocopherol transfer protein
γ-TmT	γ-enriched tocopherol mixture
ACI	August Copenhagen Irish
Apaf-1	Apoptotic protease activating factor 1
ARE	Anti-oxidant-responsive element
ATBC	Alpha-Tocopherol, Beta-Carotene
BAX	Bcl-2-associated X protein
Bcl2	B cell lymphoma 2
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
c-Casp	Cleaved-caspase
c-PARP	Cleaved-Poly (ADP-ribose) polymerase
CDK	Cyclin dependent kinase
CDMDHC	Carboxydimethyldecyl hydroxychromans
CEHC	Carboxyethyl hydroxychromans
CMBHC	Carboxymethylbutyl hydroxychromans
COMT	Catechol o-methyltransferase
COX	Cyclooxygenase

DMBA	12-dimethylbenz(a)anthracene
E_2	17β-estradiol
EGFR	Epidermal growth factor receptor
EPIC	European Prospective Investigation into Cancer and Nutrition
ER	Estrogen receptor
ERE	Estrogen response element
ErbB	Epidermal growth factor
GAB1	GRB2-associated binding protein 1
GClm	Glutamate cysteine ligase, modifier subunit
GEM	Genetically engineered mice
GPx	Glutathione peroxidase
GRB2	Growth factor receptor bound 2
GST	Glutathione s-transferases
H&E	Hematoxylin & eosin
HER2	Human epidermal growth factor receptor-2
HO-1	Heme oxygenase-1
HPLC	High performance liquid chromatography
ICAM-1	Intracellular adhesion molecule-1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
I.P.	Intraperitoneal
KEAP1	Kelch-like-ECH-associated protein 1
LH	Luteinizing hormone
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
MAPK	Mitogen-activated protein kinase

MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumor virus
MT	Metallothionein
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMU	N-methyl-N-nitrosourea
NO	Nitric oxide
NO_2	Nitrogen dioxide
NQO1	NAD(P)H dehydrogenase, quinone 1
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
Nu	Nude
p-Akt	Phospho-Akt
PARP	Poly ADP-ribose polymerase 1
PCNA	Proliferating cell nuclear antigen
PEARL	Postmenopausal Evaluation and Risk-Reduction with Lasofoxifene
PGE_2	Prostaglandin E_2
PI3K	Phosphatidylinositol 3-kinases
PKC	Protein kinase C
PPAR	Peroxisome preoliferator-activated receptor
PR	Progesterone receptor
PTEN	phosphatase and tensin homologue
PyMT	Polyoma middle T antigen
RAR	Retinoic acid receptors
Rb	Retinoblastoma
RCT	Randomized controlled trials
ROS	Reactive oxygen species
RNS	Reactive nitrogen species

RXR   Retinoid X receptors

SCID   Severe combined immunodeficiency

SD   Sprague-Dawley

SELECT   Selenium and Vitamin E Cancer Prevention Trial

SERDs   Selective estrogen receptor down-regulators

SERMs   Selective estrogen receptor modulators

SOD   Superoxide dismutase

STAR   Study of Tamoxifen and Raloxifene trial

SV40   Simian virus 40

TAG   Large T antigen

TAP   Tocopherol-associated protein

TBP   Tocopherol-binding protein

TNF-α Tumor necrosis factor α

TR   Thyroid hormone receptors

TRAMP   Transgenic adenocarcinoma of the mouse prostate

TXN   Thioredoxin

UGT   UDP-glucuronosyltransferase

VDR   Vitamin D receptor

VEGF   Vascular endothelial growth factor

WAP   Whey acidic protein

XIAP   X-linked inhibitor of apoptosis

Chapter 1: Introduction[a,b]

1. Introduction

Cancer is a major public health problem where 1 in 4 deaths in the United States is due to cancer [1]. In 2012, it was estimated that 1,638,910 new cases of cancer will be diagnosed and 577,190 deaths in the United States [1]. Breast cancer is the most frequently diagnosed malignancy and a leading cause of cancer death among women [2]. Lifestyle risk factors for breast cancer include obesity, lack of exercise, alcohol, and diet high in saturated fat [2,3]. A poor diet is estimated to be responsible for 15% to 35% of all cancer deaths [4]. Vitamins and phytochemicals from fruits and vegetables may play a significant role in the prevention of cancer [5]. Micronutrients may control intracellular events such as antioxidant activity, anti-inflammatory activity, and induction of apoptosis to reduce carcinogenesis [6]. One such dietary micronutrient is vitamin E. Vitamin E is recognized as a lipid-soluble antioxidant that has been suggested to reduce cancer risk [7].

1.1. Subtypes of breast cancer

Breast cancer is a heterogeneous disease that can be classified into subtypes based on immunohistochemical and molecular markers (Fig. 1.1). The four main breast cancer subtypes are: estrogen receptor (ER) positive luminal A, ER positive luminal B, human epidermal growth factor receptor-2 (HER2) positive, and triple negative [8]. Triple negative is referred to as lacking the expression of ER, progesterone receptor (PR) and HER2, and is also known as basal-like. Breast tumorigenesis is often hormonally driven and the majority of tumors express estrogen,

progesterone, and/or HER-2/neu receptors which increases the number of therapeutic options and have improved prognoses. However, the etiology and pathogenesis of breast cancer remains poorly understood. Recently, 510 human breast cancer tumors from 507 patients were sequenced, and identified 30,626 somatic mutations [9]. Genes that have been implicated in breast cancer (*PIK3CA, PTEN, AKT1, TP53* and *GATA3*) and novel mutated genes (*TBX3, RUNX1, CBFB, AFF2* and *NF1*) were identified [9]. Somatic mutations in *TP53, PIK3CA* and *GATA3* account for >10% incidence across all breast cancers [9]. Overall, mutated genes were considerably more diverse and recurrent within luminal A and luminal B tumors than basal-like and HER2 subtypes; however, the overall mutation rate was lowest in luminal A subtype and highest in the basal-like and HER2 subtypes [9]. Specific mutations for the different subtypes of breast cancer will be addressed in the subsequent sections.

1.1.1 Estrogen receptor (ER) positive

ER positive tumors are classified as a luminal subtype of breast cancer and are reported in 60-70% of cases [8]. Luminal tumors activate ER-responsive genes, other genes that encode characteristic proteins of luminal epithelial cells of origin, and express luminal cytokeratin 8/18 [8]. Luminal A subtype is either ER positive or progesterone receptor (PR) positive but is negative for HER2. Luminal B subtype can be classified as ER positive or PR positive and is positive for HER2 [10]. In the luminal A subtype, the most significantly mutated genes were *PIK3CA* (49%), *Cyclin D1* (14%), *MAP3K1* (14%), *GATA3*(14%), *PTEN* (13%), *TP53* (12%) and *INPP4B* (9%) [9]. In the luminal B subtype, *Cyclin D1* (58%), *TP53* (32%), *PIK3CA* (32%), *MDM2* (31%), *CDK4* (25%), *PTEN* (24%), *INPP4B* (16%) and *MAP3K1* (5%) were the most frequent mutations [9]. The prognosis for luminal A is better than luminal B and typically responds more effectively to selective estrogen receptor modulators (SERMs), such as tamoxifen [8].

Luminal A and B subtypes of breast cancer are ER positive. The interaction of ER with the estrogen response element (ERE) results in the modulation of specific gene expression, through which the physiological actions of estrogens are manifested. Estrogens acting via the ER dramatically escalates proliferative and metastatic activity in tumor cells via the induction of growth factors, proteases and basement membrane receptors [11].

There are two known ER: ERα and ERβ. ERα is widely expressed in endometrium, breast, ovarian stroma, and the hypothalamus, while expression of ERβ is located in endothelial, brain, bone, kidney, heart, lungs, intestinal mucosa and prostate cells [11]. Due to alternative splicing, at least three ERα and five ERβ isoforms are known to exist. Estrogen receptors can form both homodimers and heterodimers, although not all cells types express both receptors and thus the number of combinations is limited in specific organ cell types. A high ERα/ERβ ratio correlates with more cell proliferation, whereas higher levels of ERβ correlate with lower levels of proliferation [12]. Specifically, ERα interacts with a number of proteins including c-Src, p85 subunit of phosphatidylinositol 3-kinases (PI3K), caveolin 1, EGFR and HER2 and activates the mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways [13]. This results in cell growth and inhibition of apoptosis, especially in breast cancer cells.

Besides endogenous estrogens, chemicals, drugs and environmental pollutants are also ligands for ERα and ERβ. Xenoestrogens, synthetic or natural chemical compounds which exert estrogenic effects, can either activate or inhibit ER-mediated transcription and are termed SERMs [14]. SERMs such as diethylstilbestrol, bisphenol A and 2, 3, 7, 8-Tetrachlorodibenzodoxin (TCDD) induce mammary tumorigenesis, while resveratrol and genistein are chemopreventive compounds [15]. For example, genistein binds to ERβ with greater affinity than ERα [16]. Although some studies have shown that genistein increases the rate of cell proliferation in MCF-7 and HER2 transfected MCF-7 cells [17,18], genistein inhibited tumor multiplicity in 7,12-

dimethylbenz(a)anthracene (DMBA)-treated SD rats [19,20]. Furthermore, levels of cyclin D1, Akt and HER2 were reduced while the levels of phosphatase and tensin homologue (PTEN) increased in DMBA treated SD rats when treated prepubertal genistein dietary exposure [15,21].

Pharmaceutical SERMs are designed for their potency and selectivity. Depending on their treatment use, these SERMs can be ER agonists, antagonists, or partial agonists/antagonists (agonist in one or more tissues and antagonist in another) [22]. On the molecular level, SERMs bind to the ligand binding domain (AF-2) on the ER, and cause a conformational change which is different than the change produced by estrogens [23]. This altered conformation prevents the co-activators from binding to AF-2, which blocks the trans-activation function of the receptors [23].

Tamoxifen is an antiestrogen that is widely used in the treatment of breast cancer and has a high affinity to binding the ER [11]. The mechanism of tamoxifen is thought to exert its antitumor effects by competitively antagonizing the binding of E_2 to ER, which leads to the inhibition of gene transcription and protein synthesis [22]. As a first generation SERM, there were some unexpected side effects while using tamoxifen. Even though tamoxifen is an antagonist in breast tissue, it acts as partial agonist on the endometrium and has been linked to endometrial cancer in some women [24]. The Study of Tamoxifen and Raloxifene trial (STAR) demonstrated that the drug raloxifene, another SERM, works as well as tamoxifen in reducing breast cancer risk for postmenopausal women [24]. Furthermore, raloxifene was more effective than tamoxifen in lowering the risk for thromboembolic events and cataracts, while both drugs had similar risks for other cancers, fractures, ischemic heart disease and stroke [24]. Tamoxifen and raloxifene were shown to maintain bone density and reduce invasive breast cancer; however tamoxifen has been linked to endometrial cancer while raloxifene did not increase the incidence of endometrial cancer in human studies [12,24]. Both drugs were approved by the FDA for the treatment and prevention of osteoporosis in postmenopausal women and for the reduction of invasive breast cancer [12,24].

Recently, the development of third generation SERMs showed beneficial estrogen effects on the bone without the detrimental stimulation on the endometrium or breast tissue [12]. The Postmenopausal Evaluation and Risk-Reduction with Lasofoxifene (PEARL) study evaluated lasofoxifene and five years of treatment and concluded that a 0.5-mg dose of lasofoxifene appears to reduce the risks of both total and ER positive invasive breast cancer in postmenopausal women with osteoporosis [25].

Besides SERMs, selective estrogen receptor down-regulators (SERDs) such as fulvestrant [26], aromatase inhibitors such as aminoglutethimide [27], and leutinizing hormone releasing agonists like buserelin [28] and goserelin [29] are also utilized for the treatment of hormonal breast cancer. Aromatase inhibitors are superior to tamoxifen in both toxicity and efficacy [30]. Aromatase inhibitors block the production of estrogens from androgens, which is the main pathway of estrogen production in post-menopausal and non-pregnant women [27]. Aminoglutethimide is a first generation aromatase inhibitor, but is non-specific and can inhibit estrogen synthesis in many other tissues apart from the breast itself [27]. Second generation aromatase inhibitors (anastrozole, letrozole and exemestane) have higher potency and specificity and have promising results in advanced breast cancer studies [30]. To date, new agents with better efficacy, no effects on the endometrial tissues and bypassing hormone resistance problems are being introduced and could replace tamoxifen as the standard treatment for hormonal breast cancers [12].

1.1.2 Human epidermal growth factor receptor 2 (HER2) positive

HER2 amplification and overexpression has been reported in 18-25% of human breast cancers [31]. HER2 positive breast cancer can be characterized as HER2 positive, negative for ER, and poor differentiation [32]. The prognosis for HER2 positive is worse than luminal breast cancers. Recently, molecular mutations were observed and reported in human breast cancer

tumors. The genes most commonly mutated in HER2 breast tumors were: *TP53* (75%), *PIK3CA* (42%), *Cyclin D1* (38%), *MDM2* (30%), *INPP4B* (30%), *CDK4* (24%) and *PTEN* (19%) [9].

HER2 is a member of the epidermal growth factor (ErbB) family of transmembrane receptors which are potent mediators of normal cell growth and development [33]. The ErbB family is classified as a tyrosine kinase receptor and consists of EGFR (HER1), ERBB2 (HER2), ERBB3 (HER3), and ERBB4 (HER4). The structure consists of an extracellular domain at which the ligand binding occurs, the α-helical transmembrane segment, and the intracellular protein tyrosine kinase domain [34]. HER receptors normally exist as inactive monomers until a ligand initiates a conformational change to induce dimerization with another receptor. HER2 is unique as it already possesses an active tyrosine kinase domain and has no direct ligand while HER3 lacks an intrinsic tyrosine kinase activity and cannot form homodimers with itself [35].

The HER2-HER3 heterodimer is considered the most potent and active ErbB dimer [36-38]. HER2 signaling leads to oncogenic cell survival and proliferation through the MAPK pathway [39]. HER3 can directly bind to the p85 subunit of PI3K to stimulate the PI3K-Akt pathway while EGFR and HER2 have additional activation steps by binding to the adaptor proteins GRB2 (growth factor receptor bound 2) and GAB1 (GRB2-associated binding protein 1) [40]. Thus, the HER2-HER3 dimer leads to the MAPK pathway to stimulate angiogenesis, proliferation, and PI3K-Akt pathway to promote cell survival, suppression of apoptosis, and cell cycle control [39]. The EGFR/HER2 dimer mainly signals through the MAPK pathway enhancing cell proliferation through the upregulation Cyclin D1 and degradation of the cyclin-dependent kinase (CDK) inhibitor, p27 [32].

HER2 positive breast cancer may be treated with monoclonal antibodies such as trastuzumab which binds to domain IV on the HER2 receptor [32]. Other treatments include monoclonal antibody pertuzumab (binds to domain II of the HER2 receptor) [41], trastuzumab antibody conjugated with mertansine (DM1), which is internalized and exerts its cytotoxic effects inside the cell [42], tyrosine kinase inhibitors [43], and HSP90 inhibition which leads to

proteasomal degradation [44]. Despite the proven clinical benefit of trastuzumab, HER2 positive tumors resistance is becoming more common [45].

One theory for drug resistance includes evasion and decreased binding of the drug by the HER2 receptor itself [45,46]. Another theory for drug resistance is the upregulation of alternate pathways to compensate for the decrease in signaling from the drug [45,46]. There are numerous new agents for the treatment of HER2 positive breast cancer, most of which are modified antibodies that target the HER2 receptor [32,45,46]. There is a need to understand HER2 signaling pathway and drug resistance to further treat HER2 positive breast cancer.

1.1.3 Basal-Like

The basal-like subtype was discovered nearly a decade ago by first-generation cDNA microarrays [47]. These tumors are often referred to as triple negative breast cancers since basal-like tumors are typically negative for ER, PR and HER2, have high expression of basal stratified epithelial cytokeratins 5, 6, and 17, high expression of epidermal growth factor receptor (EGFR) and have expression of proliferation-related genes [8,48]. According to a recent analysis of the molecular mutations in basal-like breast tumors, mutations occurred in the following genes: *TP53* (84%), *MYC* (40%), *PTEN* (35%), *RB1* (20%), *MDM2* (14%), *cyclin E1* (9%) and *PIK3CA* (7%) [9]. The prognosis of basal-like tumors is poor since tumors express a high nuclear grade, large tumor size and have a high frequency of *TP53* mutations [49]. In addition to the loss of TP53, molecular analysis of basal-like tumors reconfirmed frequent loss of *RB1* and *BRCA1* [9]. BRCA1 is a tumor suppressor that is a DNA damage response protein and is responsible for repairing double-stranded DNA breaks, while another tumor suppressor, BRCA2, is a mediator of homologous recombination [48,49].

Recently, a subtype of triple negative breast cancer was identified by gene profile studies and named Claudin-low [50]. Claudin-low comprises of 25-39% of triple negative breast cancers

and is characterized by the low to absent expression of luminal differentiation markers, high expression of epithelial-to-mesenchymal transition markers, immune response genes and cancer stem cell-like features [50]. Claudin proteins are an integral part for tight junctions that link the potential space between adjacent epithelial cells and epithelial cell adhesion molecules (E-cadherin, EpCAM and mucin-1) [50].

The incidence of triple negative breast cancer may be increased by both race and age, where premenopausal African American women developed triple negative tumors (39%) compared to postmenopausal African American women (14%) and non-African American women (16%) [51]. In addition, microarray analysis revealed that younger patients of any ethnicity tend to form triple negative tumors over other types [49,51]. Other risk factors may include metabolic syndrome, obesity, low socioeconomic status and the use of oral contraceptives [50]. Basal-like breast cancer have poor overall survival, high risk of early recurrence, high rate of death during the first 5 years, rapid progression of metastasis to death, and high chemosensitivity [9,52].

Since most basal-like cancers are triple negative breast cancers, finding new drug targets for this group is critical [52]. The standard therapy is for triple negative breast cancer utilizes cytotoxic agents (cisplatin, paclitaxel and anthracycline) which have DNA-damaging effects [52,53]. As basal-like breast cancers have high metastasis rates, anti-angiogenic agents (bevacizumab) are used to inhibit breast cancer progression by targeting vascular endothelial growth factor receptor to halt the growth of blood vessels [52,54]. Poly ADP-ribose polymerase 1 (PARP1) is another targeted therapy since PARP is a nuclear protein that is activated in the presence of DNA damage and repairs single-stranded DNA breaks [52]. Drugs that inhibit PARP1 (Olaparib, Iniparib and Veliparib) cause multiple double strand breaks to form, and in tumors with BRCA1 mutations, double-strand DNA breaks cannot be efficiently repaired, leading to the death of the cells [52,55]. Triple negative breast cancer is one of the most challenging

groups of breast cancers, and other than standard chemotherapy, there are no specific treatment regimes for this disease.

1.2 Animal models of breast cancer

Animal models are important to define etiology of breast cancer as well as generating new prevention and treatment strategies. Both rat and mice models each have their advantages and disadvantages in modeling human breast cancer. Rat models more closely represent human breast cancer because of hormone subtypes, while mouse models are often associated with viral etiology [56]. However, mouse models may have an advantage over rat models since the map of the mouse genome is more well-known than the rat genome [56]. Breast cancer is both complex and heterogeneous and one single breast cancer model cannot represent all human breast cancers. Animal models for human breast cancer can be categorized into three main groups: chemically or ionizing radiation-induced models; genetically engineered mice (GEM) such as transgenic and knockout; and xenograft models. Some of the most commonly utilized chemically-induced and GEM animal models are listed in Table 1.1 and Table 1.2, respectively.

1.2.1 Carcinogen-induced breast cancer models

Chemical xenobiotics and physical agents can be utilized to induce mammary carcinomas in both rats and mice. These models have numerous advantages such as easy induction, reliability, specificity for organ site and hormone responsiveness [57]. Radiation induced tumors tend to have a longer latency and lower frequencies of occurrence when compared to chemically-induced mammary tumors [56]. A limitation of radiation-induced tumors is the occurrence of benign fibroadenomas [56]. As such, chemicals are more frequently used to induce mammary

tumors than radiation since the characterization of premalignant lesions in this system is limited [56,58].

The most commonly used chemicals to induce mammary tumors are polycyclic aromatic hydrocarbon DMBA [59] in Sprague-Dawley (SD) rats or the directly acting alkylating agent, N-methyl-N-nitrosourea (NMU) [60] in SD or Fischer 344 rats. DMBA- and NMU-induced mammary tumor models are useful for assessing anti-estrogen prevention of breast cancer [57]. After carcinogen (DMBA or NMU) exposure, anti-estrogens, such as tamoxifen, delayed tumor appearance [61]. The DMBA model is limited in that most mammary tumors are benign, and should be classified as fibroadenomas [62]. There may be a better carcinogen-induced breast cancer model than the use of DMBA to induce mammary tumorigenesis in rats unless there is a specific interest in the effects of carcinogenic hydrocarbons on the mammary gland [57,63].

Another agent, 3-methylcholanthrene (3-MC), was reported effective to produce mammary tumorigenesis when administered orally to SD rats [64,65]. However, like DMBA, the tumors that were induced are benign [63]. The use of chemical-induced mammary tumorigenesis in rats is useful for hormone dependent breast cancers; however, a major drawback is that rat mammary tumors rarely metastasize [63].

NMU is another widely utilized carcinogen to induce mammary tumorigenesis in rats [57,63,66]. There are advantages to utilizing NMU carcinogen with the simple induction methodology, carcinogenic response and experimental design [57,63,67]. For induction methodology, minimal supplies are needed, intraperitoneal (i.p.) injection is convenient for minimal animal handling, the short half-life of NMU (<2 hrs) reduces the management issues and costs associated with containing carcinogen-treated animals and the disposal of NMU is easily accomplished [63,67]. The carcinogenic response has a short latency for tumor emergence, high incidence and multiplicity of mammary carcinomas, low incidence of tumors at other organ sites, mammary tumors are easily detected by palpation and the rate of tumor growth is easily monitored [63,67]. The advantages of experimental design is the ability to distinguish between

carcinogenic initiation and promotion/progression, provide statistical power by utilizing 25-30 animals per group and the ability to complete the study in as little as 5 weeks after NMU injection [63].

In chemically induced mammary carcinomas in rats, tumors originates primarily from the terminal end bud [61]. Within 14 days of carcinogen administration, there is an enlargement of terminal end buds, and microtumors are evident after 20 days [61]. In humans, the majority of breast cancers have a ductal histogenesis [66]. Carcinomas from NMU-induced tumors and human tumors have a similar pathogenesis since tumors from NMU-induced and human breast cancer progress from ductal hyperplasia with or without atypia to ductal carcinoma *in situ*, to invasive carcinoma [66]. Other similarities between NMU model and the human disease is the occurrence of ovarian hormone-dependent and –independent carcinomas and the protection against tumor development conferred by a full-term live birth prior to carcinogenic initiation [68]. NMU-induced mammary carcinomas, like their human counterparts, have an altered expression of TGFα, ErbB2, cyclin D1 and gelsolin [68].

However, there are obvious differences between NMU-induced mammary tumorigenesis and human breast cancer. One difference between human and NMU-induced mammary tumorigenesis the *G* to *A* transition mutation in codon 12 of the H-ras gene is frequently observed in NMU-induced mammary tumorigenesis, but is rare in human cancers [56]. Another important difference is that NMU-induced mammary tumorigenesis does not appear to have a dysregulated p53 activity, while this is commonly seen in human breast cancer [66]. Overall, the NMU-induced rat mammary tumors showed similar gene expression profiles to ER-positive, low to intermediate grade of human breast cancer, making it a useful model to evaluate the efficacy of chemopreventive agents for hormone dependent mammary tumorigenesis [69].

Estrogens have been implicated in breast cancer; however, the mechanism of action still remains unclear. One theory suggests that the mechanism is dependent on the activation of the ER [70,71]. Estrogen may contribute breast cancer through stimulation of cellular proliferation,

resulting in more opportunities for accumulation of genetic damages leading to carcinogenesis [70]. Another possible mechanism of action may be through the metabolism of estrogen (Fig. 1.2), which may induce oxidative stress and play a key role in mammary cancer development [71,72]. 17β-Estradiol (E_2) and estrone are continuously interconverted by 17β-estradiol hydroxysteroid dehydrogenase (or 17β-oxidoreductase) and are the two major endogenous estrogens [73]. The carbon position of the estrogen molecules that are hydroxylated differs among various tissues in the body and each reaction is probably catalyzed by various CYP enzymes [71,73].

Estrogen may be metabolized by CYP 1A1 to form 2-hydroxyestradiol (2-OHE2) or by CYP 1B1 to form 4-hydroxyestradiol (4-OHE2). These catechols may be methylated by a phase II enzyme, catechol o-methyltransferase (COMT), and excreted out of the body [73]. The 2-OHE2 metabolite is rapidly methylated by COMT, while the 4-OHE2 metabolite is methylated more slowly and thus highly genotoxic [72].

When catechol estrogens are not conjugated (mostly 4-OHE2), it may lead to the formation of semiquinones and subsequently quinones, both of which are electrophiles capable of covalently binding to nucleophilic groups on DNA which may form DNA-adducts [70]. The protective phase II enzyme, NAD(P)H dehydrogenase, quinone 1 (NQO1), catalyzes the reduction of quinones back to catechol estrogens [74].

Under normal conditions, reactive oxygen species (ROS) or reactive nitrogen species (RNS) are neutralized by detoxifying and antioxidant enzymes [75]. Oxidative stress and/or electrophilic stress during redox cycling of catechol estrogens could contribute to nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activation. The estrogen metabolites, 4-OHE1, 4-OHE2, and 2-OHE2 were capable of activating Nrf2, while estradiol did not [76]. This suggests that a catechol structure is required for activation of Nrf2. Estrogen metabolites may exert DNA

mutations from ROS or DNA mutations which may lead to the accumulation of genomic alterations essential for mammary tumorigenesis [70].

In one study, 49 women without breast cancer were observed with larger amounts of 2-OHE2 than 4-OHE2 [77]. Whereas 28 women with breast carcinoma expressed 4-OHE2 levels that were 3.5 times more abundant than 2-OHE2 [77]. This supports the finding that estrogen and its metabolites, mainly 4-OHE2, may be carcinogenic agents in breast epithelial cells [77].

Continuous estrogen treatment induces mammary tumorigenesis in female August Copenhagen Irish (ACI) rats [78]. The ACI strain is unique in that it rarely develops spontaneous mammary tumors and resistant to chemical carcinogens, but has a high incidence of mammary tumors when exposed to synthetic estrogen, diethylstilbestrol, or naturally occurring E_2 [78]. This animal model emulates the role of estrogens in the etiology of human breast cancer. The ACI rat mammary model has a known limitation. Long-term exposure to high doses of E_2 (27 mg) induces pituitary tumors which can affect mammary tumor prevention and treatment [78]. Recently, it has been demonstrated that an ACI.COP-Ept2 rat model, still sensitive to E_2 to induce mammary tumors, shows reduced pituitary morbidities [79]. Tamoxifen treatment suppresses the growth of the estrogen-induced mammary tumors in ACI rats [80]. Based on these models, estrogen-induced mammary tumorigenesis in the ACI strain represents the most biologically relevant hormone-responsive human breast cancer [81].

1.2.2 Genetically engineered mouse models

Transgenic mouse models are useful in determining how specific genetic changes affect breast cancer tumorigenesis [82-84]. The majority of genetic changes can be divided into two categories: gain-of-function of proto-oncogenes and loss-of-function tumor suppressor genes [82]. Proto-oncogenes are involved in cell growth, proliferation and survival, while tumor suppressor genes are involved in the inhibiting cell growth and promoting apoptosis [82].

Promoters can be used to drive the expression of transgenes in the mammary epithelium and two of the most common are mouse mammary tumor virus (MMTV) [85] and whey acidic protein (WAP) [86]. Other mammary gland specific promoters include Rat prostate steroid-binding protein [C3(1)] [87], Bovine β-lactoglobulin (B-LG) [88] and metallothionein (MT) [89]. Many oncogenes have been expressed under the control of these promoters to initiate or modulate mammary tumorigenesis in mice including c-myc, HER2/neu, polyoma middle T antigen (PyMT), simian virus 40 (SV40) T antigen , Ha-Ras, Wnt-1 and TGF-α [90].

The MMTV and WAP promoters are hormonally regulated and are expressed in the mammary gland of developing virgin mice as well as during pregnancy [91]. Although these promoters are mammary gland selective, they are not specific [82,91]. The MMTV promoter is expressed in the lungs, kidneys, salivary glands and additional tissues while the WAP promoter is expressed at low levels in a variety of other tissues, including the brain [82]. One limitation is that the level of oncogene expression that is driven by the MMTV and WAP promoters may not correspond to the expression level of the same oncogene in human breast cancers [82].

In 1984, the first transgenic mouse with breast cancer was reported [92]. Transgenic mice were generated by pronuclear injection of the c-myc oncogene expression cassettes into fertilized mouse eggs [92]. MYC is often amplified and overexpressed in breast cancer [93]. The mice produced carry a normal mouse c-myc gene, but the c-myc promoter region was replaced by a hormonally inducible MMTV promoter [92]. The female transgenic mice spontaneously developed mammary adenocarcinomas during early pregnancy [92]. When the oncogene c-myc was induced by the WAP promoter, the mice expressed tumors that were poorly differentiated and solid carcinomas with a few adenocarcinomas [86,94].

Simian virus 40 large T-antigen (Tag) is a product of the early region of SV40 which is involved in cellular transformation [95]. Transformation and tumorigenesis occurs when SV40 large Tag binds and inactivates tumor suppressor genes p53 [96] and retinoblastoma (Rb) [97].

Initially, the SV40 large Tag transgene was driven by the rat prostatic steroid-binding protein C3 (1) promoter and male mice developed prostatic hyperplasia while female transgenic mice developed mammary hyperplasia by 3 months of age and adenocarcinoma by 6 months of age [98,99]. In earlier stages of mammary tumorigenesis, the tumors appear to be ER positive, however at later stages the tumors are estrogen dependent and do not express ER [87]. The mammary tumors are high-grade and poorly differentiated which more closely represents ER-negative human breast cancer [87].

There is evidence of human familial breast cancer, and many breast cancers can be predisposed to germline mutation in several tumor suppressor genes such as breast cancer type 1 susceptibility protein (BRCA1), breast cancer type 2 susceptibility protein (BRCA2), and p53 [9]. In approximately 50% of primary human breast cancers, p53 is mutated [100]. A null mutation in the p53 gene was introduced in murine embryonic stem cells and mice homozygous for the null allele were prone to spontaneous development of a variety of neoplasms by 6 months of age, however this was only rarely observed [101]. When utilizing the Cre/lox system to generate mammary specific knock out p53, mammary tumors expressed ER-positive and ER-negative tumors with a high rate of metastasis [102].

BRCA1 and BRCA2 are large nuclear phosphoproteins that are critical in DNA repair. Germline mutations of BRCA1 have been observed in about 50% of familial breast cancers [103]. In humans, BRCA1 tumors are high-grade invasive ductal carcinomas and are typically negative for ER, PR, and HER2 [103]. Heterozygous mutants of BRCA1 and BRCA2 did not share the same phenotype as human breast cancers and homozygous mice displayed embryonic lethality [104-106]. Thus, mammary tissue-specific knockout of BRCA1 was created using the Cre/lox system [91,107]. The Cre-mediated mutation of Brca1 gene in mouse mammary epithelial cells exhibited abnormal ductal development with mammary tumor formation after long latency [108].

1.2.3 Xenograft models using human breast cancer cells

Breast cancer cells share many genetic and genomic features of human breast cancers [109], and can represent several breast cancer subtypes [110]. Xenografts of cell lines allow for the examination of human breast cancer cells in the *in vivo* environment to recapitulate the complex multi-cellular interactions necessary for the involvement of cancer progression [82,109,110]. This model allows for studies to include genetic alterations for tumor growth [82]. The use of breast cancer cell lines in xenograft models were reviewed in detailed [82,110,111].

Among these cell lines, MCF-7 is the most widely used human breast-cancer cell line available, and requires estrogenic supplementation for the proliferation *in vitro* and successful establishment as xenografts [112]. The MCF-7 human breast cancer cells were derived from a pleural effusion in a woman who had already received radiotherapy and endocrine therapy and the cells express receptors for several steroid hormones and growth factors such as ER and PR [112]. There are other endocrine-responsive human breast cancer cells that are used for xenografts. T47D and ZR-75-1 breast cancer cells produce tumors in estrogen supplemented nude mice [113,114].

Most ER positive xenografts are nonmetastatic [115]. However, there are MCF-7 variants that appear to have an increased metastatic potential [115]. There was an occasional observance in lymphatic and hematogenous metastases, although, the incidence was too low to provide a useful model of metastatic ER positive breast cancer [116]. Others have generated highly angiogenic MCF-7 variants by overexpressing fibroblast growth factors, and the tumors produced have a high incidence of detectable micrometastases [117]. To date, the most aggressive xenografts are ER negative [115].

The estrogen unresponsive phenotype involves xenografts that do not require or respond to hormonal manipulations. The best characterized and most widely used ER negative cells are MDA-MB-231 and MDA-MB-435 cells into nude mice [110,115]. Xenografts generated by

MDA-MB-435 cells are metastatic when the cells are injected into the mammary fat pads of athymic nude mice [118]. The MDA-MB-435 cells were derived from a patient before they received systemic therapy, and as a result the cells exhibit a pattern of response to cytotoxic drugs *in vivo* that appears to closely reflect those seen in patients [119].

There are two common rodent hosts for human breast cancer xenografts. The most widely used hosts are mice homozygous for the nude (*nu*) mutation [115]. This genetic mutation results in a deteriorated or absent thymus, leading to an inhibited immune system [120]. The nude mice are functionally athymic exhibiting major defects in B-cell maturation and the production of T-lymphocytes, resulting in a greatly reduced number of T-cells [120,121]. Another immunodeficient mouse model has the mutation for severe combined immunodeficiency (SCID) [122]. The SCID mutation disrupts the process of rearrangement of genes encoding antigen-specific receptors on B- and T-cells [123]. Because the immunoglobulin V(D)J recombination does not occur, the cellular immune system fails to mature, and as a result the mice fail to make B or T lymphocytes [122].

Although the xenograft model provides a whole organism environment for tumor growth, there are still limitations. First most, the experiments are carried out in immunodeficient mice. The compromised immune system can have a major impact on tumor formation and progression [82,124]. In addition, the site of cell implantation is important. For breast cancer studies, it would be more biologically relevant to inject the cells in the mammary fat pad rather than subcutaneous injections [82]. Furthermore, the stroma of human and mouse mammary tissue is different and puts doubts into the relevance of xenograft models [125]. Another common problem is metastasis, as metastatic breast cancer cells preferentially colonize in the lungs of mice, and fail to grow at other common sites that occur in human breast cancers such as bone, lymph node, liver and brain [82,126]. More in-depth knowledge of the immune system, microenvironment and genetics of the different subtypes of breast cancer is needed to improve xenografts as a preclinical model.

1.3 Natural compounds in chemoprevention of breast cancer

Animal models are an invaluable tool for breast cancer research and have provided much information to understand the molecular and cellular mechanisms of the different subtypes of breast cancers. These models are used to evaluate the preventive activity of drugs alone or in combination. Chemoprevention was first introduced by Sporn [127] in 1976, and refers to the use of agents to block initiation or reverse the promotion stage of tumorigenesis. Since then, there have been significant advances in utilizing vitamins and phytochemicals to prevent cancers. The chemoprevention by dietary phytochemicals was reviewed by Surh [6].

Cancer chemoprevention uses agents that slow the progression, reverse, or inhibit carcinogenesis in healthy subjects, thereby lowering the risk of developing significant disease [128]. Consequently, an effective chemopreventive agent should intervene early in the process of carcinogenesis to eliminate premalignant cells before they become malignant, or protect normal cells from undergoing transformation [128]. This may prove difficult to implement since otherwise healthy individuals would perhaps need a lifetime of exposure to the chemopreventive agent to achieve efficacy. The same benefit may be achieved from avoiding exposure to known cancer-causing agents and consuming a balanced diet [6,128].

Many chemopreventive agents that suppress the promotion and/or progression of premalignant cells are believed to do so by modulating cell proliferation or differentiation [129,130]. It has been suggested that chemopreventive agents should be administered chronically to individuals with an increased risk of developing cancer since the agents are supposed to encourage cytostasis in epithelial cells that have already sustained DNA damage [130]. There may be minor adverse side effects which would be unacceptable [130]. For example, chemoprevention trials employing NSAIDS such as celecoxib [131] which inhibit the inflammatory mediator COX-2, or selective growth regulators like tamoxifen [132], have been

rather disappointing because of the increased risk of adverse cardiovascular events in some subjects following the prolonged use celecoxib.

Besides long-term toxicity, another concern of adverse side effects from chemopreventive agents may be the development of chemoresistance, which could limit a chronic application strategy in the chemoprevention for many cancers [133]. An alternate approach may involve the use of agents that can eliminate transformed cells in an expeditious manner through the induction of apoptosis. By shifting the mechanism of chemoprevention from cytostasis or differentiation to apoptosis, chronic exposure to a particular chemopreventive agent would not be necessary, thereby eliminating the risk of long-tern toxicity or chemoresistance [134]. For example, the eradication of premalignant lesions could be achieved by agents that trigger apoptosis in transformed cells [134]. Dietary constituents such as polyphenols, vanilloids and isothiocyanates have been shown to trigger apoptosis in tumor cells both *in vitro* and *in vivo* [6]. Moreover, these agents initiate mitochondria-mediated apoptosis via their pro-oxidant effects on transformed cells. The resulting ROS-induced mitochondrial membrane permeabilization cause cytocrome c release from the mitochondria [135,136].

Common prevention strategies include avoiding exposure to known cancer-causing agents, enhancement of those defense mechanisms against cancer and lifestyle modifications [133]. Cancer prevention trials are studies that involve healthy people [133]. Although the participants do not have cancer, they may be at a high risk for developing the disease or have had cancer and at a risk for developing a new type of cancer [133]. Cancer prevention studies look at cancer risk and try to figure out ways to reduce that risk [133].

In human studies, the National Cancer Institute divides cancer prevention studies into two types: action studies and agent studies (http://www.cancer.gov/). Action studies are when participants change their behaviors. Examples of behavioral change include an increase in exercise or the cessation of smoking to determine whether or how healthier lifestyles habits help prevent cancer. In agent studies, participants will take an agent such as medicines, vitamins or

food supplements alone or in combination. These agents are believed to lower a person's risk of developing a specific type of cancer. In agent studies, there are different groups of which a personally will be randomly selected. They may be in the placebo group (no medicine), the standard agent (medicine already used to prevent a certain cancer) or the study agent (new medicine that is believed to be more effective than the current medicines available).

In interventions studies for colorectal cancer, the clinical effectiveness and cost-effectiveness of drug and micronutrient interventions were assessed and recently reviewed [137]. The interventions included non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin and cyclo-oxygenase-2 (COX-2) inhibitors; folic acid; calcium; vitamin D and antioxidants (including vitamin A, vitamin C, vitamin E, selenium and beta-carotene [137]. The results for NSAIDs agents (aspirin and celecoxib) may reduce recurrence of adenomas and incidence of advanced adenomas in individuals with an increased risk of colorectal cancer [137]. These trials that utilized NSAIDs for the primary prevention of polyps lasted 14.5 years [138], 23 years [139] and up to 45 years [140]. However, both aspirin and NSAIDs are associated with adverse effects, thus it is important to consider the risk–benefit ratio for each population before these agents can be recommended for chemoprevention [137]. Furthermore, calcium may also reduce adenoma recurrence in individuals with a history of adenomas [137]. Folic acid and antioxidants (vitamins A, C, E, beta-carotene and selenium) were not shown to reduce adenoma or colorectal cancer incidence, and recent studies have questioned the potential harms as well as benefits of these agents when given as dietary supplements [137]. A major concern with chemoprevention trials are the length of the studies. For examples, chemoprevention may take 10 or 20 years, although the majority of treatment in clinical trials is typically shorter than this time period. Thus, the effectiveness of taking chemopreventive agents over this long time frame is unknown.

People who consumed about five servings of fruit and vegetables a day had approximately half the risk of developing cancer when compare to those who consumed fewer than two servings [6]. Micronutrients, such as antioxidant vitamins, are most commonly found in

dark, leafy vegetables and yellow/orange fruit and vegetables and have been utilized in *in vitro*, *in vivo*, and human intervention trials [6].

To date, about 35 vegetables and fruits have been shown to provide excellent sources for cancer preventive activities and include garlic, soybeans, grapes, ginger, onion, turmeric, tomatoes and broccoli [6]. Chemopreventive phytochemicals can block or reverse the premalignant stage (initiation and promotion) of multistep carcinogenesis [127,141]. Suppressing agents inhibit the malignant transformation of initiated cells [141]. Some of the active compounds of vegetables and fruits are cancer-blocking agents ellagic acid, indole-3-carbinol, sulphoraphane and flavonoids, and cancer suppressing agents triterpenoids, β-carotene, curcumin, EGCG, genistein, resveratrol, (6)-gingerol and capsaicin [6]. The cellular events that are modulated by these phytochemicals may include carcinogen activation/detoxification by xenobiotic metabolizing enzymes, DNA repair, cell-cycle progression, cell proliferation, differentiation, apoptosis, regulation of oncogenes or tumor-suppressor genes, angiogenesis, metastasis or hormonal and growth factor activity [6].

Foods rich in vitamin A include liver, sweet potatoes, carrots, milk, egg yolks and mozzarella cheese [142]. Vitamin A is found in three main forms: retinol, 3,4-didehydroretinol and 3-hydroxy-retinol [142]. Retinoids are vitamin A analogues and bind to retinoic acid receptors (RARs) and retinoid X receptors (RXRs) [143]. RXR proteins can form heterodimers with different partners such as thyroid hormone receptors (TR), vitamin D receptor (VDR), peroxisome proliferator-activated receptors (PPARs) and numerous orphan receptors [144]. As a result, the binding of retinoids to these receptors leads to regulation of cell growth, differentiation and apoptosis [144]. Mammary tumorigenesis was inhibited by the naturally occurring retinoid, 9-cis-retinoic acid (9cRA), in NMU-induced mammary tumors [145] and C3(1)-SV40 Tag transgenic mice [146].

However, in human clinical trials, 9cRA exhibited high toxicity [147]. Rexinoids were developed to be ligand specific for RXRs and have been found equal or more effective at

preventing mammary tumors with less toxicity. In C3(1)-SV40 Tag, MMTV-HER2 and p53^{-/-} animal models, the rexinoid Bexarotene/LGD1069 was able to inhibit tumor growth [148-150]. In HER2-overexpresssing mice, rexinoids such as LG100268 [151,152] and NRX194204 [153] inhibited mammary tumor growth.

Vitamin D can be obtained from dietary sources such as salmon, cod liver oil, egg yolk or from fortified foods such as milk, juice and cheese [154]. Vitamin D_3 is synthesized via the UV irradiation of 7-dehydrocholesterol to previtamin D_3 in the skin of animals at UVB wavelengths of 290–320 nm, with a further thermal isomerization step to form vitamin D_3 [154]. Vitamin D is a small lipophillic molecule which binds to VDR [155]. The active metabolite of vitamin D, 1α,25-dihydroxyvitamin D3 (1,25(OH)2D3) has been shown to reduce growth inhibition and induce differentiation in human breast adenocarcinoma [156-159].

However, naturally occurring vitamin D can induce hypercalcemia. Recently, noncalcemic vitamin D analogues have been developed to target VDR for cancer prevention. Vitamin D analogues, EB1089, Gemini 0097 and Gemini 0072, inhibited mammary tumor growth in the NMU-induced model [160,161]. In a transgenic model of luteinizing hormone (LH) overexpressing mice, EB1089 was demonstrated to decrease the growth rate of hormone-induced mammary tumors [162]. These less toxic vitamin D analogues may be promising agents to prevent breast cancers.

1.4 Vitamin E

1.4.1 Discovery and structures of vitamin E

Vitamin E was first discovered in 1922 where it was noted to restore fertility function [163,164]. In 1936, vitamin E was aptly named "tocopherol" from the Greek, *tocos* which means "childbirth" and *phero* which means "to bring" [164]. Tocotrienols were not discovered until 1964 [165], and since then, vitamin E has consisted of eight structurally related compounds; four

tocopherols and four tocotrienols (Fig. 1.3). Both tocopherols and tocotrienols have the same denotation for the number and position of methyl groups on the chromanol ring, designated as α, β, γ, and δ [166]. α-Tocopherol is trimethylated at the 5-, 7-, and 8-positions, β-tocopherol is dimethylated at the 5- and 8-positions, γ-tocopherol is dimethylated at 7- and 8-positions, and δ-tocopherol is monomethylated at the 8-postion on the chromanol ring. Tocopherols have a saturated 16-carbon side-chain while tocotrienols have an unsaturated 16-carbon side-chain with double bonds at 3', 7', and 11' positions [166,167].

1.4.2 Vitamin E in the human diet

The most significant sources of vitamin E are from plant oils and fats. α-Tocopherol is most commonly found in wheat germ, almond, and sunflower oil [168]. However, γ-tocopherol is more prominent than α-tocopherol in the American diet and is found in vegetable oils such as soybean, corn, and cottonseed [167]. δ-Tocopherol is primarily found in soybean and castor oils, and to a lesser extent, in wheat germ oil [169]. A γ-tocopherol enriched mixture containing 58% γ-tocopherol, 24% δ-tocopherol, 13% α-tocopherol, and 0.5% β-tocopherol (γ-TmT) can be easily available as a by-product of refining vegetable oil [170,171]. Tocotrienols are found primarily in palm and annatto oils and more readily consumed in East-South Asian diets [172].

1.4.3 Transport and metabolism of vitamin E

Vitamin E is taken up by intestinal cells and released into circulation in chylomicrons, where they reach the liver [173]. Three proteins have been identified to specifically bind to tocopherols: α-tocopherol transfer protein (α-TTP), tocopherol-associated protein (TAP), and tocopherol-binding protein (TBP) [174-177]. In the liver, the α-TTP, a 30-35 kDa protein [175],

preferentially transfers α-tocopherol from the liver to the blood [178]. The relative affinities of α-TTP for the variants of vitamin E as determined *in vitro* were 100% for α-tocopherol, 38% for β-tocopherol, 9% for γ-tocopherol, 2% for δ-tocopherol, and 12% for α-tocotrienol [179]. Thus, α-tocopherol is the most abundant form found in human tissues and serum.

Another protein involved with vitamin E is TAP which is a cystolic lipid-binding and transfer protein [176]. TAP is a 46-kDa protein and has the highest levels in the liver > prostate > whole brain > spinal cord > kidney > mammary gland > stomach [176]. TBP is a 15 kDa cystolic protein initially found in rat liver and heart [180] and later found in human placenta [181]. TBP is involved in intracellular transport and metabolism for α-tocopherol [177]. The first non-antioxidant function of vitamin E determined that α-tocopherol inhibited the activity of smooth muscle proliferation and protein kinase C [182,183].

Vitamin E is metabolized in the liver by cytochrome P450 4F2. CYP4F2 catalyzes the initial step in the vitamin E-ω-hydroxylase pathway followed by β-oxidation, which removes 2 carbons from the side chain in each cycle ending in the short chain metabolite, carboxyethyl hydroxychromans (CEHC) [178,184]. Since α-tocopherol is preferentially transferred to the blood by α-tocopherol transfer protein, γ-tocopherol and δ-tocopherol are more readily metabolized in the liver [178]. Due to the abundance in the serum and tissue, α-tocopherol has been termed the "classic" vitamin E and been the primary variant used in dietary supplements and in studies over the years [185].

1.4.4 Vitamin E toxicity

The recommended dietary allowance (RDA) of vitamin E is 15 mg/d and the tolerable upper intake level (UL) of alpha-tocopherol is 1000 mg/d [186]. This upper limit is not likely to cause adverse health effects to most people in the general population [187]. Short-term

supplementation with up to 1600 IU of vitamin E appears to be well tolerated and have minimal side effects [187]. Many adults take relatively large amounts of vitamin E (α-tocopherol 400 to 800 mg/day) for months to years without any apparent harm [188]. However, with chronic high intake of vitamin E supplementation (more than 1000 mg/day), there could be an increase in incidence of heart failure, hemorrhagic stroke, and mortality, especially in patients with chronic diseases [187]. Dietary supplements may alter hemostasis by a variety of mechanisms, such as reducing platelet aggregation or inhibiting arachidonic acid, a cellular signaling messenger and inflammatory intermediate [189]. Tocopherol was shown to inhibit platelet adhesion, aggregation and platelet release reactions in humans and decrease plasma production of thrombin, a protein which binds to platelets and induces aggregation in endothelial cells *in vitro* [189]. However, the number of controlled studies in the literature is too limited to demonstrate consistent anticoagulant effects of dietary supplements such as vitamin E [189].

1.5 Possible mechanisms of cancer prevention by vitamin E

Each subtype of breast cancer responds differently to current treatments and therapy. To date, there is limited *in vitro*, *in vivo*, and human data which connect individual tocopherols for prevention or treatment for each subtype of breast cancer. Chemoprevention is an approach to prevent cancer before a series of genetic and epigenetic events establish which otherwise could lead to malignancies. Thus, prevention of breast cancer is essential, and the success of prevention strategies depends on understanding the molecular mechanism of breast cancer initiation and progression.

The mechanisms of anti-cancer activity of vitamin E have been investigated for many years [190-192] and include the induction of apoptosis, modulation of nuclear receptors, inhibition of cell growth, anti-oxidative and anti-inflammatory activities [75,190,192-196]. More specifically, vitamin E has been shown to interact with PKC, Akt/PKB, MAPK family, cell cycle

related kinases, and activation of nuclear factor κB [197-201]. Table 1.3 lists molecular targets by vitamin E *in vitro* and Table 1.4 lists molecular targets by vitamin E *in vivo.*

1.5.1 Regulation of apoptosis and cell proliferation by vitamin E

Apoptosis is defined as programmed cell death with distinct morphological and biochemical changes [202,203]. During the earlier stages, the apoptotic cell shrinks in volume and the nuclear DNA condenses, while the cellular membrane remains intact [203,204]. Apoptotic bodies are formed and the tightly packed organelles leave the cell through "budding" [205]. There are two distinct apoptotic pathways: extrinsic and intrinsic [206].

The extrinsic pathway has extrinsic inducers such as toxins, hormones, growth factors, nitric oxide or cytokines that either cross the plasma membrane or transduce to affect a response [204]. The extrinsic pathway can be triggered from outside the cell by the activation of death receptors on the cell surface [204]. Killer lymphocytes can induce apoptosis by producing a protein called Fas ligand, which binds to the death receptor protein Fas on the surface of the target cell [207]. The clustered Fas proteins then recruit intracellular adaptor proteins that bind and aggregate procaspase-8, which cleave and activate one another [207]. The intrinsic pathway is when a damaged or stressed cell triggers procaspase aggregation and activation from within the cell [204].

The intrinsic pathway is initiated by intracellular response to stress such as binding of nuclear receptors by glucocorticoids, heat, radiation, nutrient deprivation, vital infection, hypoxia and increased intracellular calcium [204]. Under stress, the mitochondria are induced to release the electron carrier protein cytochrome C into the cytosol, where it binds and activates an adaptor protein called Apaf-1 [206]. Apaf-1 binds and aggregates procaspase-9 molecules which leads to the cleavage of these molecules and triggers a caspase cascade [206]. DNA damage can trigger

intrinsic apoptosis by stimulating p53 and cause the activation of transcription genes that promote the BCL-2 family [206].

The BCL-2 family of intracellular proteins helps regulate the activation of procaspases. Inhibitors of apoptosis include Bcl-2 and BCl-X_L which can partly block the release of cytochrome c from the mitochondria [208]. Bad is a promoter of apoptosis by binding and inactivating death-inhibiting members of the family, whereas Bax and Bak stimulate the release of cytochrome c from the mitochondria [208]. Another important regulator of intracellular apoptosis is the inhibitor of apoptosis (IAP) family. X chromosome-linked IAP (XIAP) is a direct inhibitor of caspase whereas cellular IAP proteins block the assembly of pro-apoptotic protein signaling complexes and mediate the expression of anti-apoptotic molecules [209].

Caspases have proteolytic activity and are able to cleave proteins. There are ten major caspases with three main sub groups: initiators (-2, -8, -9, and -10), effectors (-3, -6, and -7), and inflammatory (-1, -4, and -5) [207,210]. Caspase 3 is of particular interest since it has the ability to cleave over a hundred different substrates that lead to apoptosis [5]. Dietary compounds that induce apoptosis are highly beneficial since apoptosis has the ability to remove cells with neoplastic transformation when upstream cellular defenses have failed.

γ-Tocopherol has been shown to induce apoptosis in breast, colon, and prostate cancer cells [211-215]. Yu *et al.* showed that apoptosis was induced by δ-tocopherol in MCF-7 and MDA-MB-435 breast cancer cells [212]. More specifically, γ-tocopherol, but not α-tocopherol, induced cleaved-caspase (c-Casp) 8 and 9 in MDA-MB-435 human breast cancer cells [215]. Nu/Nu mice injected with MDA-MB-231 cells reduced tumor burden and increased apoptosis when administered γ-tocopherol [216].

In breast, colon, lung, and prostate cancer cell lines, γ-tocopherol was shown to be more effective at inhibiting cell growth than α-tocopherol [170,213,214,217]. Treatment with γ-TmT, γ-tocopherol, and δ-tocopherol inhibited cell proliferation in MCF-7 breast cancer cells in a dose-

dependent manner, while α-tocopherol did not [170]. In addition, a colony growth inhibition assay utilizing MDA-MB-435 breast cancer cells showed that γ-tocopherol and δ-tocopherol showed potential to inhibit colony formation, whereas α-tocopherol was not active [190]. γ-Tocopherol and to a greater extent, δ-tocopherol, were shown to inhibit tumor growth in a lung xenograft model, while α-tocopherol did not [218].

Mammary tumor growth and burden was decreased by administration of γ-TmT diet in SD rats induced with a synthetic carcinogen NMU [170,219]. Proliferating cell nuclear antigen (PCNA) was decreased in mammary tumors when administered γ-TmT [219]. Administration of γ-TmT increased the levels of c-Casp-3 increased in mammary tumors [170]. At higher doses, tocopherols may induce DNA damage leading to apoptosis.

1.5.2 Direct anti-oxidative activities of vitamin E

Vitamin E is an important dietary anti-oxidant which prevents the propagation of free radical reactions [167,220]. The anti-oxidant properties are mostly due to the phenolic hydrogens in the chromanol ring that are donated to lipid free radicals [221]. Due to the structural differences on the chromanol ring , α-tocopherol with two *ortho*-methyl groups is expected to be a more potent hydrogen donor than either γ-tocopherol (one *ortho* methyl group) and δ-tocopherol (zero *ortho* methyl group) [222]. Although α-tocopherol may be a better antioxidant, α-tocopherol consequently has a greater capacity than γ-tocopherol and δ-tocopherol to act as a pro-oxidant when present in high concentrations in vegetable oils, and with transition metal ions, lipid peroxides, and other oxidizing agents [222,223]. Furthermore, γ-tocopherol is more effective in trapping reactive nitrogen species than α-tocopherol [193,224-228].

The stability of tocopherol and nitrogen species derivative depends on the structure of the chromanol ring [228]. The tocopherols with a free 5 position on the chromanol ring (γ- and δ-

tocopherol) are expected to react with nitrogen species forming C-nitroso derivatives at this position [228]. Both α-tocopherol and γ-tocopherol react with nitrogen dioxide (NO_2); α-tocopherol forms an intermediate tocopheroxide analogue while γ-tocopherol may form nitric oxide (NO) or a stable nitro derivative (5-nitro-γT) [228].

α-Tocopherol is trimethylated, and consequently, the nitrosating agent only has the possibility to add to the *para*-position on the chromanol ring of α-tocopherol, forming a highly unstable compound and may form toxic N-nitroso-derivatives from amines [228]. α-Tocopherol may react with nitrous acid to yield α-tocopherol quinone and nitrogen monoxide gas [222]. This may lead to highly instable derivatives which may act as nitrosation catalysts for secondary amines. The high hydrogen donation ability by α-tocopherol may cause undesirable side effects, such as pro-oxidant and toxic nitro derivatives [223].

1.5.3 Indirect anti-oxidative activities of vitamin E

Vitamin E may also be an indirect anti-oxidant by activating Nrf2 and related enzymes. Nrf2 is a transcription factor that is a key regulator of cellular anti-oxidant and detoxification enzymes [229]. Kelch-like-ECH-associated protein 1 (KEAP1) inhibits Nrf2 in the cytoplasm and thus, Nrf2 is marked for degradation through the proteasomal pathway [229]. Chemopreventive agents or oxidative stress may modify the cysteine bonds on KEAP1, which allows the release and the consequential activation of Nrf2 [6,229,230]. Nrf2 translocates into the nucleus, dimerizes with small Maf proteins, and binds to the anti-oxidant-responsive element (ARE) to stimulate gene expression of anti-oxidant enzymes (thioredoxin [TXN], superoxide dismutase [SOD], catalase, glutathione peroxidase (Gpx), and heme oxygenase-1 [HO-1]), and phase II detoxification enzymes (glutathione s-transferases [GSTs], UDP-glucuronosyltransferases, sulfotransferases [UGT], and NQO1) [6,75,229-231]. The stimulated detoxifying and anti-oxidant

enzymes are able to protect the cells from neoplastic transformation by maintaining oxidative stress homeostasis [75,232]. In Nrf2–knockout mice, the induction of cyclooxygenase-2 (COX-2), 5-lipoxygenase, prostaglandin E_2 (PGE_2), and leukotriene B_4 (LTB_4) were significantly higher when compared to the wild type mice [233]. A loss of Nrf2 may lead to a decrease in cellular defense against oxidative stress which may result in tumorigenesis [234].

Oxidative stress and/or electrophilic stress during redox cycling of catechol estrogens could contribute to the Nrf2 activation. The estrogen metabolites, 4-OHE1, 4-OHE2, and 2-OHE2 were capable of activating Nrf2, while estradiol does not [76]. This suggests that a catechol structure is required for activation of Nrf2. In addition, it was reported that catechol estrogen-induced ARE activation depends on Nrf2, not ER [235]. *Sumi et al* found that Nrf2 is activated from electrophilic quinones rather than ROS produced from redox cycling of catechol estrogens [76]. Oxidative stress and chemopreventive agents can activate Nrf2. Further investigation is warranted to determine the effects of tocopherol in activating Nrf2 and inhibiting oxidative stress in the chemoprevention of breast carcinogenesis.

In human retinal pigment epithelial cells, pretreatment with α-tocopherol inhibited ROS generation, increased Nrf2 expression, and, induced phase II enzymes (glutamate cysteine ligase, NQO1, HO-1, GST, and SOD) [236]. The expression of Nrf2 was suppressed in prostate tumors [237], and treatment with γ-TmT upregulated the expression of Nrf2 and detoxifying enzymes, and inhibited tumor development in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice [75,237].

In E_2-treated ACI rats, administration of γ-TmT increased the protein expression level of Nrf2 in mammary gland and liver, and phase II enzymes were increased in the liver [238]. This may indicate that γ-TmT induces the transcription of Nrf2-ARE-target genes and exhibits protective defense against estrogen induced oxidative stress.

1.5.3 Regulation of estrogen receptors by vitamin E

ER is a nuclear receptor that consists of two different subtypes (α and β) and stimulates cell growth and proliferation [71]. Upon ligand activation, ER dimerizes, enters the nucleus and binds to the estrogen response element sequence. The DNA binding domains of ERα and ERβ are highly homologous while the ligand binding domain is 60% homologous [239]. ERα and ERβ are both present in breast tissue, however, the ratio of ERα to ERβ is increased in breast tumors [239]. The role of ERβ in breast tumorigenesis is not well understood. Some studies have shown that activation of ERβ in breast cancer cell lines inhibits cell growth, and the dimerization of ERβ with ERα silences the growth-promoting effects of ERα [196,239].

In ER-positive breast cancer cell lines MCF-7 and T47D, vitamin E has been shown to inhibit proliferation and work as antagonists of estrogen signaling [194]. MCF-7 cells were treated with γ-TmT, and the expression of ERα was down-regulated [170]. In mammary tumors, ERα mRNA and protein levels were down-regulated by the treatment of γ-TmT [170]. Treatment by γ-TmT reduced ERα mRNA and protein levels in hyperplastic mammary tissues in estrogen-treated ACI rats, while mRNA levels of ERβ were increased [238]. The circulating levels of E_2 in the serum were decreased when administered γ-TmT, suggesting that γ-TmT may modify the response to estrogen [238].

1.5.4 Activation of peroxisome proliferator activated receptor γ by vitamin E

Another nuclear receptor of importance in cancers is PPAR. This nuclear hormone receptor superfamily, comprises of 3 subtypes (α, γ, and δ) which are ligand-regulated transcription factors [240]. PPARγ forms a heterodimer with the retinoid X receptor after ligand activation [241]. One known PPARγ ligand is troglitazone [242], and the chromanol ring of

vitamin E is structurally similar. However, vitamin E is not a direct ligand of PPARγ, but rather γ-tocopherol was shown to induce the formation of 15-S-hydroxyeicosatetraenoic acid (15-S-HETE), an endogenous PPARγ ligand [243]. PPARγ is known to be involved in fatty acid uptake and transport and acts to control inflammation by inducing apoptosis and inhibiting cell proliferation cell survival [241,244]. PPARγ signaling is connected to the inhibition of inflammatory markers (COX-2, cytokines, and inducible nitric oxide synthase [iNOS]), PI3K/Akt pathway, and angiogenesis while inducing CDK inhibitors, differentiation and apoptosis markers in cancers [244]. Particularly in breast cancer, stimulation of PPARγ increases the degradation of cell cycle genes (Cyclin D1), interferes with estrogen receptor signaling, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling cascades [243,245].

γ-Tocopherol displayed the strongest activity in inducing the mRNA and protein levels of PPARγ in SW480 colon cancer cells [242] and transcriptional activity in NCTC 2544 keratinocytes cell line [246]. In MCF-7 and T47D breast cancer cells, γ-TmT, γ-tocopherol, and more strongly δ-tocopherol enhances the transactivation of PPARγ [170]. In an NMU-induced breast cancer model in SD rats, γ-TmT induced PPARγ mRNA and protein levels [170].

1.5.5 Inhibition of cyclooxygenase-2 by vitamin E

There are two major types of COX, constitutive (COX-1) and inducible (COX-2). COX-1 is a house-keeping protein in most tissues, and does not change in response to stimuli [247]. COX-2 is an inducible prostaglandin synthase which is upregulated by growth factors, tumor promoters, and cytokines [248], and responsive to several oncogenes, such as HER2 [249,250]. Prostaglandin synthesis is increased in inflamed and neoplastic tissues [249]. High levels of COX-2 is associated with about 40% of aggressive human breast cancers and correlates with large tumor sizes, high proliferation rates, and metastases [250]. Celecoxib, a COX-2 inhibitor,

was administered to HER2/neu transgenic mice and found that there was a 50% reduction in mammary PGE_2 levels and delayed tumor onset [251]. When stimulated with cytokines, PGE_2 and COX-2 production was accompanied by an increase in NO and corresponding iNOS enzyme in A549 human lung adenocarcinoma cell line [252].

In various model systems, vitamin E supplementation can inhibit COX-2 activity [193,253,254]. Tocopherols are known anti-inflammatory agents, and γ-tocopherol is more effective in inhibiting the activity of COX-2 than α-tocopherol [193,224]. In addition, γ-tocopherol was shown to reduce PGE_2 synthesis in macrophages and human epithelial cells [193], and the inhibitory effect was due to the decrease of COX-2 activity [193,198]. Serum levels of PGE_2 and 8-isoprostane, a marker of oxidative stress, were reduced when E_2-induced ACI rats were treated with γ-TmT, and COX-2 levels decreased in the mammary gland when treated with dietary γ-TmT [238].

1.5.6 Suppression of nuclear factor κB by vitamin E

NF-κB is a transcription factor that is closely linked to inflammation. Under normal conditions, NF-κB is located in the cytoplasm and bound to inhibitor IκB proteins. Once activated, IκB proteins are degraded which leads to the activation and translocation of NF-κB into the nucleus [255]. Activation of NF-κB can be a response to environmental stimuli, inflammatory cytokines and stress, and is responsible for regulating the activity of pro-inflammatory genes such as tumor necrosis factor (TNF-α), interleukin (IL) IL-1, IL-6, IL-8, matrix metalloproteinase (MMP)-9, vascular endothelial growth factor (VEGF), and 5-lipoxygenase [255]. Constitutive activation of NF-κB may be found in breast, colon, prostate, ovary, liver, pancreas, leukemia, and lymphoma cancers and correlates with recurrence, poor survival, aggressiveness, and tumor progression [256].

In lipopolysaccharide (LPS)-activated RAW 264.7 macrophages, apigenin blocked LPS-induced NF-κB activation, and also suppressed the promoter activity of COX-2 [257], thus shows another potential mechanism by which inhibition of COX-2 gene expression is by the alteration of the NF-κB pathway. Furthermore, it was recently shown that NF-κB could repress Nrf2 by competing for the transcription co-activator CREB binding protein and by recruiting histone deacetylase 3 to cause local hypoacetylation to hamper Nrf2 signaling [258]. Further studies are needed to understand the relationship between NF-κB, COX-2, and Nrf2.

TNF-α was used to stimulate A549 human type II alveolar epithelial cell line and treatment with α-tocopherol resulted in decreased levels of intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1, IL-8, NF-κB, p-Erk, and p38 [259]. A vitamin E analogue, α-tocopheryl succinate was shown to inhibit NF-κB translocation and phosphor-Akt (p-Akt) in MDA-MB-453 and MCF-7 breast cancer cell lines [260]. NF-κB was inhibited by α-tocopheryl succinate in KU-19-19 and 5637 bladder cancer cells lines as well as KU-19-19 pre-established tumors [261]. In androgen-independent PC-3, DU-145, and CA-HPV prostate cancer cells, α-tocopheryl succinate inhibits NF-κB, reduces the expression of IL-6, IL-8, and VEGF, suppresses cell adhesion molecule, ICAM-1, and augments AP-1 [262].

1.6 Human studies on vitamin E and cancer

The cancer preventive activity of vitamin E has been investigated in numerous epidemiological studies and recently been reviewed [263,264]. Results have been inconclusive where some epidemiological studies suggest a protective effect by vitamin E, while many large-scale interventions studies with α-tocopherol did not demonstrate cancer preventive effects.

1.6.1 Case-control and cohort studies

Numerous case-control studies utilized vitamin E and 11 studies found a risk reduction [265-275], however, 13 studies did not find an association with preventing breast cancer (Table 1.5) [276-288]. In the Shanghai Breast Cancer Study, they suggest that vitamin E supplement may reduce the risk of breast cancer among women who have low dietary intake [275].

To date, 12 cohort studies did not find any relation between vitamin E and prevention of breast cancer risk (Table 1.6) [289-300]. In one cohort study, the European Prospective Investigation into Cancer and Nutrition (EPIC) trial observed that vitamin E did not reduce breast cancer risk, but there was a weak risk reduction in post-menopausal women [298]. While investigating vitamin supplement during breast cancer treatment and survival, Nechuta *et al.* determined that vitamin E supplementation in the first 6 months after diagnosis may reduce risk of mortality and recurrence [301].

Previously, detailed assessments revealed that vitamin E (α-tocopherol) supplements did not protect against breast cancer [302,303]. Recently, Fulan *et al.* performed a meta-analysis on 38 studies between vitamin E and breast cancer [304]. For case-control studies, dietary vitamin E and total vitamin E reduced breast cancer risk by 18% and 11%, respectively [304]. When the cohort studies were pooled with the case-control studies, dietary vitamin E and total vitamin E both became non-significant [304]. Thus, a conclusion remains elusive between breast cancer and vitamin E. The term "vitamin E" is used loosely, and a distinction in these case-control and cohort studies need to clarify which variant of vitamin E is utilized.

1.6.2 Intervention studies

The Alpha-Tocopherol, Beta-Carotene (ATBC) Cancer Prevention Study examined the prevention of lung and other cancers with supplementation of all-racemic-α-tocopherol acetate

(50 mg/day) and β-carotene (20 mg/day) daily, which did not have an effect on lung or colorectal cancer [305,306]. However, the ATBC study found that males supplemented with α-tocopherol acetate (50 mg daily) had 32% lower prostate cancer incidence and 41% reduction in prostate cancer deaths [307]. The Physicians' Health Study II gave supplements of 400 IU of α-tocopherol every other day or 500 mg of vitamin C daily and concluded that neither vitamin E nor C reduced the risk of prostate cancer [308]. The Selenium and Vitamin E Cancer Prevention Trial (SELECT) administered selenium (200 μg/day) and *all rac*-α-tocopheryl acetate (400 IU/day) and revealed that selenium or vitamin E, alone or in combination, did not prevent prostate cancer [309]. These previous clinical and epidemiological studies have been primarily utilized α-tocopherol, and not a mixture of tocopherols or other variants of tocopherols for chemoprevention [190,310-312].

There have been 3 breast cancer randomized controlled trials (RCT), which administered supplemental natural-source vitamin E (either 400 IU or 600 IU), and concluded that there was no overall benefit of vitamin E supplementation [311,313,314]. Only one RCT specified using the variant α-tocopherol [314], but in most cases, the studies do not identify which variant of vitamin E was utilized. Thus, epidemiological evidence between vitamin E and breast cancer is limited and inconsistent [315]. There are four tocopherols and four tocotrienols that comprise vitamin E, each which differ in chemical structure, bioavailability, and activity. Results will remain inconclusive unless the specific variant is identified for each study.

1.7 Summary

α-Tocopherol has been the most widely studied form of vitamin E for the prevention and treatment of cancer [190,310-312]. Although the biological effects of α-tocopherol have been investigated over many decades, our current understanding of its role in inhibiting breast

carcinogenesis remains incomplete. Higher concentrations of α-tocopherol may decrease the level of γ-tocopherol in the serum [216,316]. This may be unfavorable since γ-tocopherol has demonstrated significantly greater anti-inflammatory and anti-tumor activity than α-tocopherol in several different animal models of colon, breast, and prostate cancer [214,216,217,224,242,317]. In addition, γ-TmT is a mixture of tocopherols enriched with γ-tocopherol and is readily available and inexpensive, while individual variants remain expensive to purify. There may be synergistic effects with a combination of tocopherols, but further investigation is warranted. As a result, γ-TmT may be more beneficial rather than individual tocopherols for the prevention of breast cancer.

Table 1.1. Carcinogen-induced animal models of mammary tumorigenesis

Carcinogen/Model	Strain/Species	Time for tumor development after injection/implantation	ER Status	Reference
Radiation	Wistar rat	6-12 months	+	[56]
DMBA	SD rat	8-21 weeks	+	[59]
DMBA	BALB/c mouse	20-55 weeks	-	[318]
3-MC	SD or Wistar rat	6-21 weeks	+	[65]
NMU	SD or Fischer 344 rat	8-21 weeks	+	[60]
Estrogen	ACI rat	6-9 months	+	[78]
Estrogen	ACI.COP-Ept2 rat	6-9 months	+	[81]

12-dimethylbenz(a)anthracene (DMBA); 3-methylcholanthrene (3-MC); N-methyl-N-nitrosourea (NMU); Sprague-Dawley (SD); August Copenhagen Irish (ACI); estrogen receptor (ER)

Table 1.2 Genetically engineered mouse models of mammary tumorigenesis

Model	Promoter	Transgene	Strain/Species	Time for tumor development	ER Status	Reference
c-myc	MMTV/ WAP	c-myc	C57BL/6J mouse	4-10 months	-	[86,92-94]
Ras	MMTV/ WAP	Ha-ras	C57BL/6J mouse	5 weeks – 6 months	-	[319-322]
ErbB2	MMTV/ WAP	Neu/ErbB2	FVB mouse	5-10 months	-	[85,323]
Wnt1	MMTV	Wnt1	FVB mouse	3-6 months	+/-	[324,325]
IRS-1/2	MMTV	IRS-1/2	FVB mouse	12 months	-	[326]
AIB1	MMTV	AIB1	FVB mouse	16 months	+/-	[327]
RCAS	MMTV	TVA	FVB/N mouse	5-25 weeks	+	[328]
PyMT	MMTV	MT	FVB/N mouse	1-6 months	+	[329,330]
SV40	C3(1)	SV40 large Tag	FVB mouse	3-6 months	+/-	[87,95-99]
Brca1 KO	-	Mutant Brca1	C57BL/6J mouse	10-13 months	+/-	[104,106]
Brca2 KO	-	Mutant Brca2	C57BL/6J mouse	>1 year	+/-	[331,332]
p53 KO	-	Mutant p53	C57BL/6J mouse	1-2 years	+/-	[101,102,333]

Human epidermal growth factor receptor-2 (HER2/ErbB2); Mouse mammary tumor virus (MMTV); Polyoma middle T antigen (PyMT); Simian virus 40 (SV40); Breast cancer type 1 susceptibility protein (BRCA1); Breast cancer type 2 susceptibility protein (BRCA2); Whey acidic protein (WAP); estrogen receptor (ER)

Table 1.3 Biological events and molecular targets modulated by vitamin E *in vitro*

Vitamin E	Cell Type/Cancer Model	Result	References
Apoptosis and Cell Proliferation			
γ-Tocopherol	Prostate cancer cells (LNCaP and PC-3) and lung cancer cells (A549)	↓Proliferation, ↑Apoptosis	[211]
Combination of Tocopherols, γ-Tocopherol, δ-Tocopherol	Prostate cancer cells (LNCaP)	↓Proliferation, ↑Apoptosis	[214]
γ-Tocopherol	Colon cancer cells (SW480, HCT-15, HCT-116, HT-29)	↓Proliferation, ↑Apoptosis	[213]
γ-Tocopherol	Prostate cancer cells (DU-145 and LNCaP)	↓Proliferation, ↓Cyclin D1, ↓Cyclin E	[217]
γ-Tocopherol	Prostate cancer cells (LNCaP)	↓Proliferation, ↑Apoptosis	[211]
δ-Tocopherol	Breast cancer cells (MCF-7 and MDA-MB-435)	↑Apoptosis	[212]
γ- Tocopherol	Breast cancer cells (MCF-7 and MDA-MB-435) and murine 66cl-4	↓Proliferation, ↑Apoptosis	[215]
γ-Tocopherol, δ-Tocopherol, γ-Tocotrienol, δ-Tocotrienol	Prostate cancer cells (PC-3 and LNCaP)	↓Proliferation	[334]
γ-Tocotrienol	Prostate cancer cells(PC-3)	↑Apoptosis	[334]
α-Tocotrienol, γ-Tocotrienol, δ-Tocotrienol	Breast cancer cells (MCF-7 and MDA-MB-231)	↓Proliferation	[195]
γ-Tocotrienol, δ-Tocotrienol	Breast cancer cells (MCF-7 and MDA-MB-231)	↓Proliferation	[335]
γ-Tocotrienol	Breast cancer cells (MCF-7)	↓Proliferation	[336]
γ-Tocotrienol	Colon cancer cells (HT-29)	↓Proliferation, ↑Apoptosis	[337]
γ-Tocotrienol	Gastric cancer cells (SGC-7901)	↑Apoptosis	[338]
Direct and Indirect Anti-oxidant Activities			

γ-Tocopherol	Human plasma	↓RNS, ↓peroxynitrite	[227]
Tocotrienol rich fraction from palm oil	Human monocytic cells (THP-1) induced by LPS	↓NO	[339]
α-Tocopherol	Human retinal pigment epithelial cells (ARPE-19)	↑Nrf2 protein levels, ↑glutamate cysteine ligase, ↑NQO1, ↑HO-1, ↑GST, ↑SOD	[236]
γ-Tocotrienol, δ-Tocotrienol	Breast cancer cells (MDA-MB-231)	↓KEAP1 levels, ↑Nrf2 protein levels, ↑catalase, ↑glutathione peroxidase, ↑quinone reductase 2	[335]
γ-Tocotrienol	Breast cancer cells (MCF-7)	↑NQO2	[336]
γ-Tocopheryl quinone	Neuronal cells (PC12)	↑Activating transcription factor 4, ↑glutathione	[340]

Nuclear Receptors

γ-TmT	Breast cancer cells (MCF-7)	↓ERα protein level	[170]
γ-Tocotrienol, δ-Tocotrienol	Breast cancer cells (MDA-MB-231)	↑ERβ, ↑*MIC-1*, ↑*EGR-1*, ↑*cathepsin D*	[196]
Tocotrienol rich fraction from palm oil	Breast cancer cells (MCF-7)	↓ERα mRNA and protein levels, ↑ERβ protein levels, ↑*MIC-1*, ↑*EGR-1*, ↑*cathepsin D*	[341]
γ-Tocopherol	Colon cancer cells (SW480)	↑PPARγ mRNA and protein level	[242]
γ-Tocopherol	Keratinocytes cells (NCTC 2544)	↑PPARγ mRNA levels	[246]
γ-TmT, γ-Tocopherol, δ-Tocopherol	Breast cancer cells (MCF-7 and T47D)	↑PPARγ transactivation	[170]
γ-Tocotrienol	Prostate cancer cells (PC-3)	↑PPARγ	[334]

Anti-inflammation

γ-Tocopherol	Macrophages (RAW264.7) and human epithelial cells (A549)	↓COX-2, ↓PGE$_2$	[193]
γ-Tocopherol, δ-Tocopherol	Human epithelial cells (A549)	↓COX-2	[198]
α-Tocopherol	Human epithelial cells (A549)	↓NF-κB, ↓ICAM-1, ↓IL-8, ↓p-Erk, ↓p38	[259]
α-Tocopheryl succinate	Breast cancer cells (MDA-MB-453 and MCF-7)	↓NF-κB, ↓p-Akt	[260]
α-Tocopheryl succinate	Bladder cancer cells (KU-19-19 and 5637)	↓NF-κB	[261]
α-Tocopheryl succinate	Prostate cancer cells (PC-3, DU-145, CA-HPV)	↓NF-κB, ↓IL-6, ↓IL-8, ↓VEGF, ↓ICAM-1 ↑AP-1	[262]
γ-Tocotrienol	Prostate cancer cells (PC-3)	↓NF-κB, ↓p38, ↓TGFβ2, ↓MKK3/6	[334]
γ-Tocotrienol	Human myeloid cells (KBM-5), Lung cells (H1299), Embryonic kidney cells (A293), Breast cancer cells (MCF-7), Multiple myeloma cells (U266), squamous cell carcinoma (SCC-4)	↓inducible and constitutive NF-κB activation, ↓p-p65, ↓p-Akt	[342]
Tocotrienol rich fraction from palm oil	Prostate cancer cells (PCa)	↓NF-κB	[343]
γ-Tocotrienol	Melanoma cells (C32)	↓NF-κB	[344]
Tocotrienol rich fraction from palm oil	Human acute monocytic leukemia cells (THP-1) induced by LPS	↓PGE$_2$, ↓TNF-α, ↓IL-4, ↓IL-8, ↓iNOS, ↓COX-2, ↓NF-κB	[339]

Reactive nitrogen species (RNS); Nitric oxide (NO); Nuclear factor (erythroid-derived 2)-like 2 (Nrf2); NAD(P)H dehydrogenase, quinone 1 (NQO1); Heme oxygenase-1 (HO-1); Glutathione s-transferases (GST); Superoxide dismutase (SOD); Kelch-like-ECH-associated protein 1 (KEAP1); Estrogen receptor (ER); Peroxisome preoliferator-activated receptor γ (PPARγ); Prostaglandin E$_2$ (PGE$_2$); Cyclooxygenase-2 (COX-2); Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB); Interleukin (IL); Vascular endothelial growth factor (VEGF); γ-enriched mixed tocopherols (γ-TmT)

Table 1.4 Biological events and molecular targets modulated by vitamin E *in vivo*

Vitamin E	Cell Type/Cancer Model	Result	References
Apoptosis and Cell Proliferation			
γ-Tocopherol	Breast cancer MDA-MB-231 xenograft in nu/nu mice	↑Apoptosis	[216]
γ-TmT	NNK-induced A/J mice and lung cancer xenograft in nu/nu mice (H1299)	↑Apoptosis	[345]
γ-Tocopherol, δ-Tocopherol	Lung cancer H1299 xenograft in nu/nu mice	↑Apoptosis	[218]
γ-TmT	NMU-induced mammary tumors in female Sprague-Dawley rats	↓Proliferation	[219]
γ-TmT	NMU-induced mammary tumors in female Sprague-Dawley rats	↑Apoptosis	[170]
γ-Tocopherol, δ-Tocopherol, γ-TmT	NMU-induced mammary tumors in female Sprague-Dawley rats	↑Apoptosis	[346]
γ-TmT	Estrogen-induced mammary hyperplasia in female ACI rats	↓Proliferation, ↑Apoptosis	[238]
γ-TmT	Colon cancer in AOM/DSS-treated mice	↑Apoptosis	[171]
Mixed tocotrienols	TRAMP mice	↑Apoptosis, ↑CDK Inhibitors, ↓Cyclins	[347]
Direct and Indirect Anti-oxidant Activities			
γ-Tocopherol	Zymosan-induced acute peritonitis in male Fischer 344 rats	↓RNS	[226]
γ-Tocopherol	Carrageenan-induced inflammation in Wistar male rats	↓RNS	[225]
γ-TmT	NNK-induced A/J mice and lung cancer xenograft in nu/nu mice (H1299)	↓8-oxo-dG, ↓γ-H2AX, ↓nitrotyrosine	[345]
γ-Tocopherol, δ-Tocopherol, γ-TmT	Lung cancer xenograft in nu/nu mice (H1299)	↓8-oxo-dG, ↓γ-H2AX, ↓nitrotyrosine	[218]
γ-TmT	Colon cancer in AOM/DSS-treated mice	↓nitrotyrosine	[171]
γ-TmT	Prostate carcinogenesis in TRAMP male mice	↑Nrf2 protein levels, ↑GSTm1, UGT1A1, SOD, HO-1, catalase,	[75]

		glutathione peroxidase	
γ-TmT	Estrogen-induced mammary hyperplasia in female ACI rats	↑Nrf2 protein levels	[238]

Nuclear Receptors

γ-TmT	NMU-induced mammary tumors in female SD rats	↓ERα mRNA and protein level	[170]
γ-TmT	Estrogen-induced mammary hyperplasia in female ACI rats	↓ERα mRNA and protein level, ↑ERβ mRNA level, ↓E$_2$ in the serum	[238]
γ-TmT	NMU-induced mammary tumors in female SD rats	↑PPARγ mRNA and protein level	[170]
γ-TmT	Estrogen-induced mammary hyperplasia in female ACI rats	↑PPARγ mRNA and protein level	[238]

Anti-inflammation

γ-Tocopherol	Carrageenan-induced inflammation in Wistar male rats	↓PGE$_2$, ↓LTB$_4$, ↓TNF-α	[225]
γ-TmT	NNK-induced A/J mice and lung cancer xenograft in nu/nu mice (H1299)	↓PGE$_2$, ↓LTB$_4$	[345]
γ-TmT	Colon cancer in AOM/DSS-treated mice	↓PGE$_2$, ↓LTB$_4$	[171]
γ-TmT	Estrogen-induced mammary hyperplasia in female ACI rats	↓COX-2, ↓PGE$_2$, ↓8-isoprostane	[238]

N-methyl-N-nitrosourea (NMU); August Copenhagen Irish (ACI); Sprague-Dawley (SD);Nicotine-derived nitrosamine ketone (NNK); Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP); azoxymethane (AOM); dextran sodium sulphate (DSS); 8-hydroxy-2'-deoxyguanosine (8-oxo-dG); Cyclin dependent kinase (CDK); Reactive nitrogen species (RNS); Nuclear factor (erythroid-derived 2)-like 2 (Nrf2); Heme oxygenase-1 (HO-1); Glutathione s-transferases (GST); Superoxide dismutase (SOD); Estrogen receptor (ER); Peroxisome preoliferator-activated receptor γ (PPARγ); Prostaglandin E$_2$ (PGE$_2$); Cyclooxygenase-2 (COX-2); UDP-glucuronosyltransferases (UGT); Tumor necrosis factor-α (TNFα); Estradiol (E$_2$); γ-enriched mixed tocopherols (γ-TmT)

Table 1.5. Case-Control Studies of Vitamin E and Breast Cancer Risk

Population	Case/Control[a]	Intake or blood levels	Relative risk (95% CI) for highest *vs.* lowest level	Conclusion	Study
Canada	223/85	Serum or adipose tissue levels of α-T: levels were not specified	Serum α-T: 0.85 (0.45-1.59) Adipose tissue α-T:1.34 (0.73-2.47)	No association	[277]
US	969/969	Serum α-T or γ-T: levels were not specified	Serum α-T: 0.79 (0.57-1.08) Serum γ-T: 0.96 (0.71-1.30)	No association	[278]
US	244/244 (1974 Study) 115/115 (1989 Study)	Serum α-T: 0.91 – 1.40 mg/dl; 0.99 - 1.65 mg/dl Serum γ-T: 0.15 – 0.32 mg/dl; 0.13 – 0.34 mg/dl	Serum α-T: 0.94 (0.52-1.73); 0.67 (0.28 – 1.62) Serum γ-T: 0.70 (0.40-1.23); 0.80 (0.33 – 1.93)	No association	[279]
US	64/64	Serum α-T: 1.31 mg/dl Serum γ-T: 0.25 mg/dl	α-T: 0.46 (0.23 – 0.64) γ-T: 0.53 (0.32 – 0.69)	No association	[280]
India	Pre-M: 28/23 Post-M: 29/19	Serum α-T: 38 *vs.* 25 µmol/L Serum γ-T: 30 *vs.* 25 µmol/L	Serum α-T: P<0.05 Serum γ-T:p<0.02	**Risk reduction**	[266]
US	27/28	Serum α-T: ≤ 20.5~ ≥35 µmol/L Serum γ-T: ≤ 2.12~ ≥7.573 µmol/L	Serum α-T: 0.76 (0.10-5.75) Serum γ-T:0.31 (0.04-1.93)	No association	[282]
Greek	Pre-M: 270/505 Post-M: 550/1041	Vit E: <5.2 ~ ≥8.6 IU/day	Pre-M:0.50 (0.25-1.02) Post-M: 0.85 (0.53-1.36)	No association	[283]
Finish	Pre-M: 119/324	Vit E: ≤7 ~ >13 mg/day	0.5 (0.2-1.0)	**Risk reduction**	[267]
Uruguay	400/405	Vit E: 4.7 ~ 9.7 mg/day	0.4 (0.26-0.62)	**Risk reduction**	[268]

Italian	Pre-M:989/841 Post-M: 1577/1745	Vit E: <8.5 ~ 11.7 mg/day	Pre-M:1.27 (0.9-1.78) Post-M:1.16 (0.92-1.46)	No association	[284]
US	105/203	Serum α-T: ≤ 21.6~ ≥31.3 μmol/L	1.2 (0.5-2.8)	No association	[285]
Italy	Pre-M: 988/843 Post-M: 1572/1742	Vit E: levels were not specified	Pre-M:0.8 (0.7-1.0) Post-M:0.75 (0.6-0.9)	**Risk reduction**	[269]
US	297/311	α-T: <6 ~ ≥11 mg/day	0.55 (0.34-0.88)	**Risk reduction**	[270]
US	Pre-M without family history:224/251	α-T: ≤ 6.3 ~ >10.4 IU/day	0.5 (0.2-1.0)	**Risk reduction**	[265]
US	Post-M: 313/349	Vit E:11 vs 5.4 mg/day (median)	0.4 (0.2-0.9)	**Risk reduction**	[271]
Malaysia	57/139	Vit E: 6.1 vs 6.9 mg/day (mean)	2.12 (1.00-4.21)	**Risk Reduction**	[272]
South Korea	362/362	Vit E: 10.6 vs 11.2	0.66 (0.41-1.08)	No association	[281]
Switzerland	289/442	Vit E: 9.4-18.1 mg/day	0.49 (0.35-0.71)	**Risk Reduction**	[273]
Italy	2569/2588	Vit E: 7.21 – 13.43 mg/day	0.75 (0.6 – 0.9)	**Risk Reduction**	[274]
Germany	310/353	Vit E: 7.1 – 12.7 mg/day	1.08 (0.58-2.03)	No association	[276]
China	3454/3474	Vit E: levels not specified	Low supplemental Vit E: 0.7 (0.5 – 1.0) High supplemental Vit E: 1.2 (0.9 – 1.6)	**Risk Reduction**	[275]
US	1498/1559 (Non-Hispanic white) 763/877 (Hispanic)	α-T: 108 – 224 mg/day β-T: 0.3 – 0.4 mg/day γ-T: 15.9 – 19.4 mg/day δ-T: 2.94 – 3.59 mg/day	α-T: 0.87 (0.73 – 1.03) β-T: 1.10 (0.89 – 1.36) γ-T: 1.13 (0.89 – 1.44) δ-T: 1.10 (0.89 – 1.35)	No association	[286]
Denmark	418/394	Dietary Vit E: 4.30 – 14.8 Supplemental Vit E: 0.94 – 78.23	Dietary Vit E: 1.13 (0.61 – 2.10) Supplemental Vit E: 1.00 (0.96 – 1.03)	No association	[287]

| South Korea | 224/250 | Dietary Vit E: 6.26 – 12.71 mg/day | Dietary Vit E: 0.71 (0.39 – 1.27) | No association | [288] |

[a] Pre-menopausal (Pre-M) or postmenopausal (Post-M) women; Tocopherols (T)

Table 1.6 Cohort Studies of Vitamin E and Breast Cancer Risk

Population	Year	Case/control[a]	Intake or blood levels	Relative risk (95% CI) for highest *vs.* lowest level	Conclusion	Study
Canada	1982-1987	519/1182	α-T: <3 *vs.*>7 mg/day	α-T: 1.05 (0.65-1.70)	No association	[289]
Sweden	1987-1990	1271/59036	Vit E: 9.3 *vs.* 3.8 mg/day (median)	0.83 (0.6-1.14)	No association	[290]
		Pre-M: 784/53938	Vit E: 10 *vs.* 5 IU/day (median)	0.81 (0.64-1.02)	No association	[291]
Netherlands		Post-M: 650/62573	Vit E: 19.8 *vs.* 6.9 mg/day (median)	1.25 (0.85-1.85)	No association	[292]
Finland		88/4697	Vit. E: levels were not specified	1.08	No association	[293]
US	1986	570/21782	Vit E: 10 *vs.* 5 mg/day	0.81 (0.64-1.02)	No association	[294]
US	1976 - 1982	1439/89494	Dietary Vit E: <3.9 ~ ≥24.1 IU/day Supplemental Vit E: 600 *vs.* 0 IU/day	Dietary Vit E: 0.90 (0.77-1.06) Supplemental Vit E: 1.01(0.69-0.49)	No association	[295]
Canada		325/628	Vit E: ~ 18 IU/day (median)	1.32 (0.85-2.05)	No association	[296]
US	1980-1987	Post-M: 344/18586	Vit E: <4.3 ~ ≥9.3 mg/day	0.86 (0.61-1.21)	No association	[297]
Europe	1992-2000	7502/334493	Vit E; 5.4-19.5 mg/day	0.92 (0.77-1.11)	No association	[298]
US	1993-1998	2879/81926	Dietary Vit E: 6.2 – 9.4 mg/day Supplemental Vit E: 0 – 424 mg/day	Dietary Vit E: 1.03 (0.91 -1.17) Supplemental Vit E: 1.01 (0.90 - 1.14)	No association	[299]
US	1991-1999	Pre-M: 714/90655	Vit E: 7-59 mg/day Dietary Vit E: 6 – 10 mg/day	Vit E: 1.13 (0.89 – 1.43) Dietary Vit E: 1.17 (0.92 – 1.50)	No association	[300]

[a] Pre-menopausal (Pre-M) or postmenopausal (Post-M) women; Tocopherols (T)
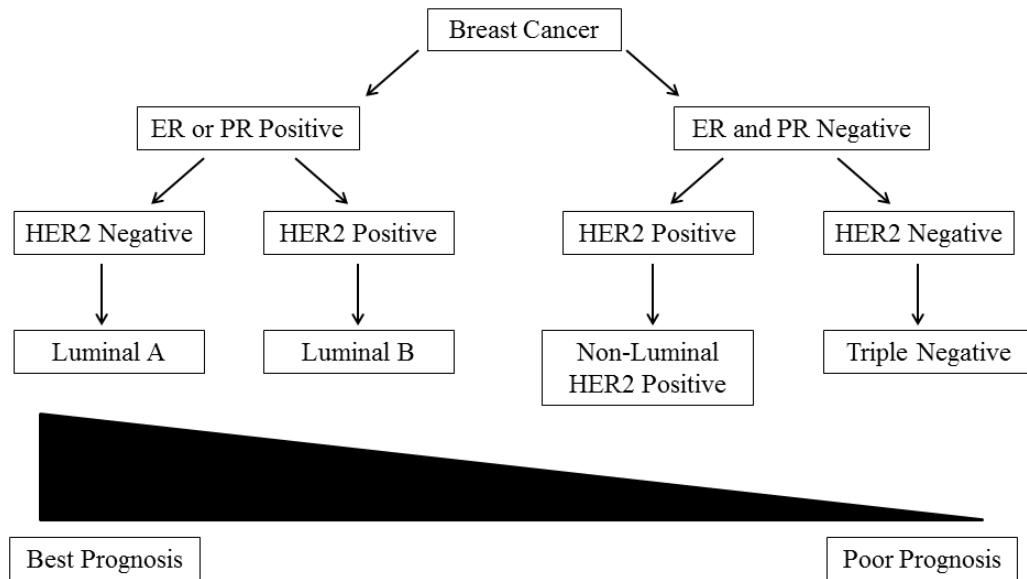
Figure 1.1 Breast cancer is a heterogeneous disease and is divided into 4 subtypes. The different subtypes are classified as estrogen receptor (ER) positive luminal A, ER positive luminal B, human epidermal growth factor receptor-2 positive (HER2 positive), and triple negative (negative for ER, progesterone receptor (PR) and HER2). Modified from [8,9].
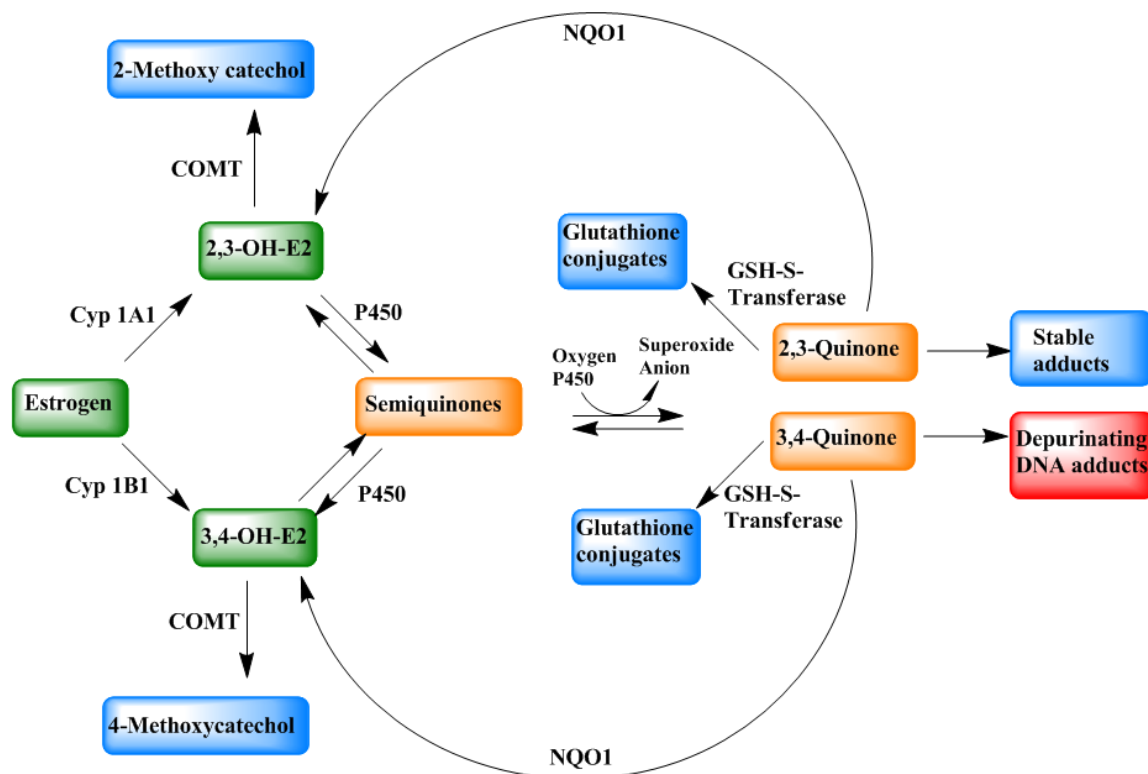
Figure 1.2 Oxidative metabolism of estrogen through the catechol pathway. Phase I and Phase II enzymes are shown. Modified from [73].
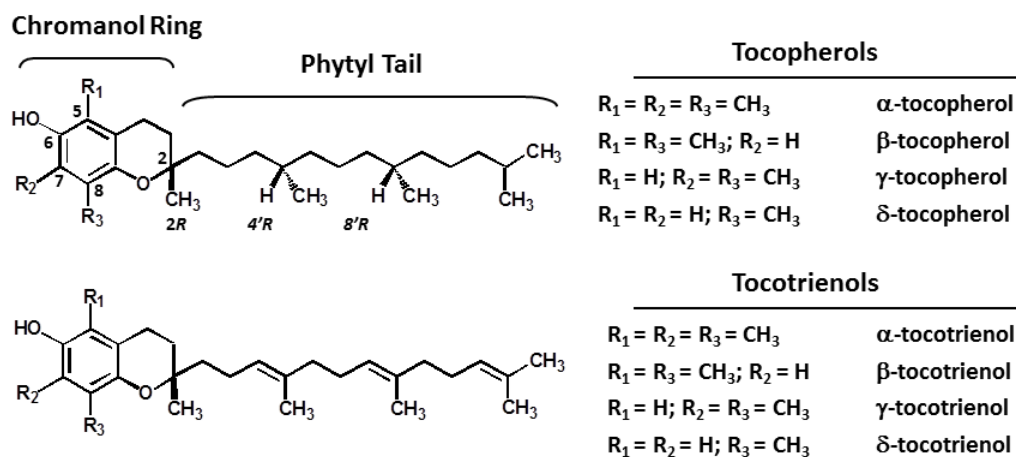
Figure 1.3 Vitamin E is comprised of 8 naturally occurring forms. These forms are different in number and position of methyl groups on the chromanol ring and presence and absence of unsaturated bonds in the phytyl tail. Structures of variants, α-, β-, γ-, δ-tocopherols and tocotrienols are shown. Adapted from [170,238,263]

Chapter 2: Dietary treatment with γ-TmT reduces estrogen-induced mammary hyperplasia[a,b]

2.1 Introduction

One risk factor for breast cancer is elevated lifetime exposure to estrogen [348]. Estrogens exert their carcinogenic effects via ER-dependent and ER-independent mechanisms [73]. The activation of ER by estrogens can increase cellular proliferation, resulting in accumulation of genetic damages leading to carcinogenesis [70]. The ER-independent pathway of estrogen-induced mammary tumorigenesis involves the metabolism of estrogen via cytochrome P450 enzymes to generate genotoxic metabolites [73]. Estrogen may be metabolized by CYP 1A1 to form 2-OHE_2 or by CYP 1B1 to form 4-OHE_2. The 4-OHE_2 metabolite is tumorigenic and undergoes oxidative metabolism to generate eletrophilic quinones, and reacts with DNA to produce depurinating adducts and ROS [70,73].

ACI rats exposed to elevated levels of estrogen provide a more natural representation of human breast carcinomas. The ACI rat strain is known to be sensitive to estrogen treatment and serves as an appropriate model to study estrogen-induced mammary carcinogenesis [78] and is similar to human ER positive breast cancer [349]. Female ACI rats rarely develop spontaneous mammary tumors [78] and show a high incidence (80-100%) of mammary ductal adenocarcinomas within 24-36 weeks when exposed to low doses of exogenous E_2 [80,350].

Estrogen is a risk for ER positive breast cancer, and there are limited agents that are safe and effective to prevent and treat this disease. Tocopherols are antioxidants and have chemopreventive effects. The purpose of this chapter is to determine whether γ-TmT inhibits estrogen-mediated events during the development of mammary hyperplasia in a physiologically

relevant ER positive ACI rat model. We hypothesize that γ-TmT may inhibit estrogen-mediated events in mammary hyperplasia in ACI rats by decreasing cell proliferation and increasing apoptosis via the activation of PPARγ. We investigated molecular markers of cell proliferation (PCNA), apoptosis (c-Casp-3), nuclear receptors (ERα, ERβ and PPARγ), inflammation (PGE$_2$ and COX-2) and the Nrf2 pathway by Western blot, IHC and/or quantitative PCR analysis.

2.2 Material and Methods

2.2.1 Animals and experimental procedures

Female ACI rats were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN) at 7-8 weeks of age. The procedure to implant the pellet was adapted from a previously described method [350]. Briefly, an incision was made in a shaved area in the middle of the back between the scapulas. The animals were implanted subcutaneously with either a control pellet containing 20 mg of cholesterol or a pellet containing 2.5 mg of E$_2$ and 17.5 mg of cholesterol. The pellets were purchased from Hormone Pellet Press (Shawnee Mission, KS). The animals were sacrificed 2 or 10 weeks after the surgical implantation of the pellet. Blood was collected; serum was prepared and stored in -80°C. Mammary glands from each animal were harvested at necropsy and fixed in 10% formalin or flash frozen and stored in -80°C. The liver was weighed and stored in -80°C for further analysis.

2.2.2 Tocopherol Diet

γ-TmT was supplied by the Cognis Corporation (Cincinnati, OH) and contained 58% γ-tocopherol, 24% δ-tocopherol, 13% α-tocopherol, and 0.5% β-tocopherol. Semipurified modified AIN-93M diet was obtained from Research Diets Laboratory (New Brunswick, NJ) and

used as the control diet. The test diet was prepared by Research Diets Laboratory by adding either 0.3% or 0.5% γ-TmT to the AIN-93M diet. The diets were separated into sealed bags after they were flushed with nitrogen gas to prevent oxidation of the diet while they were stored at 4°C. The food cups were replenished with fresh pellets twice weekly.

2.2.3 Analysis of tocopherols in rat serum and mammary glands

The levels of tocopherols (α-, γ-, δ-) and their metabolites in the serum and mammary gland were analyzed by high performance liquid chromatography (HPLC) using methods as previously described [170,171,351]. Reference samples were prepared as previously reported [170,351]. Briefly, fat-soluble vitamins were extracted from 150 µL of plasma with ethanol and hexane, and then dissolved in a mixture of ethanol and acetonitrile. A HPLC system was developed using a Supelcosil LC18 column, 5 µm (4.6 x 150 mm) with ethanol:acetonitrile (45:55) as the mobile phase. A Waters 490 multiwavelength detector (Waters-Millipore) was used to detect absorbance at 292 nm (α-, γ-, δ-tocopherol).

2.2.4 Serum estradiol levels

The E_2 levels in the serum were analyzed using an EIA kit from Enzo Life Sciences International, Inc (#ADI-900-174; Plymouth Meeting, PA). Serum samples were mixed with diethyl either, vortexed and allowed to separate. The organic layer was collected and dried using a Speed Vacuum Evaporator (VWR International, Inc, West Chester, PA). Samples were then reconstituted in EIA buffer supplied by the company and the assay was performed as described in the manufacture's protocol. Briefly, the standards and samples were added to the wells coated with a DxS IgG antibody. A blue solution of estradiol-17β conjugated to alkaline phosphatase is

then added, followed by a yellow solution of sheep polyclonal antibody to estradiol-17β. During a simultaneous incubation at room temperature the antibody binds in a competitive manner, the estradiol-17β in the sample or conjugate. The plate is washed, leaving only bound estradiol-17β. pNpp substrate solution is added and the substrate generates a yellow color when catalyzed by the alkaline phosphatase on the estradiol-17β conjugate. The stop solution is added, and the plate is read at 405 nm. The amount of signal is indirectly proportional to the amount of estradiol-17β in the sample.

2.2.5 Enzyme immunoassays for prostaglandin E_2 and 8-isoprostane

EIA kits from Cayman Chemicals (Ann Arbor, MI) were used to analyze the levels of PGE_2 (#514010) and 8-isoprostane (#516351). Serum samples were prepared as previously described [171]. Briefly, the serum samples were mixed with ethyl acetate, vortexed and then centrifuged. The organic layer was collected and dried using a Speed Vacuum Evaporator (VWR International, Inc, West Chester, PA). Samples were then reconstituted in EIA buffer and the assay was performed as described in the manufacture's protocol. Briefly, the plate was pre-coated with goat polyclonal anti-mouse IgG (for PGE_2 EIA) or mouse anti-rabbit IgG (8-isoprostane EIA), and tracer, antibody/anti-serum, and either standard or sample was incubated. Next the plate was washed to remove all unbound reagents. Ellman's Reagent (contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm, and the plate was read at 405nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of PGE_2 or 8-isoprostane tracer bound to the well, which is inversely proportional to the amount of free PGE_2 or 8-isoprostane present in the well during incubation.

2.2.6 Histopathological analyses and immunostaining

Mammary glands from the 4th position on the left side were collected at necropsy, fixed in 10% formalin for 24 hours, and transferred to 70% ethanol. The mammary glands were embedded in paraffin (Electron Microscopy Sciences, Hatfield, PA) and then sectioned at 4 μm thickness. Tissue sections mounted on slides were stained with hematoxylin & eosin (H&E) for histopathological analysis. For immunohistochemistry, the sections were immunostained with antibodies to PPARγ (1:200 diluted, Santa Cruz Biotechnology, Santa Cruz, CA), ERα (1:50 diluted, ThermoScientific, Lafayette, CO), PCNA (1:1000 diluted, BD Pharmingen, San Diego, CA), c-Casp-3 (1:200 diluted, Cell Signaling, Beverly MA), Nrf2 (1:2000 diluted, Epitomics, Burlingame, CA) or COX-2 (1:500 diluted, Cayman Chemicals, Ann Arbor, MI).

The slides were incubated with 3-diaminobenzamine substrate (Vector Labs, Burlingame, CA) and counterstained with Harris hematoxylin (Sigma, St. Louis, MO). Histopathological images were taken randomly with Zeiss AxioCam HRc camera fitted to a Zeiss Axioskop 2 Plus microscope (Carl Zeiss Microimaging, LLC, Thornwood, NY) at 200x total magnification. Immunohistochemical images were taken randomly with Nikon Eclipse E800 (Melville, N.Y) fitted to Nikon digital sight Ri1 at 600x total magnification. Quantification was performed by randomly selecting three animals per group and counting three sections from each animal for over 4000 cells per animal. Positive staining for PCNA, ERα, and PPARγ is indicated by brown staining in the nucleus. Positive staining for Nrf2 is found both in the cytoplasm and nuclei of the cells. Positive staining for COX-2 is found in the cytoplasm. The staining density for nuclear or cytoplasmic positive cells was determined by using Aperio® Scan Scope (Vista, CA). Positive c-Casp-3 staining is indicated by light brown to dark brown precipitate in the cytoplasm or

perinuclei area of the cells. Quantification of c-Casp-3 was determined by NIS-Elements software (Melville, N.Y) where over 3000 cells were counted per animal (three animals/treatment group).

2.2.7 Western blot analysis

Liver tissue was homogenized; protein was extracted and electrophoresed as previously described [170]. The protein samples were separated on 4-15% SDS-PAGE gels (Biorad, Hercules, CA) followed by transfer to a polyvinylidene fluoride membrane (PALL, Ann Arbor, MI). The membranes were blocked with 5% milk in Tris buffer for 1 h and then incubated with the appropriate primary antibody solutions overnight at 4°C. The membranes were washed with Tris buffer, and incubated with horseradish peroxidase conjugated secondary antibody solutions for 1 h at room temperature. The protein bands were visualized using a chemiluminescence based kit from Amersham Biosciences (Buckinghamshire, UK). The primary antibodies against Nrf2, Keap1, NQO1, (Santa Cruz Biotechnology, Santa Cruz, CA), UGT (Cell Signaling, Beverly MA), HO-1 (Epitomics, Burlingame, CA), β-actin (Sigma, St. Louis, MO), and secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Quantification of western blots was analyzed by ImageJ program (NIH, USA).

2.2.8 Liver microsome analysis

Liver microsomes were made as described previously [352]. The final pellet was resuspended in 0.25 M sucrose by homogenization in a glass homogenizer with a Teflon pestle. Microsomal proteins, 10-20 μg per lane, were resolved on SDS-gels Criterion XT -10% BisTris and transferred to nitrocellulose BA83 pore 0.2 μm from Schleicher & Schuell Biosciences, Inc. (Keene, NH). Blots were probed with selected anti-cytochrome P450 monoclonal antibodies

produced in mouse or polyclonal antibodies produced in rabbit as described previously [352]. Recognition of certain cytochrome P450 enzymes with these antibodies was revealed by probing with the appropriate secondary antibody coupled to alkaline phosphatase and visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

2.2.9 mRNA expression analysis using quantitative PCR

Mammary glands from the 4th position on the right side and liver tissue were harvested at necropsy, placed in 1 ml of trizol and stored in -80°C until RNA extraction. RNA extraction, reverse transcription, and quantitative PCR were carried out as previously reported [353]. Labeled primers for glyceraldehyde-3-phosphate dehydrogenase, ERα, ERβ, PPARγ, Nrf2, Keap1, Cyp 1A1, Cyp 1B1, UGT1A1, NQO1, GSTmu 1, LOC5011 (rat orthologue of GST A1), COMT, Glutamate cysteine ligase, modifier subunit (GClm), SOD1, GPX1, HO-1, TXN1, and catalase were obtained from Applied Biosystems (Carlsbad, CA).

2.2.10 Statistical analysis

Data was analyzed using Graph Pad Prism 4.0 (GraphPad Software Inc.). Statistical significance was evaluated by using one-way analysis of variance (ANOVA) followed by Dunnett's post-test. The data presented represents the mean ± S.E.M. P values <0.05 were considered significant.

2.3 Results

2.3.1 Estrogen increases body and liver weight.

As shown in Table 2.1, there was no difference in the body weight among treatment groups at the 2-week time point. However, at the 10-week time point, the average body weight of

the control pellet group was significantly lower than the E_2 control group; no changes of body weight were shown with γ-TmT treatment. When administered E_2, it is well documented in ACI and other rodent models that the liver weight increases [354]. Estrogen can induce proliferation and regulate MAPK activities, which result in cell survival and regeneration [354]. Thus, increased liver weight can be attributed to estrogen-induced growth. As seen in our study, E_2 treated rats showed an increase in liver weight for both 2- and 10-week time points. At the 10-week time point, the liver weight of the 0.5% γ-TmT fed group was significantly less than the E_2 control group.

2.3.2 Administration of γ-TmT increases levels of γ- and δ-tocopherol in the serum and mammary glands.

We examined the levels of α-, γ-, δ-tocopherol and their respective metabolites, carboxydimethyldecyl hydroxychromans (CDMDHCs), carboxymethylbutyl hydroxychromans (CMBHCs), and carboxyethyl hydroxychromans (CEHCs) in the serum and mammary gland (Table 2.2). The longer chain metabolites, α-, γ-, δ-CDMDHCs and α-, γ-, δ-CMBHCs, showed negligible levels in the serum and mammary gland (data not shown). When administered 0.3% and 0.5% γ-TmT diet for either 2 or 10 weeks of treatment, α-CEHC was increased, while the level of α-tocopherol remained relatively constant in both the serum and mammary gland. After the treatment with 0.3% and 0.5% γ-TmT for 2 weeks, serum levels of γ- and δ-tocopherols and their short chain metabolite, CEHC, were significantly increased (Table 2.2): γ-tocopherol (6.4- and 7.4-fold, respectively), δ-tocopherol (5.0- and 6.0-fold, respectively), γ-CEHC (59- and 85-fold, respectively), and δ-CEHC (39- and 54-fold, respectively). After 10 weeks of 0.3% and 0.5% γ-TmT treatment, serum levels of tocopherols and CEHCs were higher than those of 2 weeks: γ-tocopherol (6.4- and 14-fold, respectively), δ-tocopherol (7.0- and 18-fold,

respectively), γ-CEHC (91- and 108-fold, respectively), and δ-CEHC (40- and 54-fold, respectively). The levels of tocopherols in the mammary gland showed a similar trend of changes to the serum data. After 2 weeks of 0.3% and 0.5% γ-TmT treatment, levels of tocopherols and CEHCs in the mammary gland were increased: γ-tocopherol (4.2- and 6.4-fold, respectively), δ-tocopherol (24- and 37-fold, respectively), γ-CEHC (37- and 49-fold, respectively) and δ-CEHC (20- and 26-fold, respectively). After 10 weeks of 0.3% and 0.5% γ-TmT treatment, levels of tocopherols and CEHCs in the mammary gland increased: γ-tocopherol (8.0- and 11-fold, respectively) and δ-tocopherol (14- and 21-fold, respectively) and γ-CEHC (28- and 32-fold, respectively) and δ-CEHC (45- and 60-fold, respectively).

2.3.3 Serum levels of E_2 are decreased by the administration of γ-TmT in rats.

Circulating E_2 levels were determined at 2- and 10-week time points in rats treated with the control pellet or with E_2 pellets and their respective diets (Fig. 2.1). We chose 2 weeks as a time point because previous experiments showed a peak of circulating E_2 levels in the serum in E_2 treated ACI rats (data not shown). In addition, *Turan et al.* showed that after 10 weeks of low dose E_2 treatment, circulating levels of E_2 remained constant [350]. In the present study, 2 weeks after implanting the pellets the average serum E_2 levels of the control pellet group were 21.1 ± 2.1 pg/ml, whereas average serum E_2 levels in the E_2 control group were increased to 60.4 ± 2.8 pg/ml ($p<0.01$). Treatment with 0.5% γ-TmT for 2 weeks significantly decreased E_2 serum levels to 45.3 ± 4.3 pg/ml ($p<0.05$). After 10 weeks of pellet implantation, the serum E_2 level in the control pellet group (28.0 ± 3.6 pg/ml), E_2 control group (25.8 ± 5.1 pg/ml), and 0.3% γ-TmT fed group (34.4 ± 5.1 pg/ml) were similar, suggesting that the serum E_2 level spike shown at 2 weeks in the E_2 control group reduced to the basal level by 10 weeks. Furthermore, treatment with 0.5% γ-TmT for 10 weeks showed a significant decrease in E_2 levels to 11.6 ± 2.3 pg/ml ($p<0.05$).

2.3.4 Hyperplasia is evident in the mammary gland in the E_2 treated groups.

H&E staining was performed on mammary gland sections (Fig. 2.2). The control pellet group showed normal mammary glands at both the 2- and 10-week time points. Based on histological evaluation, mild lobular hyperplasia was evident in the mammary gland in the E_2 control group. Treatment with 0.3% and 0.5% γ-TmT at both 2- and 10-week time points showed no apparent effect on E_2-induced mammary hyperplasia. Further evaluation was carried out to determine if γ-TmT had an effect on cell proliferation in mammary hyperplasia. Immunohistochemical evaluation of the hyperplastic mammary gland, as described below, revealed that cell proliferation was significantly decreased, while a significant increase of apoptotic cells was observed when administered γ-TmT.

2.3.5 Treatment with γ-TmT reduces proliferating cell nuclear antigen (PCNA) but increases cleaved-caspase 3 (c-Casp-3) in the mammary gland.

PCNA expression in the mammary gland was reduced after 2 and 10 weeks of treatment with γ-TmT (Fig. 2.3). After 10 weeks of treatment, 0.3% or 0.5% γ-TmT-fed groups resulted in a 54% or 56% decrease in proliferation, respectively ($p<0.05$). As shown in Fig. 2.4, after 2 weeks of treatment with 0.3% or 0.5% γ-TmT, c-Casp-3 expression in the mammary gland showed 108% ($p<0.01$) or 54% ($p<0.05$) increase above the E_2 control group, respectively. After 10 weeks of treatment, 0.3% or 0.5% γ-TmT diet-fed group showed an increase in c-Casp-3 expression by 47% ($p<0.05$) and 66% ($p<0.01$), respectively, indicating an induction of apoptosis in the mammary gland by treatment with γ-TmT.

2.3.6 γ-TmT treatment decreases estrogen receptor α (ERα) but increases peroxisome proliferator activated receptor γ (PPARγ) protein levels.

As shown in Fig. 2.5, after treatment of 0.3% and 0.5% γ-TmT for 2 weeks, there was a 10% and 29% decrease in percentage of ERα positive cells in the mammary gland, respectively. After 10 weeks with treatment of 0.3% and 0.5% γ-TmT, there was a 49% ($p<0.05$) and 50% ($p<0.05$) decrease in ERα expression, respectively. After 2 weeks of treatment, there was a 66% ($p<0.05$) increase above the E_2 control group in expression of PPARγ in the 0.5% γ-TmT treated group (Fig. 2.6). When the rats were treated with 0.5% γ-TmT for 10 weeks, the expression of PPARγ was increased by 67% ($p<0.01$) in the mammary gland.

2.3.7 γ-TmT treatment suppresses the expression of ERα mRNA while inducing the expression of ERβ and PPARγ mRNA in mammary glands.

The mRNA levels of ERα, ERβ, and PPARγ were significantly changed by γ-TmT in mammary glands (Fig. 2.7). When given γ-TmT, the mRNA level of ERα was decreased: the 0.5% γ-TmT treated group for the 2- and 10-week time points were reduced by a fold change of 3.6 ± 0.1 ($p<0.05$) and 2.5 ± 0.1, respectively (Fig. 2.7A). The mRNA expression for ERβ showed fold increase of 2.3 ± 0.5 ($p<0.05$) for the 0.3% γ-TmT treated group at 10 weeks (Fig. 2.7B). After treatment with 0.3% and 0.5% γ-TmT for 2 weeks, PPARγ mRNA level was increased by a fold change of 3.9 ± 0.6 ($p<0.05$) and 2.5 ± 0.3, respectively (Fig. 2.7C). After γ-TmT treatment for 10 weeks, there was further fold increase of 5.0 ± 1.1 ($p<0.05$) and 3.2 ± 0.6 when fed 0.3% and 0.5% γ-TmT, respectively.

2.3.8 Treatment with γ-TmT increases the expression of Nrf2 in mammary glands and upregulates protein levels of Nrf2, NQO1, UGT, and HO-1 in the liver.

As shown in Fig. 2.8, after 2 weeks of treatment with 0.3% or 0.5% γ-TmT, Nrf2 levels in the mammary gland showed 12% ($p<0.05$) or 20% ($p<0.01$) increase above the E_2 control group, respectively. After 10 weeks of treatment, 0.3% or 0.5% γ-TmT diet-fed group showed an increase in Nrf2 levels by 11% ($p<0.05$) and 17% ($p<0.05$), respectively. In the liver, the protein level of Nrf2 is increased when administered 0.3% and 0.5% γ-TmT in the diet for 2 weeks, while Keap1 levels remain constant (Fig. 2.9). At 10 weeks of γ-TmT treatment, NQO1, UGT, and HO-1 protein levels were increased in the liver.

To determine whether the levels of hepatic cytochrome P450 isozymes were altered by implanting estrogen pellets or concurrent feeding of γ-TmT, we also examined microsomal preparations by SDS-PAGE and Western blots using antibodies specific for 7 individual P450 isozymes. At the 2-week time point, the levels of P450 2B1/2 and 2C7 decreased 10-15% in the group treated with 0.5% γ-TmT compared to E_2 control group but this change was not statistically significant (data not shown). The levels of P450 2E1, 2A1 and 2C12, a female specific isozyme, were unchanged by estrogen pellet implants or dietary γ-TmT. At the 10-week time point, the levels of P450 2B1/2, 2A1, 2C12 and 2C7 were not altered by the treatments; however, levels of P450 2E1 decreased 10-20% by 0.3% and 0.5% γ-TmT treatment, but these changes were not significant (data not shown). Neither cytochrome P450 3A1 nor 3A2 were detected at either the 2- or 10-week time points regardless of treatment. This was not unexpected since P450 3A2 is male specific and P450 3A1 levels are very low and not easily detected unless the rats have been exposed previously to a P450 3A inducer such as dexamethasone, other steroids, or barbiturates like phenobarbital [352]. However, this suggests that the E_2 released from the implanted pellets nor the γ-TmT in the diet at 0.3% or 0.5% were sufficient to elevate P450 3A1 levels which is the rat homolog of human P450 3A4.

2.3.9 Administration of γ-TmT induces the expression of phase II detoxifying enzymes mRNA in the mammary gland and the liver.

Nrf2 activation leads to induction of enzymes involved in anti-oxidative functions and phase II detoxification, such as GST, NQO1, HO-1, and UGT to reduce cellular stress, which may contribute to prevention of carcinogenesis. Quantitative RT-PCR was performed on mammary gland (Table 2.3) and liver tissues (Table 2.4) for Nrf2 and related markers. In the mammary gland after 10 weeks of treatment, the mRNA level of Cyp 1A1 was significantly reduced when fed 0.3% γ-TmT. In the mammary gland, UGT1A1, GSTm1, and LOC5O11 were induced by γ-TmT treatment. Antioxidant enzymes did not show a significant change when given γ-TmT diet (Table 2.3). In the liver, phase II enzymes, UGT1A1, NQO1, GSTm1, and GClm, were increased with γ-TmT treatment. Among the antioxidant enzymes, only HO-1 was significantly increased in the liver when administered γ-TmT diet (Table 2.4).

2.3.10 Inflammatory markers are reduced by the treatment with γ-TmT.

Immunohistochemical staining for COX-2 was performed on mammary hyperplastic tissues (Fig. 2.10). After treatment of 0.3% and 0.5% γ-TmT for 2 weeks, there were 39% ($p<0.01$) and 45% ($p<0.01$) decrease in COX-2 positive cells in the mammary gland, respectively. After 10 weeks with treatment of 0.3% and 0.5% γ-TmT, there were 24% ($p<0.05$) and 30% ($p<0.05$) decrease in levels of COX-2, respectively. PGE_2 is a downstream product of the enzyme COX-2. Serum levels of PGE_2 were decreased by γ-TmT treatment, with which significant reduction of serum PGE_2 was shown by 10 week treatment with 0.5% γ-TmT by 71% ($p<0.05$) (Fig. 2.11). The serum level of another inflammatory marker, 8-isoprostane, was also examined.

At 10 weeks, the E_2 control group had serum 8-isoprostane levels of 637 ± 107 pg/ml, and treatment with 0.5% γ-TmT decreased by 64% to 230 ± 31 pg/ml (p<0.05) (Fig. 2.11).

2.4 Discussion

In the present study, increased levels of γ- and δ-tocopherol and their short chain metabolites were evident in both the serum and mammary gland of the γ-TmT treatment groups, while α-tocopherol levels remained relatively constant in both the serum and mammary gland of all treatment groups. The α-tocopherol-transfer protein preferentially transfers α-tocopherol from the liver to the blood, while γ- and δ-tocopherol remain in the liver and are metabolized [178]. Cytochrome P450 4F2 catalyzes the initial step in the vitamin E-ω-hydroxylase pathway followed by β-oxidation, which removes 2 carbons from the side chain in each cycle ending in the short chain metabolite, CEHC [178]. When administered γ-TmT diet, γ-tocopherol, γ-CEHC, δ-tocopherol, and δ-CEHC showed a significant increase in the serum and mammary gland. We believe the cancer preventive activity of γ-TmT treatment may be mostly due to the increased levels of γ- and δ-tocopherol. The activity of individual metabolites remains to be determined.

Estrogens have been implicated in breast cancer. Vitamin E has been shown to inhibit ER-positive cell proliferation and work as antagonists of estrogen signaling [194]. Previously, we reported that γ- and δ-tocopherols, but not α-tocopherol, inhibited proliferation of ER-positive MCF-7 human breast cancer cells [170]. In this study, we found that γ-TmT decreased the expression of ERα in hyperplastic mammary tissues. In addition, γ-TmT in the diet reduced circulating levels of E_2 in the serum, suggesting that γ-TmT could modify the response to estrogen. Similar to the finding in the NMU-induced breast cancer model in SD rats [170], PPARγ was increased at both the protein and mRNA level in the mammary gland of ACI rats when treated with γ-TmT while ERα expression was decreased. Since PPARγ transactivation can

be suppressed by ERα binding to the PPAR response element [355], the inhibition of ERα expression by tocopherols may result in the activation of PPARγ.

PPARγ is expressed in breast, prostate, and colon epithelium, and involved in lipid and glucose metabolism, cell proliferation and apoptosis, differentiation, and cell survival [241]. PPARγ signaling in cancer has been shown to upregulate differentiation markers, CDK inhibitors (p21 and p27), and apoptosis markers while PPARγ has been shown to inhibit inflammatory markers (COX-2, cytokines, and inducible nitric oxide synthase), PI3K/Akt activity, and angiogenesis [244]. Specifically in breast cancer, stimulation of PPARγ increases the degradation of cell cycle genes (cyclin D1), interferes with estrogen receptor signaling, and NF-κB signaling cascades [243,245]. Thus, activation of PPARγ by tocopherols in breast tissue may have anti-estrogenic effects, inhibit cell cycle progression, and induce apoptosis to prevent breast cancer.

Nrf2 is a transcription factor which regulates cellular antioxidant and detoxification enzymes. The expression of Nrf2 was suppressed in prostate tumors [237], and treatment with γ-TmT upregulated the expression of Nrf2 and detoxifying enzymes, and inhibited tumor development in TRAMP mice [75]. Yao *et al.* reported that inhibition of estrogen signaling activates the Nrf2 pathway in breast cancer [356]. In our study with estrogen-treated ACI rats, the protein expression level of Nrf2 was increased in the mammary gland and liver. Protein levels of phase II enzymes were increased in the liver by γ-TmT treatment. The mRNA expression in the mammary gland and liver showed that phase II detoxifying enzymes were also induced by γ-TmT treatment, suggesting that γ-TmT increases the transcription of Nrf2-ARE-target genes and exhibits protective defense against estrogen-induced oxidative stress.

In the present study, serum levels of PGE_2 and 8-isoprostane as well as COX-2 levels in the mammary gland were reduced when treated with γ-TmT. In a lung tumor model using A/J mice, *Lu et al*. reported a decreased level of PGE_2 and LTB_4 in the serum when given γ-TmT diet [345]. In a colon cancer model with azoxymethane/dextran sulfate sodium treated mice, treatment

with γ-TmT reduced the levels of PGE_2, LTB_4, and 8-isoprostane in the serum [171]. Consistent with anti-inflammatory effects of tocopherols over several cancer models, γ-TmT treatment may reduce inflammation in an estrogen-induced model of mammary hyperplasia and tumorigenesis.

2.5 Summary

When administered γ-TmT diet, γ-tocopherol and δ-tocopherol levels were significantly increased in the serum and mammary gland, while levels of α-tocopherol were similar to that of the control samples. The cancer preventive activity of γ-TmT treatment is most likely due to the enriched levels of γ- and δ-tocopherol. This could indicate that γ- and δ-tocopherols, not α-tocopherol, are mostly responsible for the different expression of markers performed in this study. We have shown that treatment with dietary γ-TmT inhibits cell proliferation, down-regulates the levels of ERα and circulating serum E_2, suppresses serum levels of inflammatory markers, while upregulates PPARγ and Nrf2 levels in mammary hyperplasia. This suggests that γ-enriched mixed tocopherols may be a promising agent for hormone dependent human breast cancer prevention.

Table 2.1 Body and liver weights of female ACI rats at 2 and 10 weeks

Treatment Group	Treatment Period (weeks)	No. of rats per group	Body Weight (g)	Liver Weight (g)	Liver/Body Wt Ratio
Control Pellet	2	6	155.6 ± 3.5	$5.3 \pm 0.3^{\dagger}$	$3.4 \pm 0.1^{\dagger}$
E_2 Control	2	7	151.8 ± 3.6	7.0 ± 0.2	4.6 ± 0.1
$E_2 + 0.3\%$ γ-TmT	2	6	155.0 ± 2.5	7.0 ± 0.2	4.5 ± 0.2
$E_2 + 0.5\%$ γ-TmT	2	6	149.4 ± 5.3	6.7 ± 0.3	4.5 ± 0.1
Control Pellet	10	6	$177.7 \pm 2.4^{\dagger}$	$5.5 \pm 0.1^{\ddagger}$	$3.1 \pm 0.1^{\ddagger}$
E_2 Control	10	7	190.7 ± 1.8	9.0 ± 0.3	4.7 ± 0.1
$E_2 + 0.3\%$ γ-TmT	10	6	191.0 ± 3.0	8.6 ± 0.3	4.5 ± 0.1
$E_2 + 0.5\%$ γ-TmT	10	6	184.9 ± 3.8	$8.2 \pm 0.2*$	$4.4 \pm 0.1*$

Rats were treated with a control pellet, E_2 pellet, E_2 pellet and 0.3% γ-TmT diet, or E_2 pellet and 0.5% γ-TmT diet for 2 or 10 weeks. Values presented are mean ± S.E.M (n=6-7). Statistical significance, $*p<0.05$, $^{\dagger}p<0.01$, $^{\ddagger}p<0.001$. p-Values are compared to the E_2 control of their respective time point.

Table 2.2 Analysis of tocopherol levels in the serum and mammary gland of ACI rats

Serum

	α-T (μM)	γ-T (μM)	δ-T (μM)	α-CEHC (μM)	γ-CEHC (μM)	δ-CEHC (μM)
2 weeks						
E$_2$ Control	33.8 ± 1.9	0.5 ± 0.1	0.1 ± 0.0	7.5 ± 1.0	0.3 ± 0.1	1.0 ± 0.2
E$_2$ + 0.3% γ-TmT	44.6 ± 4.8*	3.2 ± 0.2‡	0.5 ± 0.1‡	26.8 ± 2.6‡	17.8 ± 2.1‡	39.8 ± 6.2‡
E$_2$ + 0.5% γ-TmT	34.2 ± 2.1	3.7 ± 0.5‡	0.6 ± 0.1‡	22.0 ± 1.1‡	25.6 ± 2.1‡	54.4 ± 4.2‡
10 weeks						
E$_2$ Control	33.5 ± 1.5	0.5 ± 0.1	0.1 ± 0.0	4.7 ± 0.0	0.2 ± 0.0	0.6 ± 0.0
E$_2$ + 0.3% γ-TmT	36.2 ± 0.6	3.2 ± 0.1‡	0.7 ± 0.1‡	14.0 ± 0.0‡	18.3 ± 0.0‡	32.3 ± 0.0‡
E$_2$ + 0.5% γ-TmT	39.1 ± 1.7	7.0 ± 0.6‡	1.8 ± 0.2‡	16.1 ± 1.2‡	21.6 ± 2.8‡	44.1 ± 2.1‡

Mammary Gland

	α-T (μM)	γ-T (μM)	δ-T (μM)	α-CEHC (μM)	γ-CEHC (μM)	δ-CEHC (μM)
2 weeks						
E$_2$ Control	25.5 ± 0.8	2.1 ± 1.2	0.1 ± 0.0	0.9 ± 0.1	0.0 ± 0.0	0.3 ± 0.1
E$_2$ + 0.3% γ-TmT	37.1 ± 6.4	8.9 ± 2.4†	2.4 ± 0.6†	4.0 ± 0.9‡	3.7 ± 0.9‡	6.1 ± 1.6‡
E$_2$ + 0.5% γ-TmT	47.0 ± 12.5	13.4 ± 2.8‡	3.7 ± 0.9‡	3.5 ± 0.3‡	4.9 ± 0.4‡	7.8 ± 0.5‡
10 weeks						
E$_2$ Control	26.9 ± 0.8	0.8 ± 0.1	0.2 ± 0.1	0.4 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
E$_2$ + 0.3% γ-TmT	25.4 ± 0.8	6.4 ± 0.5‡	2.8 ± 0.2‡	1.4 ± 0.1‡	2.8 ± 0.2‡	4.5 ± 0.2‡
E$_2$ + 0.5% γ-TmT	25.7 ± 1.6	9.1 ± 0.7‡	4.3 ± 0.3‡	1.8 ± 0.1‡	3.2 ± 0.2‡	6.0 ± 0.2‡

Serum and mammary gland samples were collected at necropsy and analyzed for α-, γ-, δ-tocopherol (α-, γ-, δ-T) and their short chain metabolites, carboxyethyl hydroxychromans (α-, γ-, δ-CEHC). β-Tocopherol is not shown because β-tocopherol and its metabolites were below the limit for detection. The data is expressed as the mean ± S.E.M (n = 6). Statistical significance, *p<0.05, †p<0.01, ‡p<0.001. p-Values are compared to the E$_2$ control of their respective time points.

Table 2.3 Analysis of mRNA expression levels in the mammary gland of ACI rats

	2 weeks			10 weeks		
	E_2 Control	$E_2 + 0.3\%$ γ-TmT	$E_2 + 0.5\%$ γ-TmT	E_2 Control	$E_2 + 0.3\%$ γ-TmT	$E_2 + 0.5\%$ γ-TmT
Nrf2 Pathway						
Nrf2	1.0 ± 0.4	1.2 ± 0.3	1.2 ± 0.5	1.0 ± 0.1	0.7 ± 0.1	1.0 ± 0.1
Keap1	1.0 ± 0.1	0.8 ± 0.2	0.7 ± 0.2	1.0 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
Phase I Enzymes						
Cyp 1A1	1.0 ± 0.1	1.0 ± 0.3	0.8 ± 0.1	1.0 ± 0.1	0.3 ± 0.2*	0.5 ± 0.2
Cyp 1B1	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.3	1.0 ± 0.3	0.8 ± 0.1	0.8 ± 0.4
Phase 2 Enzymes						
UGT1A1	1.0 ± 0.2	1.8 ± 0.1*	1.6 ± 0.3	1.0 ± 0.1	1.8 ± 0.1[†]	1.6 ± 0.2
NQO1	1.0 ± 0.1	1.2 ± 0.1	1.1 ± 0.2	1.0 ± 0.2	1.1 ± 0.2	1.1 ± 0.1
GSTm1	1.0 ± 0.1	1.9 ± 0.3*	2.4 ± 0.2[‡]	1.0 ± 0.1	1.3 ± 0.3	1.7 ± 0.4
LOC5O11	1.0 ± 0.2	2.8 ± 0.1[‡]	2.3 ± 0.3*	1.0 ± 0.1	1.2 ± 0.2	1.4 ± 0.2
COMT	1.0 ± 0.1	1.5 ± 0.1*	1.6 ± 0.1*	1.0 ± 0.2	1.2 ± 0.2	1.6 ± 0.5
GClm	1.0 ± 0.1	0.9 ± 0.2	0.9 ± 0.2	1.0 ± 0.3	1.0 ± 0.5	1.4 ± 0.5
Antioxidant Enzymes						
SOD1	1.0 ± 0.2	1.2 ± 0.1	0.7 ± 0.2	1.0 ± 0.1	1.1 ± 0.3	1.2 ± 0.2
GPX1	1.0 ± 0.2	1.2 ± 0.2	1.7 ± 0.5	1.0 ± 0.1	1.0 ± 0.1	1.5 ± 0.6
HO-1	1.0 ± 0.2	1.2 ± 0.1	1.0 ± 0.2	1.0 ± 0.2	1.2 ± 0.2	1.2 ± 0.3
TXN1	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.2	1.0 ± 0.1	0.9 ± 0.2	1.2 ± 0.3
Catalase	1.0 ± 0.2	0.8 ± 0.2	0.7 ± 0.1	1.0 ± 0.1	0.7 ± 0.2	0.7 ± 0.2

ACI rats treated with estrogen pellets were fed the control, 0.3%, or 0.5% γ-TmT diet. A mammary gland from each rat was analyzed for mRNA levels by quantitative PCR (n = 2-6). The values are represented as mean ± S.E.M. Statistical significance, *$p<0.05$, [†]$p<0.01$, [‡]$p<0.001$.

Table 2.4 Analysis of mRNA expression levels in the liver of ACI rats

	2 weeks			10 weeks		
	E₂ Control	E₂ + 0.3% γ-TmT	E₂ + 0.5% γ-TmT	E₂ Control	E₂ + 0.3% γ-TmT	E₂ + 0.5% γ-TmT
Nrf2 Pathway						
Nrf2	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.8 ± 0.1
Keap1	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	1.0 ± 0.1
Phase I Enzymes						
Cyp 1A1	1.0 ± 0.2	1.4 ± 0.2	1.1 ± 0.2	1.0 ± 0.2	0.5 ± 0.1	1.8 ± 0.7
Cyp 1B1	1.0 ± 0.2	0.7 ± 0.1	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.5	3.2 ± 0.5*
Phase 2 Enzymes						
UGT1A1	1.0 ± 0.2	1.4 ± 0.2	1.3 ± 0.1	1.0 ± 0.1	$1.8 \pm 0.1^{\ddagger}$	$1.6 \pm 0.1^{\ddagger}$
NQO1	1.0 ± 0.1	$2.8 \pm 0.2^{\ddagger}$	$2.6 \pm 0.3^{\ddagger}$	1.0 ± 0.1	1.3 ± 0.3	1.5 ± 0.8*
GSTm1	1.0 ± 0.1	$2.0 \pm 0.2^{\ddagger}$	$1.8 \pm 0.2^{\dagger}$	1.0 ± 0.1	1.5 ± 0.2	1.7 ± 0.2
LOC5O11	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
COMT	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	1.2 ± 0.2
GClm	1.0 ± 0.1	$2.0 \pm 0.3^{\dagger}$	$2.3 \pm 0.2^{\ddagger}$	1.0 ± 0.1	1.4 ± 0.2	1.3 ± 0.9
Antioxidant Enzymes						
SOD1	1.0 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.3 ± 0.1
GPX1	1.0 ± 0.2	1.5 ± 0.2	1.3 ± 0.2	1.0 ± 0.1	0.8 ± 0.1	1.2 ± 0.0
HO-1	1.0 ± 0.1	0.9 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	1.2 ± 0.3	1.3 ± 0.0*
TXN1	1.0 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.3	1.3 ± 0.0
Catalase	1.0 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.2 ± 0.3	1.3 ± 0.1

ACI rats treated with estrogen pellets were fed the control, 0.3%, or 0.5% γ-TmT diet. Liver from each rat was analyzed for mRNA levels by quantitative PCR (n=2-6). The values are represented as mean ± S.E.M. Statistical significance, *p<0.05, †p<0.01, ‡p<0.001.
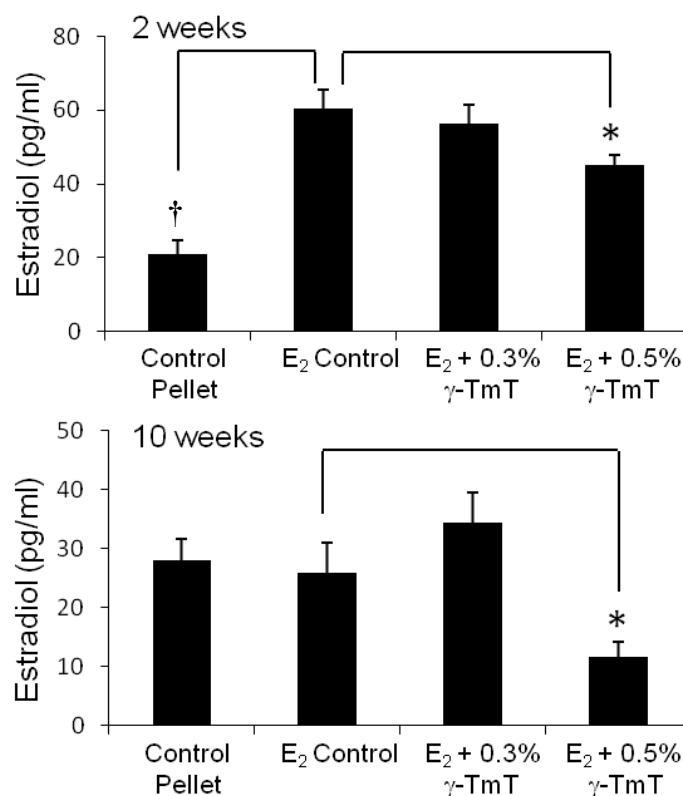
Figure 2.1 ACI rats treated with estrogen pellets were fed the control, 0.3%, or 0.5% γ-TmT diet for either 2 or 10 weeks. Serum samples were analyzed for E_2 (pg/ml) (n = 6). The data are presented as the mean ± S.E.M. Statistical significance, *p<0.05, †p<0.01. p-Values are compared to the E_2 control of their respective time point.
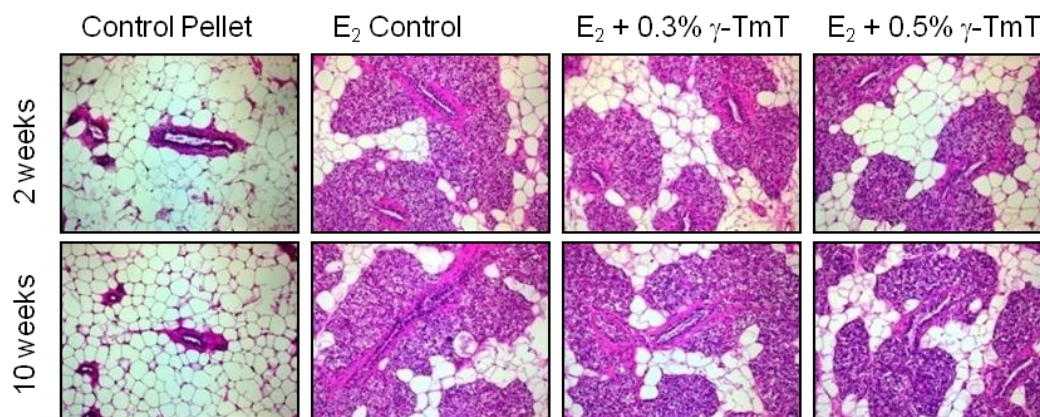
Figure 2.2 ACI rats treated with estrogen pellets were fed the control, 0.3%, or 0.5% γ-TmT diet for either 2 or 10 weeks. Mammary glands were stained with H & E (200x total magnification). Representative sections from the control pellet, E_2 control, and E_2 treated animals fed either 0.3% or 0.5% γ-TmT diet are shown.
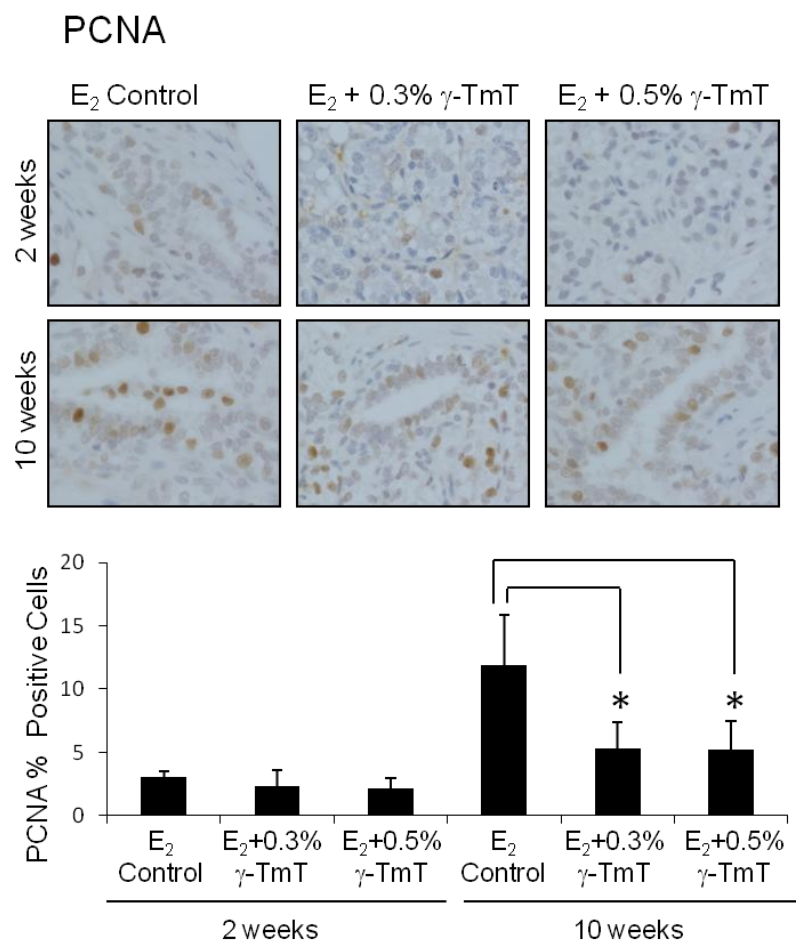
Figure 2.3 ACI rats treated with estrogen pellets were fed the control, 0.3%, or 0.5% γ-TmT diet. A representative immunostaining of PCNA in the mammary gland is shown (600x). Three mammary glands from each group were randomly selected, and three representative areas from each mammary gland were analyzed for PCNA expression. Positive staining of PCNA is found in the nuclei of the cells. The data are presented as the mean ± S.E.M (n=3). Statistical significance, *p<0.05, †p<0.01.
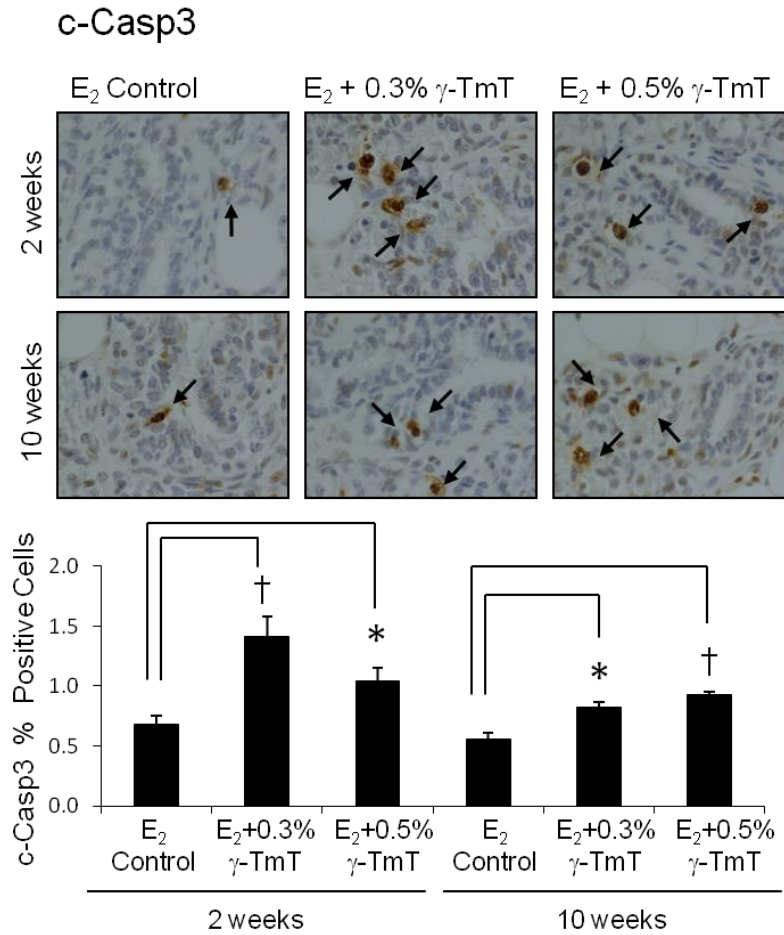
Figure 2.4 ACI rats treated with estrogen pellets were fed the control, 0.3%, or 0.5% γ-TmT diet. A representative immunostaining of c-Casp3 in the mammary gland is shown (600x). Three mammary glands from each group were randomly selected, and three representative areas from each mammary gland were analyzed for c-Casp3 expression. Positive staining of c-Casp3 is shown as a light brown to dark brown precipitate in the cytoplasm or perinuclei of the cells. The arrows indicate positive staining for c-Casp3. The data are presented as the mean ± S.E.M (n=3). Statistical significance, *p<0.05, †p<0.01.
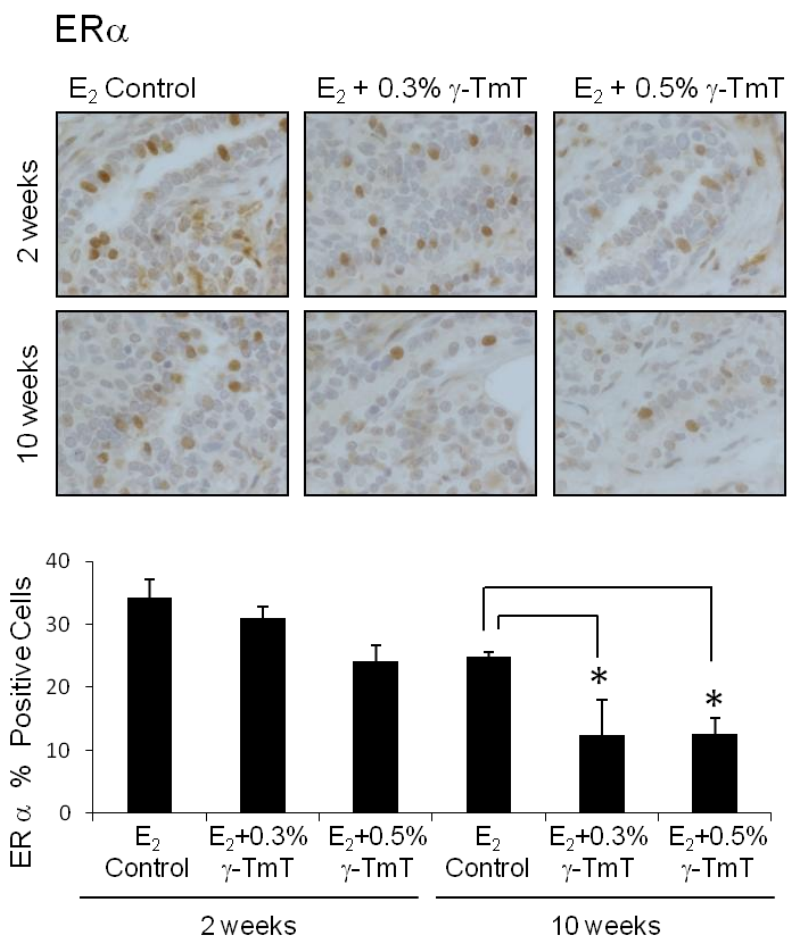
Figure 2.5 ACI rats treated with estrogen pellets were fed the control, 0.3%, or 0.5% γ-TmT diet. A representative immunostaining of ERα in the mammary gland is shown (600x). Three mammary glands from each group were randomly selected, and three representative areas from each mammary gland were analyzed for ERα expression. Positive staining of ERα is found in the nuclei of the cells. The data are presented as the mean ± S.E.M (n=3). Statistical significance, *p<0.05, †p<0.01.
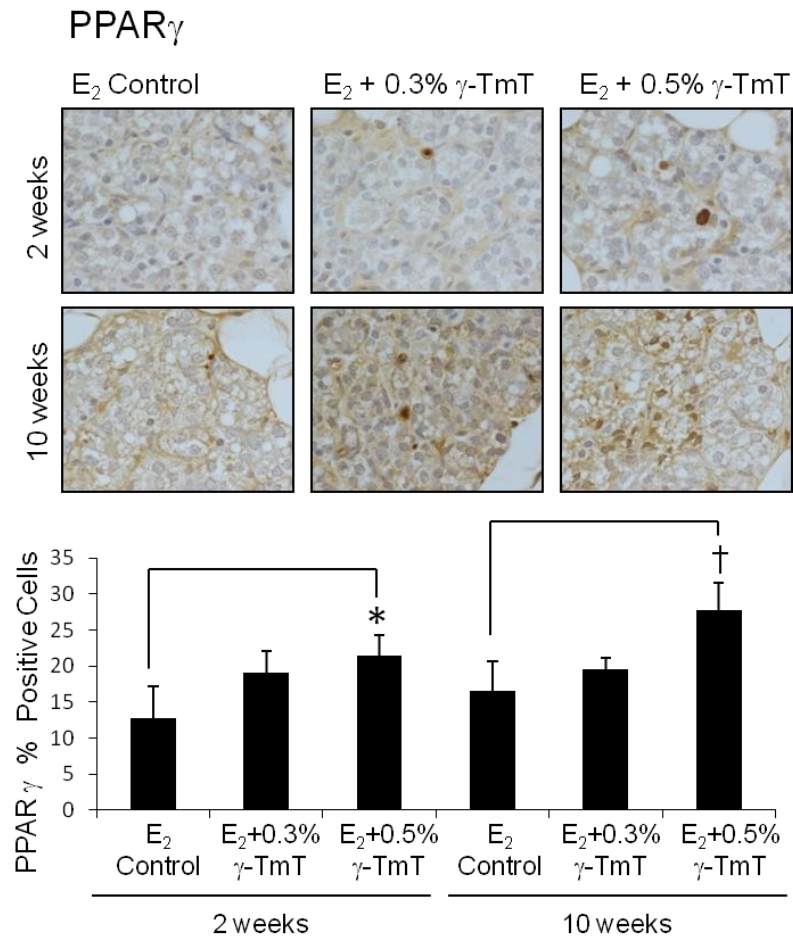
Figure 2.6 ACI rats treated with estrogen pellets were fed the control, 0.3%, or 0.5% γ-TmT diet. A representative immunostaining of (B) PPARγ in the mammary gland is shown (600x). Three mammary glands from each group were randomly selected, and three representative areas from each mammary gland were analyzed for PPARγ expression. Positive staining of PPARγ is found in the nuclei of the cells. The data are presented as the mean ± S.E.M (n=3). Statistical significance, *p<0.05, †p<0.01.
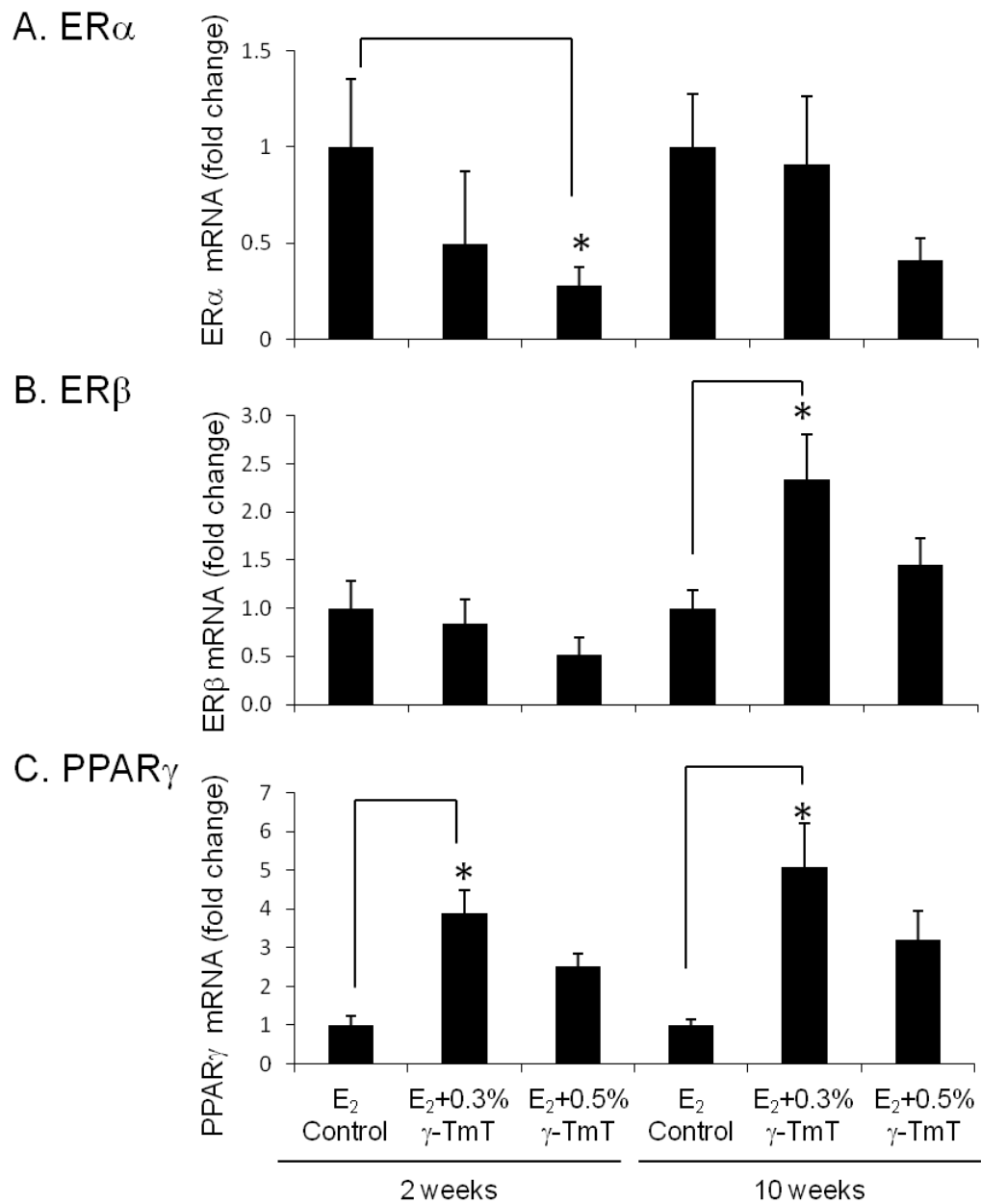
Figure 2.7 ACI rats treated with estrogen pellets were fed the control, 0.3%, or 0.5% γ-TmT diet. At least four mammary glands from each treatment group were analyzed for mRNA levels of (A) ERα, (B) ERβ, and (C) PPARγ by quantitative PCR (n = 4 - 6). The data are presented as the mean ± S.E.M. Statistical significance, *p<0.05.
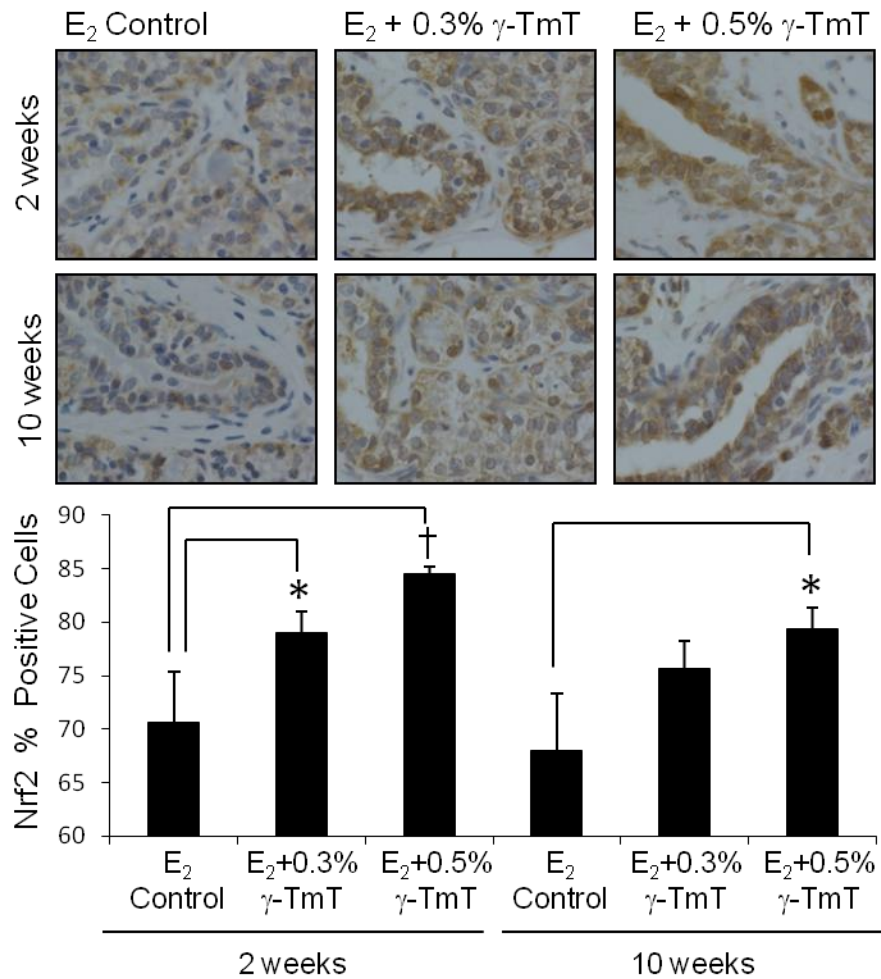
Figure 2.8 ACI rats treated with estrogen pellets were fed the control, 0.3%, or 0.5% γ-TmT diet. A representative immunostaining of Nrf2 in the mammary gland is shown (600x)**.** Three mammary glands from each group were randomly selected, and three representative areas from each mammary gland were analyzed for levels of Nrf2. Positive staining for Nrf2 is found both in the cytoplasm and nuclei of the cells. The data are presented as the mean ± S.E.M (n=3). Statistical significance, *p<0.05, †p<0.01.

Liver Proteins
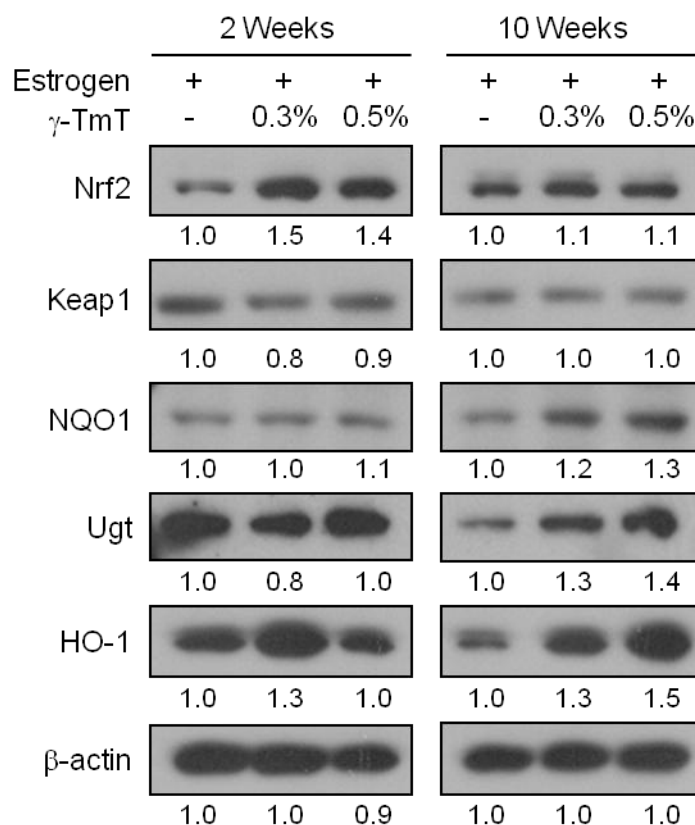
Figure 2.9 ACI rats treated with estrogen pellets were fed the control, 0.3%, or 0.5% γ-TmT diet. Regulation of liver protein expression by the treatment with γ-TmT. Liver samples were homogenized and pooled together (n = 3). Western blots are shown. Quantification of western blot was performed by ImageJ 1.45s (NIH), and the numbers are provided at the bottom of each western blot.
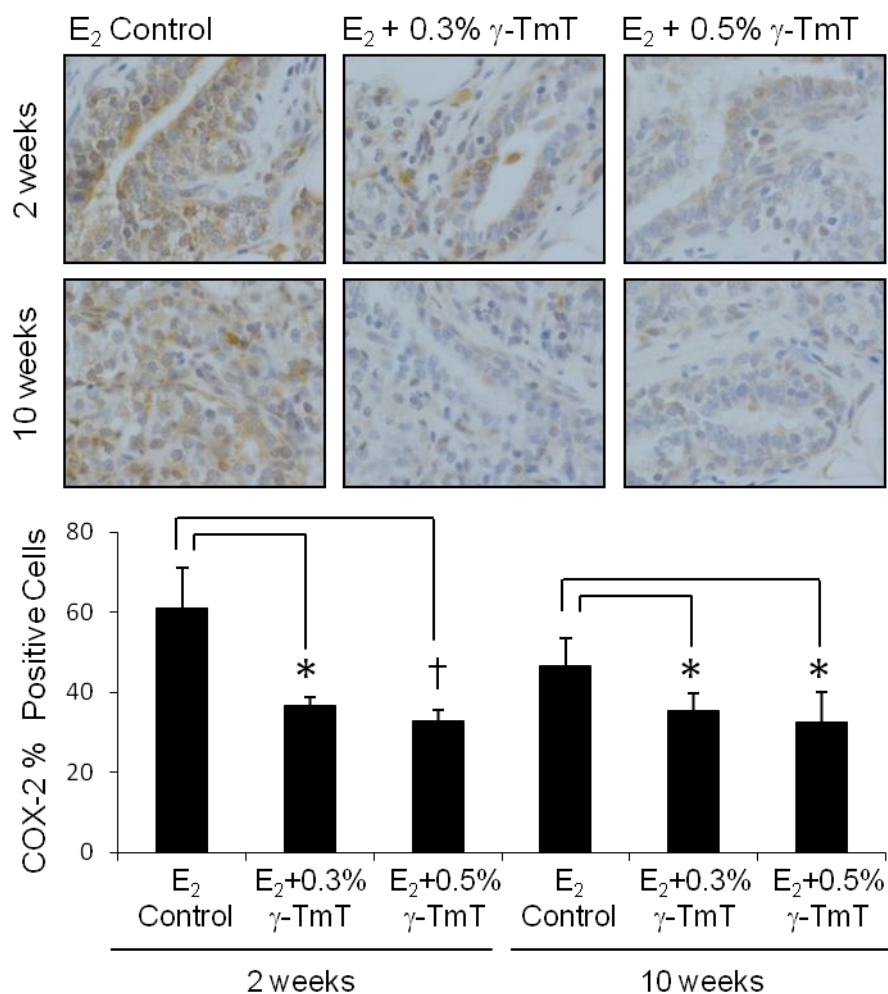
**Figure 2.10** ACI rats treated with estrogen pellets were fed the control, 0.3%, or 0.5% γ-TmT diet. (A) A representative immunostaining of COX-2 in the mammary gland is shown (600x). Three mammary glands from each group were randomly selected, and three representative areas from each mammary gland were analyzed for levels of COX-2. Positive staining for COX-2 is found in the cytoplasm. The data are presented as the mean ± S.E.M (n=3). Statistical significance, *$p<0.05$, †$p<0.01$.

Serum Inflammatory Markers
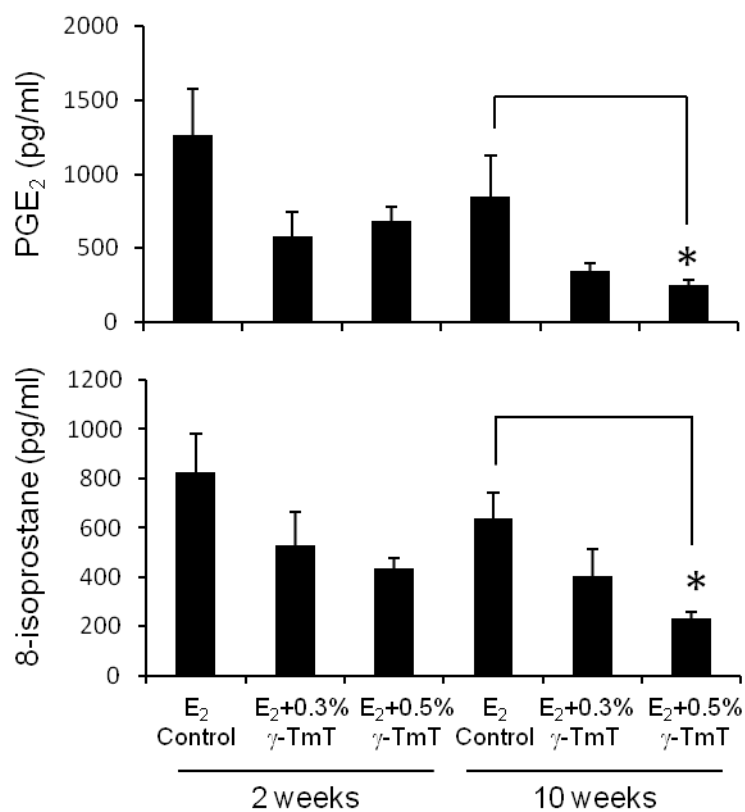
Figure 2.11 ACI rats treated with estrogen pellets were fed the control, 0.3%, or 0.5% γ-TmT diet. Serum was collected at necropsy and analyzed for PGE_2 and 8-isoprostane (n = 6). The data are presented as the mean ± S.E.M. Statistical significance, *p<0.05, †p<0.01.

Chapter 3: Administration of γ- and δ-tocopherols inhibits tumor growth in carcinogen-treated rats[a,b]

3.1 Introduction

Chemical carcinogen-induced mammary carcinogenesis in rats is a conventional model for the investigation of ER-positive breast cancer. Tumors that are induced by a single i.p. injection of 20-50 mg/kg body weight of NMU have latencies that range between 8-21 weeks with final tumor incidences close to 100% [357]. The histology of the tumor is also influenced by the dose of the chemical carcinogen. In SD rats given single NMU dose of 10 mg/kg, 42% of tumors were malignant, while when administered 35-50 mg/kg if NMU, 86-94% of tumors were malignant [358].

The NMU-induced mammary tumors are similar to human breast cancers in the respect that there is altered expression of TGFα, ErbB2, cyclin D1 and gelsolin [56]. The NMU-induced rat mammary tumors showed similar gene expression profiles to ER-positive, low to intermediate grade of human breast cancer, making it a useful model to evaluate the efficacy of chemopreventive agents for hormone dependent mammary tumorigenesis [69].

We have previously reported that administration of a diet containing 0.3% or 0.5% γ-TmT suppressed mammary tumor growth in NMU-induced rat model [170]. To expand on this area of research, we assessed the chemopreventive activities of individual (α-, γ-, δ-) tocopherols against breast cancer in NMU-treated rats. By using individual tocopherols, we are able to determine the relative potency of individual tocopherols and possible mechanism of action to inhibit tumor burden. We hypothesize that the structural differences of γ-tocopherol and

δ-tocopherol may be more effective at trapping nitrogen species than α-tocopherol, which may contribute to better chemopreventive activity. Furthermore, γ-tocopherol and δ-tocopherol, but not α-tocopherol, may reduce tumor burden by reducing DNA damage and increasing apoptosis. We investigated molecular markers of cell proliferation (PCNA, PKCα, p-Akt and PTEN), apoptosis (c-Casp-3 and BAX), nuclear receptors (ERα, ERβ and PPARγ), ROS/RNS and the Nrf2 pathway by Western blot, IHC and/or quantitative PCR analysis.

3.2 Material and Methods

3.2.1 Animals and experimental procedures

Female SD rats were purchased from Taconic Farms (Hudson, NY) at 21 ± 1 days of age and were treated with a single intraperitoneal injection of the carcinogen NMU (50 mg/kg body weight). One week after NMU injection, rats were fed AIN-93M control diet or AIN-93M diets containing 0.3% tocopherols (α-, δ-, γ-, or γ-TmT) (n=30 per group). Body weight and tumor volume were measured weekly. The rats were sacrificed 11 weeks after NMU injection. The mammary glands and mammary tumors were harvested, fixed in 10% formalin and transferred to 70% ethanol or flash frozen and stored in -80°C. Blood was collected by cardiac puncture immediately prior to necropsy; serum was prepared and stored at -80°C. All animal studies were done in accordance with an institutionally-approved protocol.

3.2.2 Animal diets

Semipurified modified AIN-93M diet was obtained from Research Diets Laboratory (New Brunswick, NJ) and used as the control diet. The test diets were prepared by Research Diets Laboratory by adding 0.3% α-tocopherol, δ-tocopherol, γ-tocopherol or γ-TmT to the AIN-93M

diet. γ-TmT was supplied by the Cognis Corporation (Kankakee, IL) and contained 57% γ-tocopherol, 24% δ-tocopherol, 13% α-tocopherol and 1.5% β-tocopherol. γ-Tocopherol was purified from γ-TmT by silica gel chromatography to a purity of 97%, with no detectable α- and δ-tocopherol. δ-Tocopherol (containing 94% δ-tocopherol, 5.5% γ-tocopherol and 0.5% α-tocopherol) and α-tocopherol (containing 69.7% α-tocopherol, 2.6% γ-tocopherol and 0.2% δ-tocopherol) were purchased from Sigma-Aldrich (St. Louis, MO). The diets were stored at 4°C and the food was replenished with fresh pellets twice weekly.

3.2.3 Serum estradiol levels

E_2 levels in the serum were analyzed using an EIA kit from Enzo Life Sciences International, Inc (Plymouth Meeting, PA). Serum samples were purified and the assay was performed according to the manufacturer's protocol.

3.2.4 Analysis of tocopherols in rat serum, mammary glands and mammary tumors

Serum, mammary glands and mammary tumors were analyzed by high performance liquid chromatography for tocopherol (α-, δ-, γ-) and short chain metabolite, CEHC, levels as previously described [170,351].

3.2.5 Immunohistochemical analysis

Mammary glands and tumors were processed and stained as previously described [238]. Sections were immunostained with antibodies to 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) (JaICA/GENOX Corporation, Baltimore, MD), nitrotyrosine (Millipore, Billerica, MA), PCNA

(BD Pharmingen, San Diego, CA) and c-Casp-3 (Cell Signaling, Beverly MA). Images were taken randomly with Nikon Eclipse E800 (Melville, N.Y) fitted to Nikon digital sight Ri1. The staining density was determined by using an Aperio® Scan Scope (Vista, CA). Quantification was performed where three mammary glands or tumors from each treatment group were selected randomly and three areas from each gland or tumor were analyzed for over 1000 cells/mammary gland or 4000 cells/mammary tumor.

3.2.6 mRNA expression analysis using quantitative polymerase chain reaction (PCR)

RNA was extracted from mammary tumors, and reverse transcription and quantitative PCR were carried out as previously reported [359]. Labeled primers for glyceraldehyde-3-phosphate dehydrogenase, Bcl-2-associated X protein (Bax), B cell lymphoma 2 (Bcl2), x-linked inhibitor of apoptosis (XIAP), PCNA, protein kinase C α (PKCα), PTEN, Myc, p53, p21, p27, Cyclin D1, ERα, ERβ, PPARγ, Nrf2, KEAP1, NQO1, GCLm, GSTm1, UGT1A1, COMT, SOD, HO-1, GPx, TXN and catalase were obtained from Applied Biosystems (Carlsbad, CA).

3.2.7 Western blot analysis

Mammary tumors were homogenized and the protein extracts were analyzed by Western blotting as previously described [170]. The primary antibodies against apoptotic protease activating factor 1 (Apaf-1), XIAP, c-Casp-9, c-Casp-8, c-Casp-3, cleaved-Poly (ADP-ribose) polymerase (c-PARP), PKCα, p-Akt, PTEN, p53, cyclin E, CDK2, CDK4, CDK6, TXN and UGT were from Cell Signaling (Beverly, MA); Bcl-2, ERα, PPARγ, c-Myc, p21, p27, cyclin D1, Nrf2, KEAP1 and NQO1 were from Santa Cruz Biotechnology (Santa Cruz, CA); Bax, SOD, GCLm and GPx were from Abcam (Cambridge, MA); ERβ was from Affinity BioReagents

(Golden, CO); PCNA was from BD Pharmingen (San Diego, CA); GSTm1, catalase and HO-1 were from Epitomics (Burlingame, CA); and β-actin was from Sigma-Aldrich (St. Louis, MO). Secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Tumor samples from different animals for each group (n=3) were pooled for analysis by Western blot.

3.2.8 Statistical analysis

Statistical significance was evaluated using ANOVA with Dunnett's adjustment, preserving the overall type-1 error at the 5% level. Tumor incidence was calculated by the Log Rank (Mantel-Cox) Test using Graph Pad Prism 4.0 (GraphPad Software Inc.). The data presented represent the mean ± S.E. p-Values of <0.05 were considered significant.

3.3 Results

3.3.1 δ- And γ-tocopherols inhibit tumor growth and multiplicity in NMU-treated mammary tumorigenesis.

Female SD rats were either fed the control diet or the diets containing 0.3% α-tocopherol, 0.3% δ-tocopherol, 0.3% γ-tocopherol, or 0.3% γ-TmT. After 7 weeks of treatment, the tumor incidence for the control group was 80% and similar in the α-tocopherol group (73.3%). However, the corresponding tumor incidence at week 7 for the δ-tocopherol, γ-tocopherol, and γ-TmT groups was markedly lower at 50.0% (p<0.05), 56.7% (p=0.057), and 53.3% (p<0.05), respectively. At the conclusion of the 11-week study, the overall tumor incidence was not significantly different among the groups (Fig. 3.1). As compared to the control group, dietary administration of δ- and γ-tocopherol reduced tumor burden by 32% (p<0.01) and 33% (p<0.01), respectively, but α-tocopherol-enriched diet had no effect (Fig. 3.2). Tumor multiplicity for the control group was 5.0 ± 0.1 and was decreased by treatment with δ-tocopherol (2.9 ± 0.1), γ-

tocopherol (3.4 ± 0.1), and γ-TmT (3.9 ± 0.1) which translates to 42% (p<0.001), 32% (p<0.01), and 22% (p<0.05) inhibition, respectively (Fig. 3.2). All subsequent serum and tissue analyses reported are for the activity of individual tocopherols in NMU-treated rats.

3.3.2 Serum levels of estradiol were decreased by the administration of tocopherols.

Circulating endogenous serum levels of E_2 were analyzed to determine changes between the treatment groups (Fig. 3.3). Average E_2 serum levels in the control group were 86.6 ± 13.0 pg/ml, whereas average serum E_2 levels in the α-, δ-, γ-tocopherol, and γ-TmT groups were significantly decreased by 37% (p<0.05), 38% (p<0.05), 42% (p<0.05), and 35% (p<0.05), respectively.

3.3.3 Levels of tocopherols and metabolites were increased in serum, mammary glands and mammary tumors when treated with α-, γ-, δ-tocopherol and γ-TmT diets.

Serum, mammary gland, and mammary tumor samples were collected at necropsy and analyzed for the levels of α-, γ-, δ-tocopherol and the short chain metabolite, CEHC (Table 3.1). In general, the levels of individual tocopherols and CEHCs differed among serum, mammary glands and mammary tumors. The highest levels of the hydrophobic parent tocopherols were found in the adipose-rich mammary gland, whereas comparable levels of tocopherols relative to control were found in both serum and mammary tumor. Due to the selective transport of α-tocopherol by the α-tocopherol transport protein in the liver, α-tocopherol is the major form found in serum. The water-soluble short chain metabolites were more prevalent in serum and mammary tumor compared to mammary gland.

More specifically, levels of α-tocopherol and α-CEHC were significantly increased in serum, mammary glands and mammary tumors of rats fed with 0.3% α-tocopherol. Levels of γ-tocopherol and γ-CEHC were significantly increased in serum, mammary glands and mammary tumors of rats fed with 0.3% γ-tocopherol; however the levels of α-tocopherol decreased in the serum ($p<0.05$), mammary glands ($p<0.001$) and mammary tumors ($p<0.01$) by treatment with γ-tocopherol. Administration of 0.3% δ-tocopherol diet increased levels of δ-tocopherol, γ-tocopherol, and δ-CEHC in serum, mammary glands and mammary tumors. Furthermore, 0.3% γ-TmT diet increased levels of δ-tocopherol, γ-tocopherol, α-CEHC, δ-CEHC and γ-CEHC in serum, mammary glands and mammary tumors, while α-tocopherol levels only increased in mammary glands.

3.3.4 Treatment with δ-tocopherol, γ-tocopherol and γ-TmT induced apoptosis and inhibited cell proliferation and cell cycle in mammary tumors.

As shown in Fig. 3.4, the levels of pro-apoptotic proteins Bax, c-Casp-3 and c-PARP were increased by δ-tocopherol, γ-tocopherol and γ-TmT-enriched diets. Levels of c-Casp-9 were increased by all tocopherols, whereas c-Casp-8 remained unchanged. This may indicate that apoptosis is activated through the extrinsic apoptosis pathway. Furthermore, anti-apoptotic proteins Bcl2 and XIAP were inhibited by δ-tocopherol, γ-tocopherol and γ-TmT.

The oncogene cMyc regulates the G1 phase of the cell cycle; dietary δ- and γ-tocopherol decreased cMyc protein levels in mammary tumors. Furthermore, protein levels of tumor suppressor p53, and CDK inhibitors p21 and p27 were increased by δ-tocopherol, γ-tocopherol and γ-TmT. In contrast, α-tocopherol upregulated only p27. Cyclin E and the corresponding CDK2 were not affected by tocopherols. However, the protein level of cyclin D1 was decreased

by γ-tocopherol and γ-TmT, CDK4 was reduced by δ-tocopherol, γ-tocopherol and γ-TmT, and CDK6 was down-regulated by each of the tocopherol-containing diets.

All tocopherol diets affected the Nrf2 pathway (Fig. 3.5). Although levels of KEAP1 remained unchanged, the treatment increased the protein levels of Nrf2. Furthermore, the levels of down-stream phase II detoxifying and antioxidant enzymes TXN, GCLm, GSTm1, GPx and HO-1 were increased by all tocopherols. NQO1 was induced by δ-tocopherol, γ-tocopherol and γ-TmT, but not by α-tocopherol. Protein levels of SOD, catalase, and UGT remained unchanged regardless of tocopherol treatment. Levels of nuclear receptors were examined in mammary tumors (Fig. 3.6). Protein levels of ERα were decreased in samples from rats treated with α-, δ-, γ-tocopherol and γ-TmT, whereas levels of ERβ remained relatively unchanged. Interestingly, the protein level of PPARγ was increased by δ-tocopherol, γ-tocopherol and γ-TmT-containing diets, but not by α-tocopherol.

The cell cycle pathway plays a major role in regulating cancer development for the continuation of cell proliferation and survival. Cell proliferation markers, PCNA and PKC, were inhibited by treatment with δ-tocopherol, γ-tocopherol and γ-TmT, but not by α-tocopherol (Fig. 3.7). More specifically, in the cell survival pathway, PTEN was upregulated whereas p-Akt was down-regulated by δ-tocopherol, γ-tocopherol and γ-TmT. The protein level of total Akt was examined in responding and non-responding animals (Fig 3.8). Compared to the control animals, there were no changes observed for total Akt level, which means that δ- and γ-tocopherol may inhibit the activation of Akt, rather than reducing total Akt levels.

3.3.5 There are molecular differences between animals that responded and did not respond to individual tocopherol treatment.

Further analysis was conducted on the tumor of animals that did or did not respond to tocopherol treatment (Fig. 3.8). Based on the growth rate of the tumors, we were able to distinguish animals as responding and non-responding to the treatments. In mammary tumors of responding animals, treatment with δ- and γ-tocopherol modulated apoptosis (increased c-Casp3), cell proliferation (decreased PCNA and PKCα), cell survival (increased PTEN and decreased p-Akt), nuclear receptors (increased PPARγ and decreased ERα), cell cycle (increased p53 and p21) and the Nrf2 pathway (increased Nrf2 and HO-1). However, the mammary tumors of the animals that did not respond to individual tocopherol treatment had similar protein levels as the control. Based on these data, the animals that respond to δ- and γ-tocopherol treatment have modifications in PCNA, PKCα, PTEN, p-Akt, p53 and PPARγ. This indicates that the chemopreventive mechanism of action for δ-tocopherol and γ-tocopherol, but not α-tocopherol, may be related to the cell survival and cell cycle pathways.

In Fig 3.9, we compared the fold response between 5 different individual tumor samples to verify tumor variability. Levels of p21, c-Casp-3, PPARγ and PTEN were increased when treated with δ-tocopherol, γ-tocopherol and γ-TmT. PKCα and PCNA levels was down-regulated by δ-tocopherol, γ-tocopherol and γ-TmT, while α-tocopherol did not change when compared to the control.

3.3.6 Oxidative and nitrosative stress markers in mammary glands were reduced by δ- and γ-tocopherols.

Nitrotyrosine is a known marker for nitrosative stress. In the mammary gland, the dietary administration of δ-tocopherol, γ-tocopherol and γ-TmT resulted in a decrease of nitrotyrosine levels by 14% (p<0.01), 18% (p<0.01), and 19% (p<0.05), respectively (Fig. 3.10). The level of 8-oxo-dG, a marker of oxidative stress, was also decreased by treatment with δ-tocopherol (10%),

γ-tocopherol (21%) and γ-TmT (14%); however the results were not statistically significant (Fig.3.11). Levels of 8-oxo-dG and nitrotyrosine were not reduced by α-tocopherol in the mammary gland. In mammary tumors, markers of oxidative and nitrosative stress were markedly lower than mammary glands, and tocopherol treatment did not significantly change the levels of 8-oxo-dG and nitrotyrosine in mammary tumors.

3.3.7 Treatment with δ-tocopherol, γ-tocopherol and γ-TmT reduced PCNA and increased c-Casp-3 in mammary tumors.

In mammary glands, levels of PCNA and c-Casp-3 did not change (data not shown). However, in mammary tumors, PCNA expression was reduced by δ-tocopherol, γ-tocopherol and γ-TmT, but not by α-tocopherol (Fig. 3.12). Administration of δ-tocopherol, γ-tocopherol and γ-TmT resulted in a 24% ($p<0.05$), 21% ($p<0.05$), and 27% ($p<0.05$) decrease in PCNA level, respectively. Furthermore, treatment by δ-tocopherol, γ-tocopherol and γ-TmT increased c-Casp-3 level in the mammary tumor by 89% ($p<0.01$), 107% ($p<0.01$), and 141% ($p <0.001$) above the control group, respectively (Fig. 3.13). α-Tocopherol increased c-Casp-3 level by 47% above the control, but it was not statistically significant.

3.3.8 The mRNA levels for apoptotic, cell proliferation, cell survival, and cell cycle markers, nuclear receptors, and Nrf2 pathways are regulated by tocopherols.

The mRNA levels of apoptotic markers, Bax, Bcl2, and XIAP, were examined in mammary tumors (Table 3.2). The mRNA levels of Bcl2 were decreased by α-tocopherol ($p<0.01$), δ-tocopherol ($p<0.05$), and γ-tocopherol ($p<0.05$) and γ-TmT ($p<0.01$), whereas BAX and XIAP were unchanged. Changes in mRNA levels of proliferation, survival, and cell cycle pathway markers were examined in mammary tumors (Table 3.2). The tumor mRNA levels of

PTEN increased in rats treated with δ-tocopherol (p<0.05), γ-tocopherol (p<0.05) and γ-TmT (p<0.05), while α-tocopherol did not. A CDK inhibitor, p21, was increased with δ-tocopherol (p<0.05), γ-tocopherol (p<0.05) and γ-TmT (p<0.05) treatment; p27 was increased by α-tocopherol (p<0.05), δ-tocopherol (p<0.05), γ-tocopherol (p<0.01) and γ-TmT (p<0.001). However, the mRNA levels of PCNA, PKCα, Myc, p53 and cyclin D1 did not change by tocopherol treatment. mRNA levels for ERα were significantly decreased by δ-tocopherol (p<0.05) and γ-TmT (p<0.05) treatment; levels of ERβ did not decrease. Interestingly, δ-tocopherol, γ-tocopherol and γ-TmT increased mRNA levels of PPARγ (p<0.05, p<0.01, p<0.05, respectively) (Table 3.2). Both Nrf2 and KEAP1 mRNA levels were unchanged by tocopherol treatment (Table 3.2). The phase II detoxifying enzymes GCLm, GSTm1 and Ugt1A1 were increased by all tocopherol treatment, while NQO1 and COMT mRNA levels were not affected. The mRNA levels of most of the antioxidant enzymes (SOD-1, HO-1, TXN1 and catalase) were unaltered.

3.4 Discussion

In this study, we utilized the carcinogen NMU to induce mammary tumors in female SD rats. NMU-induced mammary tumors mainly represent estrogen-dependent and locally invasive phenotypes that are similar to human breast cancer [66,360]. Both δ- and γ-tocopherol, but not α-tocopherol, inhibited mammary tumor development and a possible schematic of chemompreventive activity by δ-tocopherol and γ-tocopherol is shown in Fig. 3.14.

Tocopherols are known antioxidants and may reduce ROS and RNS to prevent cellular injury and mutations [263]. ROS and RNS may promote tumor onset and progression by affecting DNA mutations, cell proliferation, and survival [361]. γ-Tocopherol has been shown to be more effective at trapping reactive nitrogen species than α-tocopherol [193,224-228]. Previously, in a

lung xenograft tumor model, δ-tocopherol, γ-tocopherol and γ-TmT administration reduced 8-oxo-dG and nitrotyrosine levels, whereas α-tocopherol did not [218]. In the NMU-induced breast cancer model, treatment with δ-tocopherol, γ-tocopherol and γ-TmT reduced 8-oxo-dG and nitrotyrosine levels in the mammary gland, whereas α-tocopherol did not. Interestingly, low levels of ROS and RNS markers were observed in mammary tumors and were not changed by tocopherols. At the time of the analysis, the damage from the NMU carcinogen leading to mammary tumorigenesis already occurred and may explain why we did not see changes in ROS and RNS levels in mammary tumors.

Tocopherols may also function as an indirect antioxidant by stimulating the Nrf2 pathway. The down-stream enzymes in the Nrf2 pathway protect cells from neoplastic transformation by maintaining oxidative stress homeostasis [75,232]. More importantly, the loss of Nrf2 may lead to an increase in inflammation and to a decrease in cellular defense against oxidative stress, which may result in tumorigenesis [234]. In estrogen-induced hyperplasia model, we reported that administration of γ-TmT increased protein levels of Nrf2 and mRNA levels of UGT1A1, GSTm1 and COMT in estrogen-induced mammary hyperplasia [238]. In the NMU-induced breast tumorigenesis study, we showed that all tocopherol diets were able to increase protein levels of Nrf2 and subsequent down-stream phase II and antioxidant enzymes.

Apoptosis removes transformed cells when the upstream cellular defenses have failed. Previously, treatment with γ-tocopherol, but not α-tocopherol, induced c-Casp 8 and 9 in MDA-MB-435 human breast cancer cells [215]. In a lung xenograft model, both δ- and γ-tocopherol inhibited tumor growth and increased apoptosis, whereas α-tocopherol did not [218]. We have reported earlier that dietary γ-TmT induced c-Casp3 in mammary hyperplasia [238] and in mammary tumors [170]. In the present study, we demonstrate that δ-tocopherol, γ-tocopherol and γ-TmT increased apoptosis by inducing BAX, c-Casp-3 and c-PARP while down-regulating Bcl-

2 and XIAP. This shows that δ- and γ-tocopherol may inhibit mammary tumor growth by inducing apoptosis.

Cyclins D and E promote cell cycle progression and are regulated by cyclin-dependent kinases inhibitors (p21[CIP1] and p27[KIP1]) during the G_1-S phase [362,363]. Myc is a transcription factor and has been shown to regulate the cell cycle pathway by inducing cyclin E/CDK2 and cyclin D/CDK4/CDK6 complexes [364]. Myc was also shown to inactivate p21[Cip1], p27[Kip1] and p57[Kip2] [364]. In both prostate (LNCaP and DU-145) and colon (CaCo-2) cancer cells, γ-tocopherol was more effective than α-tocopherol at inhibiting cell proliferation and cell cycle progression by the reduction of cyclin D1 and cyclin E [217]. In the present study, we found that δ-tocopherol, γ-tocopherol and γ-TmT induced tumor suppressor p53, CDK inhibitors p21 and p27, while reducing levels of cMyc and CDK4/6, suggesting that δ- and γ-tocopherol may further inhibit cell proliferation by affecting the cell cycle pathway.

p53 is a transcription factor that responds to DNA damage and upregulates gene involved in cell cycle arrest, DNA repair, and apoptosis. Following p53, PTEN is the most common tumor suppressor that is lost or inactivated in many human cancers, including breast cancer. One role of PTEN is antagonizing PI3K preventing induction of the pro-survival Akt pathway. In mice that over-express PTEN, there was reduced cellular proliferation and increased apoptosis in the mammary glands [365]. PTEN has been shown to regulate p53 expression and transcriptional activity by either (phosphatase-dependent) inhibiting PI3K/Akt-induction of Mdm2 nuclear translocation (phosphatase-dependent) or through direct interaction with p53 (phosphatase-independent) [366]. The cross-talk between PTEN-p53 has been shown to increase cell cycle arrest through modulation of cyclins and cyclin dependent inhibitors [367].

One of the down-stream targets of p53 is the cyclin dependent kinase inhibitor, p21. p21 is a critical protein involved in the cell cycle arrest at the G_1 and G_2/M checkpoints. It has been shown *in vitro* that cancer cells that harbored inactivating mutations in PTEN exhibit reduced

expression of p53 and loss of subsequent downstream pro-apoptotic proteins [368]. Apoptosis may be activated by the induction of p53 or, in part, by blocking the activation of PI3K/Akt cell survival pathway [369]. Taken together, the upregulation of PTEN inhibits activation of Akt and MDM2, which increases the levels of p53, leading to cell cycle arrest and apoptosis.

δ- And γ-tocopherol were shown to inhibit cell proliferation in ER-positive breast cancer cells (MCF-7 and T47D) *in vitro* [170], and dietary γ-TmT decreased serum E_2 levels and the protein levels of ERα in mammary hyperplasia [238]. In the NMU-induced model, all tocopherol treatment reduced circulating E_2 serum levels and ERα protein levels in mammary tumors. Estrogen signaling is strongly associated with cell proliferation and stimulates the PI3K/Akt pathway [71,370]. ERα was shown to physically associate with PPARγ and functionally interferes with PPARγ signaling in breast cancer [355], thus crosstalk between the nuclear receptors should be taken into account for their different chemopreventive activities by tocopherols. The crosstalk between ERα and PPARγ should be taken into account for their different chemopreventive activities by tocopherols. PPARγ has been connected to multiple pathways where it inhibits PI3K/Akt activity, angiogenesis, and inflammatory markers, while inducing CDK inhibitors (p21 and p27), apoptosis and differentiation markers [244]. Initial studies demonstrated that PPARγ agonists reduced overall growth rate of breast cancer cells [371]. In MCF-7 breast and CaCo2 colon cancer cell lines, treatment with a PPARγ agonist increased PTEN expression, and inhibited phosporylation of Akt and cell proliferation. Furthermore, it was later confirmed that PPARγ agonist induced PTEN expression via a PPARγ dependent mechanism [372]. In addition, the specific binding of PPARγ to the PTEN promoter was observed and enhanced by treatment with a PPARγ agonist [355]. Our data indicate that δ-tocopherol and γ-tocopherol increase PPARγ which may decrease cell proliferation and survival in breast tumorigenesis.

PKCα may be another molecular target of interest. Another molecule of interest is PKCα. Previous studies have shown that PKC is associated with the PI3K/Akt pathway [373] and increases motility, invasion and metastasis [374]. PKCα regulates tumor growth and activation of PKCα is correlated with a more aggressive phenotype in breast cancer [375]. The overexpression of MCF-7 breast cancer cells leads to increased anchorage-independent growth, tumorigenicity and metastasis in mice [376]. Thus, the inhibition of PKCα by tocopherols indicates a decrease in cell proliferation, cell survival and aggressiveness.

Besides molecular changes in the proteins examined, there may be other reasons for the difference for responding and non-responding animals. Female SD rats are outbred and may have genetic variations, there may be differences in the animals from either the single exposure of 50 mg/kg to NMU at 21 days of age or there may be epigenetic changes. At 21 days of age, there is a larger number of terminal end buds compared to a rat at 50 days of age [57] which may be a target of carcinogenic initiation. In addition, NMU-induced mammary tumorigenesis mainly represent estrogen-dependent (~60-80%) and locally invasive phenotypes that are similar to human breast cancer [66]. Thus, the age of the rats when treated with the NMU carcinogen is significant for initiation as well as estrogen-dependency. Furthermore, NMU-induced mammary tumors show H-ras mutation by a G to A mutation in the second base of codon 12 in 70-90% of rats [377]. However, further analysis revealed that NMU did not increase the number of cells with H-ras mutations. Rather, the majority of tumors arose from cells with pre-existing mutations in H-ras [378].

Epigenetic changes between animals may include DNA methylation. In cancer cells, methylation of CpG islands is elevated. Recently, it was shown that a mixture of tocopherols inhibited CpG methylation in the Nrf2 promoter in the prostate of TRAMP mice [379]. Future studies in our laboratory could assess epigenetic changes by tocopherol treatment. Another possible epigenetic change may be DNA conformation. Exposure to NMU induces a loss of the

conformation switch in normal mammary cells and this could lead to mammary carcinogenesis [380]. Taken together, the age the carcinogen NMU was administered to the rats, epigenetic differences and the time of tocopherol treatment may play a role in why some animals responded and did not respond.

3.5 Summary

α-Tocopherol has been the primary tocopherol utilized for chemoprevention studies, and the results have been inconclusive [166,191,315]. However, the precise mechanism of action of individual tocopherols in cancer prevention is still unknown. Our findings showed that δ- and γ-tocopherol, but not α-tocopherol, reduced mammary tumor burden in NMU-treated rats. Initially, we observed that δ- and γ-tocopherol treated groups modulated cell proliferation, cell survival, and cell cycle pathways. Upon comparing responding and non-responding animals, we demonstrated that the main cancer preventive mechanism of δ- and γ-tocopherol may be through upregulating PPARγ and PTEN, and down-regulating PKCα and p-Akt. α-Tocopherol was not effective in reducing tumor burden and did not modulate PPARγ and PTEN, providing further evidence for a possible anti-cancer mechanism by δ- and γ-tocopherol. δ- And γ-tocopherol, but not α-tocopherol, may increase PPARγ and PTEN, thus modulating cell cycle and apoptosis by increasing p53 levels and inhibiting PKCα and PI3K/Akt pathway.

Table 3.1. Analysis of tocopherol and short chain metabolite levels in NMU-treated rats fed with tocopherol (α-, δ-, γ-) and γ-TmT-containing diets

	Tocopherol			Short Chain Metabolite		
	α-T	δ-T	γ-T	α-CEHC	δ-CEHC	γ-CEHC
Serum						
Control	18.0 ± 1.0	0.1 ± 0.1	0.2 ± 0.4	0.4 ± 0.1	0.1 ± 0.0	0.0 ± 0.0
0.3% α-T	72.8 ± 5.5^d	0.1 ± 0.0	0.1 ± 0.0	15.1 ± 2.0^c	0.2 ± 0.0	0.0 ± 0.0
0.3% δ-T	14.9 ± 1.3	10.6 ± 1.3^c	2.4 ± 0.2^c	0.5 ± 0.0	12.6 ± 0.7^c	0.3 ± 0.0^c
0.3% γ-T	7.0 ± 0.6^a	0.1 ± 0.0	26.8 ± 2.5^c	0.5 ± 0.1	0.1 ± 0.0	3.7 ± 0.6^c
0.3% γ-TmT	23.1 ± 1.8	2.7 ± 0.3^a	4.3 ± 0.5^c	2.9 ± 0.4^c	6.5 ± 0.3^c	2.3 ± 0.1^c
Mammary Gland						
Control	147.9 ± 3.3	1.05 ± 0.3	3.2 ± 0.5	0.3 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
0.3% α-T	215.7 ± 5.5^c	2.1 ± 0.3	3.4 ± 0.9	9.8 ± 1.1^c	0.2 ± 0.1	0.0 ± 0.0
0.3% δ-T	131.9 ± 7.5	131.3 ± 2.0^c	62.0 ± 6.4^c	0.2 ± 0.0	4.2 ± 0.7^c	0.1 ± 0.0
0.3% γ-T	95.5 ± 4.3^c	2.8 ± 0.8	155.4 ± 1.4^c	0.2 ± 0.0	0.1 ± 0.0	0.8 ± 0.2^c
0.3% γ-TmT	171.3 ± 4.0^a	96.5 ± 2.8^c	112.8 ± 4.4^c	1.2 ± 0.1^a	1.1 ± 0.1^a	0.5 ± 0.0^b
Tumor						
Control	7.2 ± 1.3	0.0 ± 0.0	0.1 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.1 ± 0.0
0.3% α-T	19.6 ± 3.1^c	0.0 ± 0.0	0.1 ± 0.0	16.1 ± 7.0^b	1.5 ± 0.6	0.0 ± 0.0
0.3% δ-T	6.0 ± 0.6	22.2 ± 1.3^c	3.2 ± 0.5^c	1.0 ± 0.4	7.6 ± 1.1^c	0.1 ± 0.0
0.3% γ-T	2.0 ± 0.6^b	0.0 ± 0.0	27.8 ± 5.6^c	1.0 ± 0.4	0.9 ± 0.5	1.2 ± 0.3^c
0.3% γ-TmT	11.8 ± 2.0	6.5 ± 0.8^c	6.2 ± 0.7^c	1.4 ± 0.3^a	1.6 ± 0.3^c	0.4 ± 0.1^b

The effects of 0.3% α-, δ-, γ-tocopherol (T), or γ-TmT supplementation on the levels of tocopherols and their metabolites (CEHC) in serum (µmol/L), mammary gland (µmol/kg), and mammary tumor (µmol/kg) in NMU-treated rats. Data are presented as the mean ± S.E (n=6-12 per group); [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$.

Table 3.2. Analysis of mRNA expression levels in the mammary tumor of NMU-treated rats

	Control	α-T	δ-T	γ-T	γ-TmT
Apoptotic Markers					
BAX	1.0 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	1.4 ± 0.2
Bcl2	1.0 ± 0.2	0.5 ± 0.1[b]	0.6 ± 0.1[a]	0.6 ± 0.1[a]	0.4 ± 0.1[b]
XIAP	1.0 ± 0.4	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
Cell Proliferation, Survival, and Cycle					
PCNA	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.7± 0.1
PKCα	1.0 ± 0.3	1.1 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.2
PTEN	1.0 ± 0.1	1.4 ± 0.2	2.0 ± 0.4[a]	2.1 ± 0.3[a]	1.6 ± 0.3[a]
Myc	1.0 ± 0.2	0.7 ± 0.2	0.9 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
p53	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	1.1 ± 0.2
p21	1.0 ± 0.1	1.2 ± 0.1	1.3 ± 0.1[a]	1.4 ± 0.1[a]	1.5 ± 0.2[a]
p27	1.0 ± 0.3	1.8 ± 0.2[a]	2.0 ± 0.4[a]	2.1 ± 0.3[b]	2.5 ± 0.3[c]
Cyclin D1	1.0 ± 0.2	1.2 ± 0.3	1.0 ± 0.3	0.5 ± 0.1	0.7 ± 0.3
Nuclear Receptors					
ERα	1.0 ± 0.1	0.9 ± 0.2	0.6 ± 0.0[a]	0.7 ± 0.1	0.5 ± 0.1[a]
ERβ	1.0 ± 0.1	0.7 ± 0.2	0.6 ± 0.1	0.8 ± 0.2	0.7 ± 0.2
PPARγ	1.0 ± 0.1	1.5 ± 0.3	1.8 ± 0.2[a]	2.1 ± 0.2[b]	1.9 ± 0.1[a]
Nrf2 Pathway					
Nrf2	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	0.8 ± 0.1
Keap1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
Phase II Detoxifying Enzymes					
NQO1	1.0 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.2	1.4 ± 0.1
GClm	1.0 ± 0.3	2.8 ± 0.9[a]	2.5 ± 0.2[a]	2.8 ± 0.5[a]	2.9 ± 0.4[a]
GSTm1	1.0 ± 0.1	1.6 ± 0.2[a]	1.5 ± 0.1[a]	1.7 ± 0.1[a]	2.0 ± 0.5[a]
Ugt1A1	1.0 ± 0.1	1.8 ± 0.3[b]	1.7 ± 0.2[a]	1.7 ± 0.2[a]	1.8 ± 0.4[a]
COMT	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.2 ± 0.3
Antioxidant Enzymes					
SOD1	1.0 ± 0.1	1.3 ± 0.2	1.2 ± 0.1	1.2 ± 0.2	1.4 ± 0.2
HO-1	1.0 ± 0.1	1.3 ± 0.2	1.4 ± 0.3	1.3 ± 0.1	1.2 ± 0.1
GPx	1.0 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.6 ± 0.2[a]	1.5 ± 0.2
TXN1	1.0 ± 0.1	1.0 ± 0.1	1.3 ± 0.2	1.3 ± 0.2	1.3 ± 0.2
Catalase	1.0 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1

NMU-treated Sprague-Dawley rats were administered 0.3% α-, δ-, γ-tocopherol (T), or γ-TmT in the diet. A mammary tumor from each rat was analyzed for mRNA levels by quantitative PCR and normalized by GAPDH. The values (fold-induction) are represented as mean ± S.E (n=6-8 per group); [a]p<0.05, [b]p<0.01, [c]p<0.001.
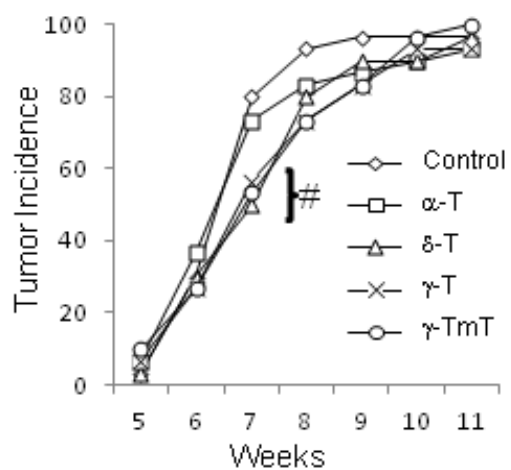
Figure 3.1 NMU-treated Sprague-Dawley rats were administered 0.3% α-, δ-, γ-tocopherol, or γ-TmT in the diet (n=30 per group). At 7 weeks of treatment, the tumor incidence for δ-tocopherol and γ-TmT was significant and denoted as [#]$p<0.05$.
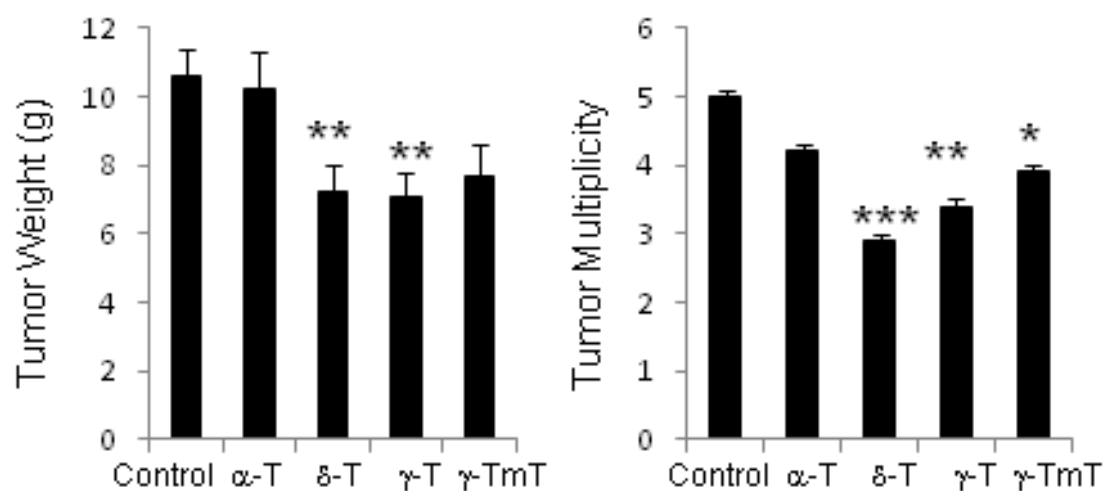
Figure 3.2 NMU-treated Sprague-Dawley rats were administered 0.3% α-, δ-, γ-tocopherol, or γ-TmT in the diet. Data are represented as mean ± S.E (n=30 per group). Statistical significance was determined by ANOVA with Dunnett's adjustment, *p<0.05, **p<0.01, ***p<0.001.
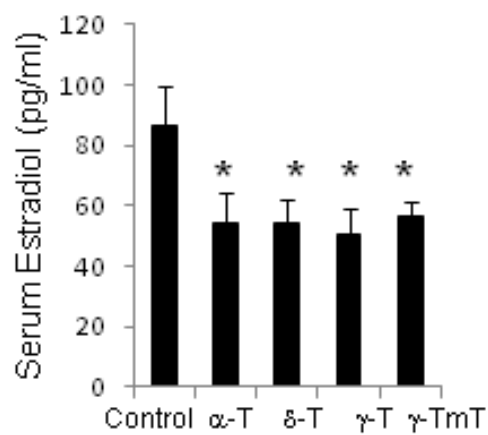
Figure 3.3 NMU-treated Sprague-Dawley rats were administered 0.3% α-, δ-, γ-tocopherol, or γ-TmT in the diet. Serum samples were analyzed for E_2 (pg/ml) (n=7). Statistical significance was determined by ANOVA with Dunnett's adjustment, *p<0.05.

Apoptotic Markers

	Control	α-T	δ-T	γ-T	γ-TmT
Apaf-1	1.0	0.9	0.9	0.9	0.7
BAX	1.0	1.0	3.2	2.2	2.0
Bcl2	1.0	0.4	0.7	0.5	0.6
XIAP	1.0	0.9	0.5	0.3	0.3
c-Casp9	1.0	4.9	4.5	5.7	3.2
c-Casp8	1.0	0.5	1.3	1.0	1.9
c-Casp3	1.0	1.6	2.3	3.5	3.5
c-PARP	1.0	3.6	5.7	5.7	7.0
B-Actin	1.0	1.3	1.3	1.2	1.3
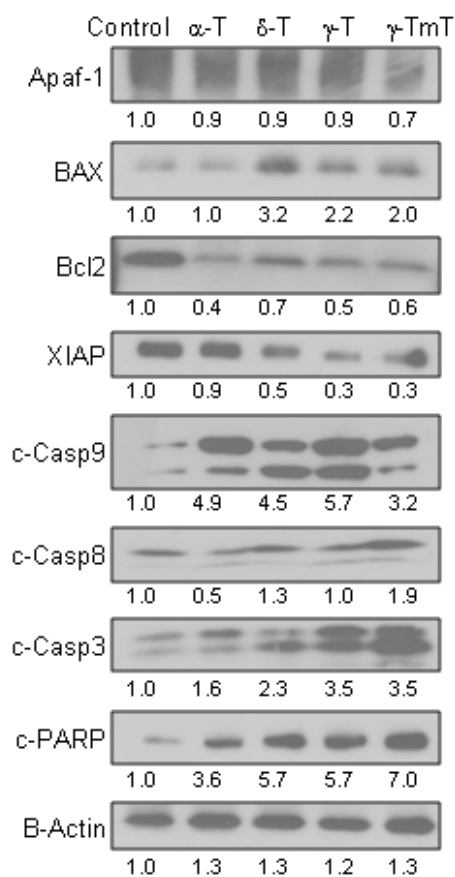
Figure 3.4 Tocopherol treatment modifies the levels of proteins in the mammary tumor of NMU-treated rats associated with apoptosis. Mammary tumors were pooled together (n=3 per group). Quantification of Western blot was performed by ImageJ 1.45s (NIH), and the numbers are provided at the bottom of each Western blot.
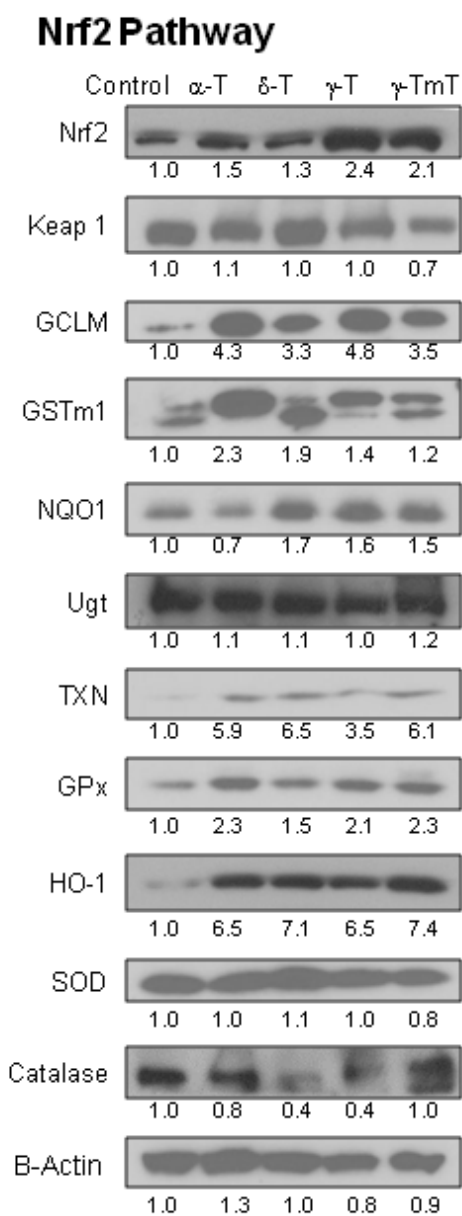
Figure 3.5 Tocopherol treatment modifies the levels of proteins in the mammary tumor of NMU-treated rats associated with Nrf2 pathway. Mammary tumors were pooled together (n=3 per group). Quantification of Western blot was performed by ImageJ 1.45s (NIH), and the numbers are provided at the bottom of each Western blot.

Nuclear Receptors

	Control	α-T	δ-T	γ-T	γ-TmT
ERα	1.0	0.6	0.6	0.3	0.6
ERβ	1.0	0.3	1.3	0.6	0.1
PPARγ	1.0	1.5	2.4	2.1	2.6
B-Actin	1.0	1.0	1.1	1.0	1.1
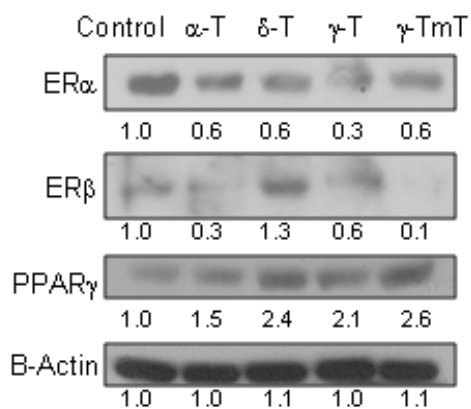
Figure 3.6 Tocopherol treatment modifies the levels of proteins in the mammary tumor of NMU-treated rats associated with nuclear receptors. Mammary tumors were pooled together (n=3 per group). Quantification of Western blot was performed by ImageJ 1.45s (NIH), and the numbers are provided at the bottom of each Western blot.

Cell Proliferation, Survival, and Cycle
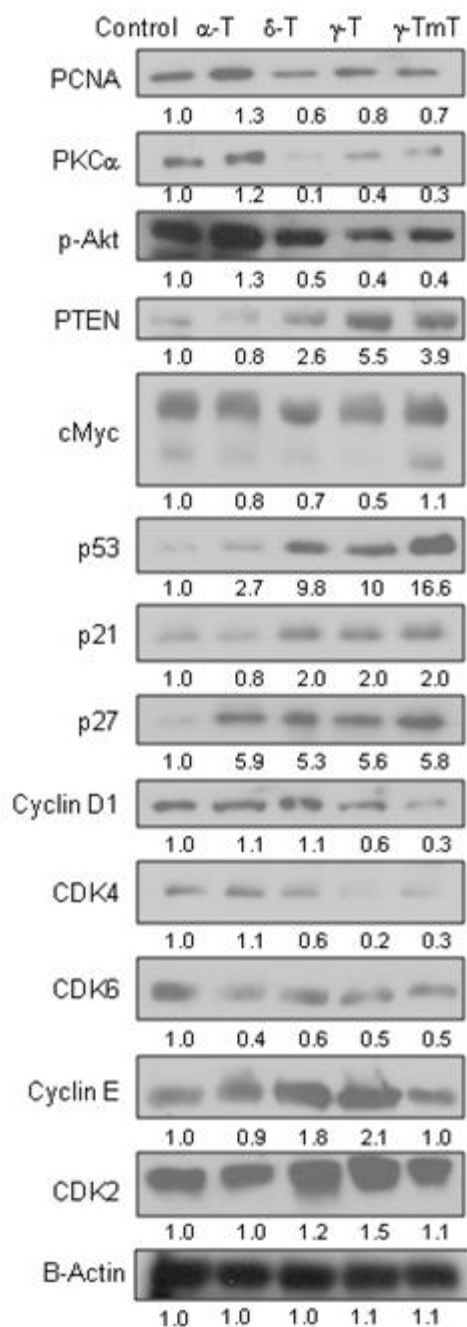
Figure 3.7 Tocopherol treatment modifies the levels of proteins in the mammary tumor of NMU-treated rats associated with cell proliferation. Mammary tumors were pooled together (n=3 per group). Quantification of Western blot was performed by ImageJ 1.45s (NIH), and the numbers are provided at the bottom of each Western blot.

Responders   **Non-Responders**

	Control	α-T	δ-T	γ-T	γ-TmT	Control	α-T	δ-T	γ-T	γ-TmT
c-Casp3	1.0	1.0	10.5	25.1	21.3	1.0	1.0	1.0	1.0	3.3
PCNA	1.0	1.0	0.6	0.2	0.7	1.2	1.3	0.7	1.7	1.9
PKCα	1.0	1.0	0.1	0.2	0.1	0.9	0.9	0.8	0.7	0.7
PTEN	1.0	1.4	6.5	7.2	7.8	1.1	1.1	1.2	1.4	0.7
p-Akt	1.0	1.3	0.7	0.6	0.6	2.1	1.8	1.9	1.3	1.6
Akt	1.0	1.0	1.0	1.1	1.1	0.9	1.0	0.9	0.8	0.8
PPARγ	1.0	1.1	3.8	3.2	3.4	1.1	1.3	1.1	1.0	1.0
ERα	1.0	0.5	0.7	0.8	0.9	1.4	1.0	1.3	1.1	1.3
p53	1.0	1.0	1.4	1.7	1.9	1.1	1.1	0.9	0.8	1.1
p21	1.0	0.8	3.2	2.1	2.6	1.0	0.9	0.7	0.6	1.2
Nrf2	1.0	3.8	9.0	8.2	6.7	2.5	2.3	1.4	1.1	2.4
HO-1	1.0	1.6	1.8	1.9	1.9	1.2	1.2	1.2	1.2	1.0
β-Actin	1.0	1.0	0.9	1.0	0.9	0.9	0.9	1.1	1.0	0.9
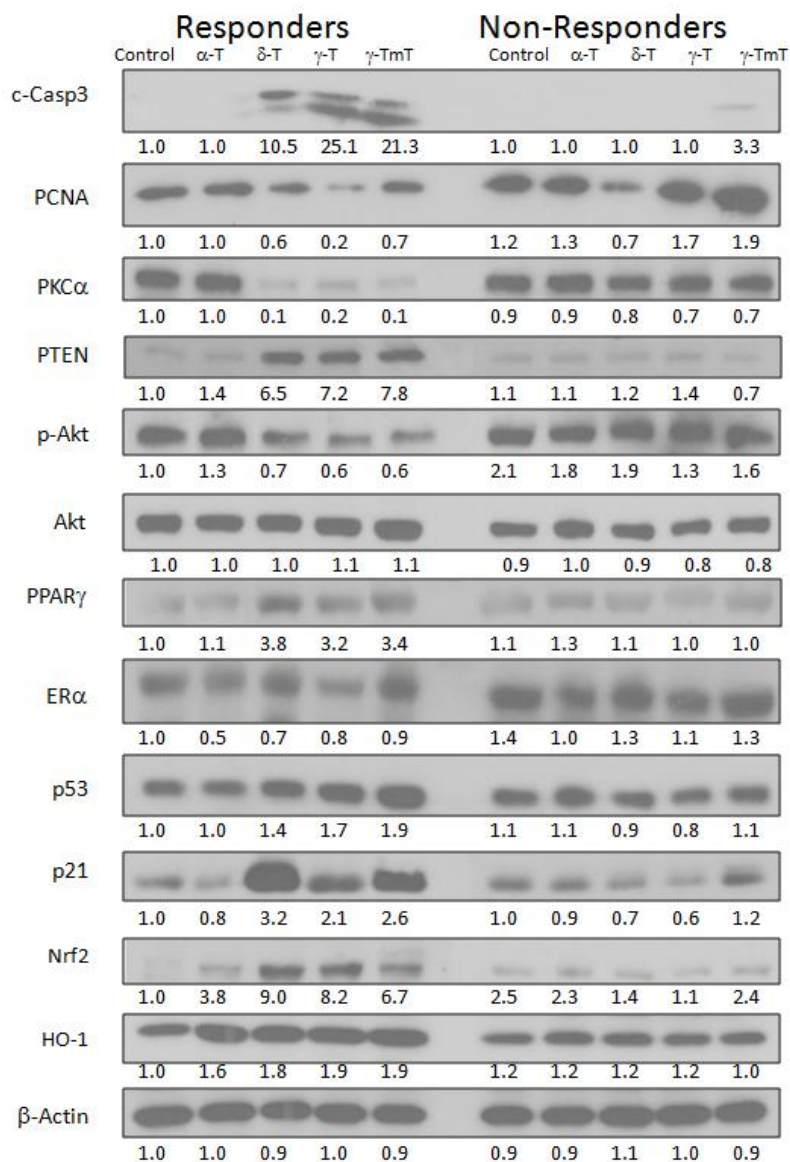
Figure 3.8 Tocopherol treatment modifies the levels of proteins in the mammary tumor of NMU-treated rats. Mammary tumors were pooled together (n=5 per group). Quantification of Western blot was performed by ImageJ 1.45s (NIH), and the numbers are provided at the bottom of each Western blot.
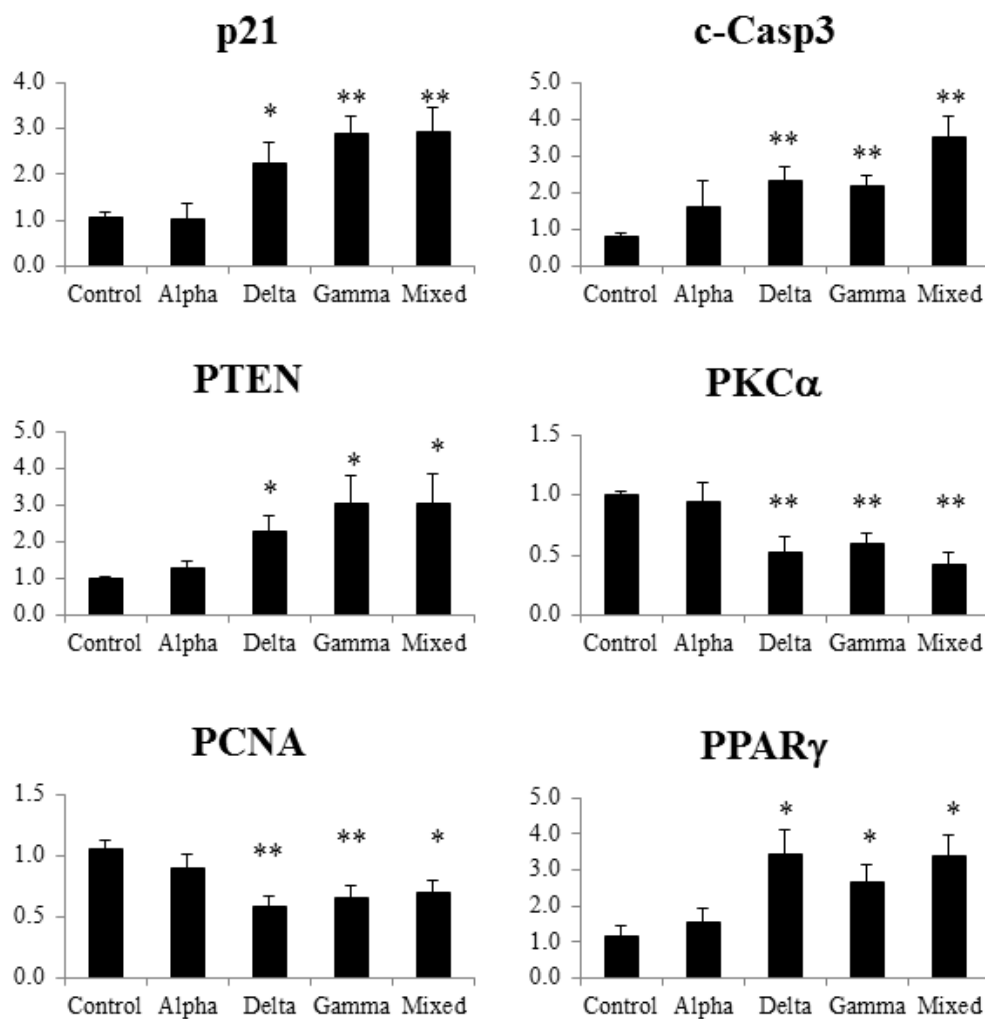
Figure 3.9 Tocopherol treatment modifies the levels of proteins in individual mammary tumor of NMU-treated rats. Five different mammary tumors from 5 different rats were analyzed for fold change in the different proteins to verify tumor variability. Quantification was performed utilizing ImageJ 1.45s (NIH). The data are presented as the mean ± S.E (n=5); *p<0.05, **p<0.01.
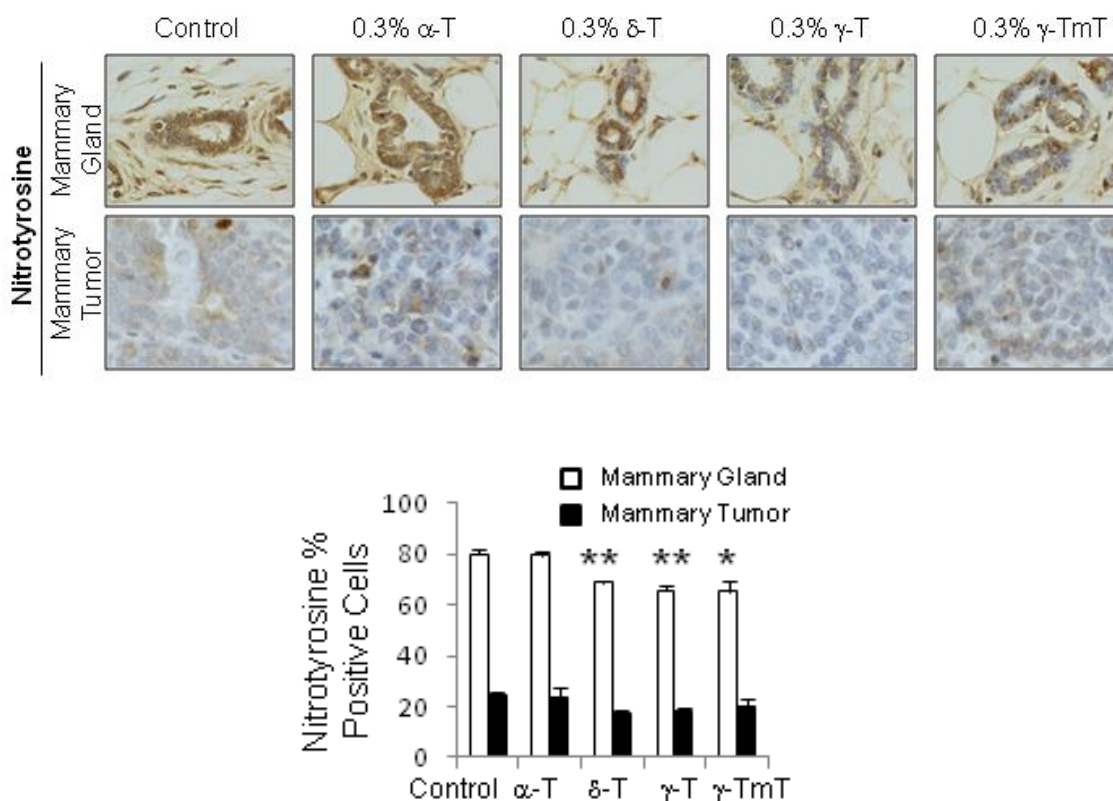
Figure 3.10 A representative immunostaining of nitrotyrosine in the mammary gland and tumor of NMU-treated rats (600x). Positive staining for nitrotyrosine is found in the cytoplasm of the cells. Quantification was performed using Aperio® Scan Scope where three mammary glands or tumors from each treatment group were selected and three areas from each gland or tumor were analyzed for over 1000 cells/mammary gland or 4000 cells/mammary tumor. The data are presented as the mean ± S.E (n=3); *p<0.05, **p<0.01.
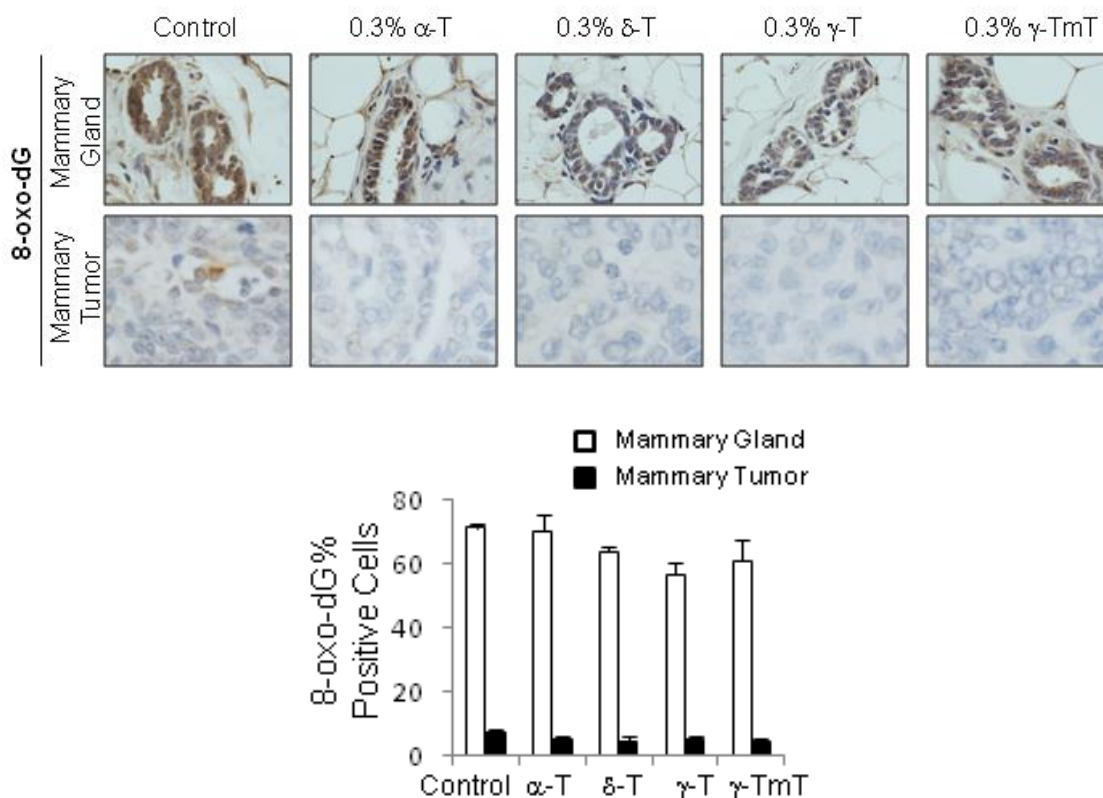
Figure 3.11 A representative immunostaining of 8-oxo-dG in the mammary gland or tumor of NMU-treated rats (600x). 8-oxo-dG shows positive staining in the nuclei of the cells. Quantification was performed using Aperio® Scan Scope where three mammary glands or tumors from each treatment group were selected and three areas from each gland or tumor were analyzed for over 1000 cells/mammary gland or 4000 cells/mammary tumor. The data are presented as the mean ± S.E (n=3).
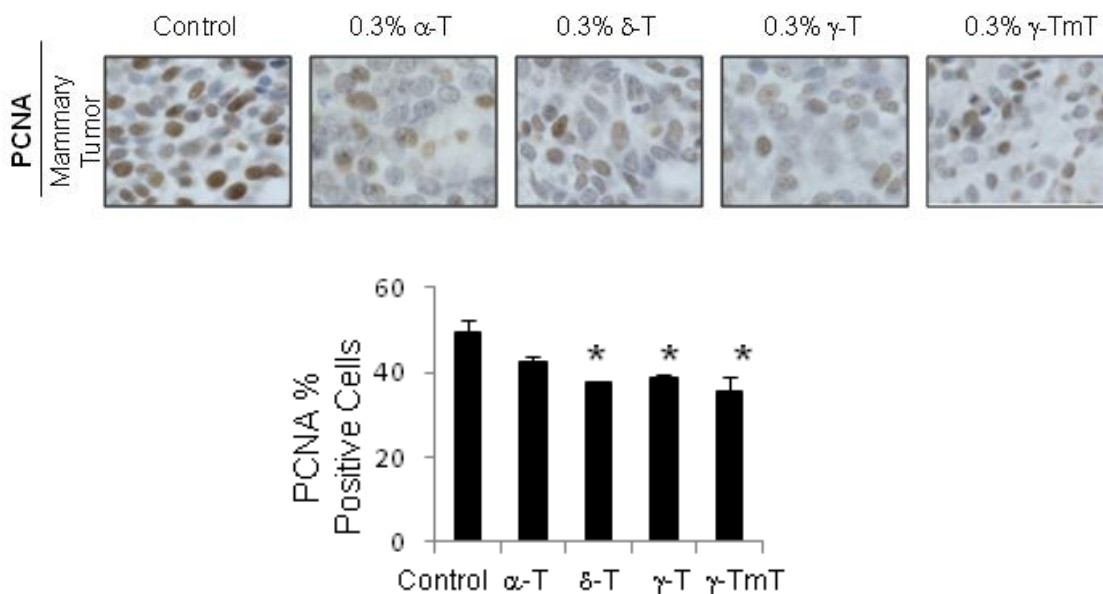
Figure 3.12 A representative immunostaining of PCNA in the mammary tumor of NMU-treated rats (600x). PCNA shows positive staining in the nuclei of the cells. Quantification was performed using Aperio® Scan Scope where three mammary tumors from each treatment group were selected and three areas from each tumor were analyzed for over 4000 cells/mammary tumor. The data are presented as the mean ± S.E (n=3); *p<0.05.
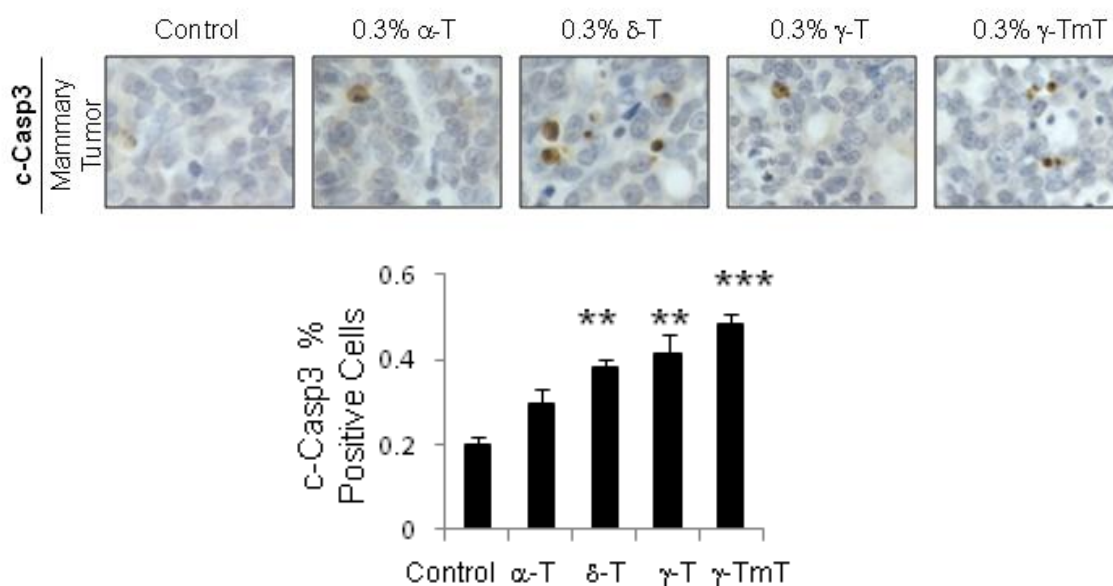
Figure 3.13 A representative immunostaining of c-Casp3 in the mammary tumor of NMU-treated rats (600x). Positive staining for c-Casp3 is shown as a light brown to dark brown precipitate in the cytoplasm and or perinuclei of the cells. Quantification was performed using Aperio® Scan Scope where three mammary tumors from each treatment group were selected and three areas from each tumor were analyzed for over 4000 cells/mammary tumor. The data are presented as the mean ± S.E (n=3); *p<0.05, **p<0.01, ***p<0.001.
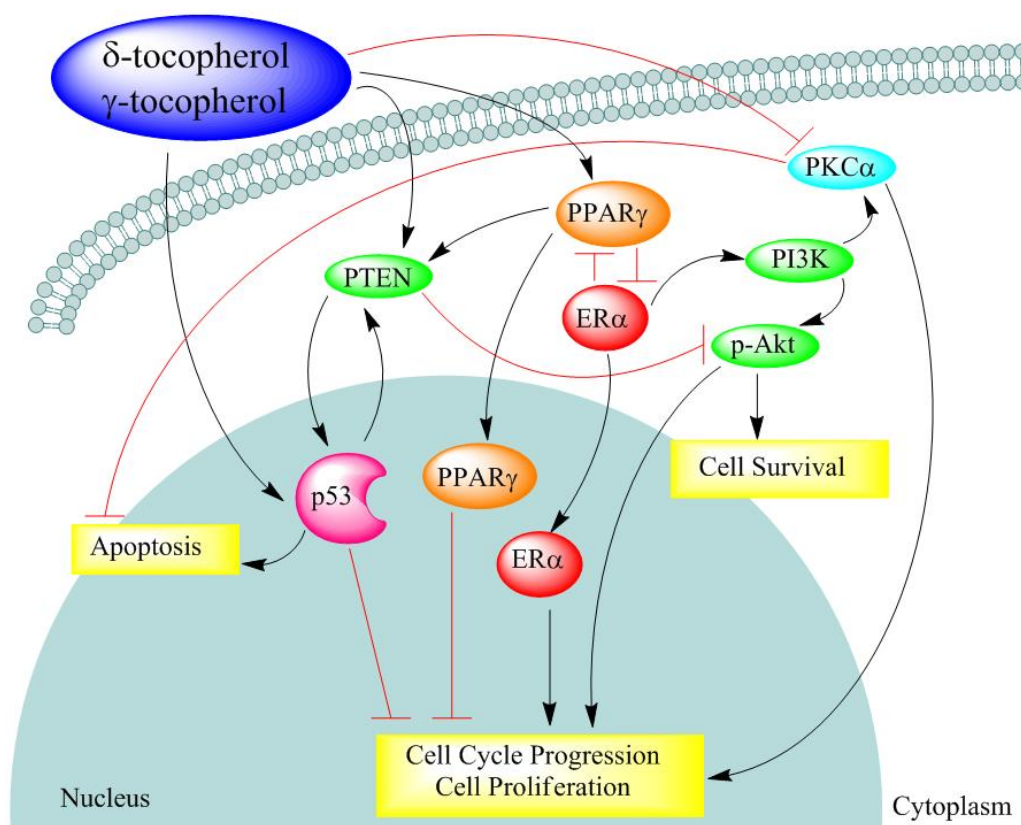
Figure 3.14 A schematic representation which shows possible mechanisms of action by δ- and γ-tocopherol in mammary carcinogenesis. δ-Tocopherol and γ-tocopherol may activate PPARγ, PTEN, p53, and decrease ERα, PKCα and p-Akt to inhibit cell cycle progression and cell growth resulting in the inhibition of mammary tumorigenesis.

Chapter 4: Dietary administration of individual tocopherols does not inhibit tumorigenesis in MMTV-ErbB2/neu transgenic mice [a,b]

4.1 Introduction

In about 18-25% of breast cancer, there is overexpression and amplification of the HER2/neu, which is a transmembrane protein and a member of epidermal growth factor receptor family [31]. The MMTV promoter controls the expression of un-activated HER2 proto-oncogene in mammary tissue [85,323]. MMTV/Neu/HER-2 transgenic mice were developed in the laboratory of William Muller [323] and tumors arise in the MMTV/Neu mice at 26-60 weeks of age. Direct evidence associating HER2 as an oncogene originates from studies of transgenic mice expressing an activated Neu (rat homologue of HER2/ErbB2). MMTV-driven overexpression of neu/HER2 oncogene resulted in mammary tumors that are hormone-independent and well-differentiated breast adenocarcinoma [85,323]. Therefore, MMTV/HER2/neu transgenic mouse model has been widely utilized for breast cancer prevention studies [381]. HER2 breast cancer can stimulate COX-2 [249] which is upregulated in response to growth factors, tumor promoters, and cytokines [248]. High levels of COX-2 are associated in about 40% of human breast cancers and correlate with aggressive breast cancer (large tumor size, high proliferation rate, and metastases) and *HER2* gene amplification [250].

Tocopherols are known antioxidants and anti-inflammatory agents and γ-tocopherol is more effective in inhibiting the activity of cyclooxygenase and trapping reactive nitrogen species than α-tocopherol [193,224-227]. Tocopherols have shown anti-inflammatory actions over

several cancer models. In a lung tumor model using A/J mice, *Lu et al*. reported a decreased level of PGE_2 and LTB_4 in the serum when given γ-TmT diet [345]. In a colon cancer model with azoxymethane/dextran sulfate sodium treated mice, treatment with γ-TmT reduced the levels of PGE_2, LTB_4, and 8-isoprostane in the serum [171]. Tocopherols may reduce tumor burden in in HER2 positive tumorigenesis by decreasing inflammation as evidenced in other cancer models.

Our recent results in chapters 2 and 3 demonstrate that δ-tocopherol, γ-tocopherol and γ-TmT inhibit mammary hyperplasia and tumorigenesis in ER positive breast cancer models via the reduction of inflammatory markers (PGE_2 and COX-2) and induction of apoptosis. Therefore, we investigated the efficacy of mixed (γ-TmT) and individual (α-, γ-, δ-) tocopherols in a HER2 positive breast cancer model. We hypothesize that tocopherols may reduce inflammation and oxidative stress via the induction of Nrf2 and related genes to reduce mammary tumorigenesis in MMTV/HER2/neu transgenic mouse model.

4.2 Material and Methods

4.2.1 Animals and experimental procedures

Female MMTV/ErbB2/neu transgenic mice at 6-7 weeks old were purchased from Jackson Laboratory (Bar Harbor, ME). At 12 weeks of age, the mice received AIN-93M control diet or AIN-93M diets containing 0.3% tocopherols (α-, δ-, γ-, or γ-TmT) (n= 28 per group). The body weight and tumor size of each animal were measured weekly. The mice were sacrificed at 55 weeks of age and the tumors were weighed at necropsy. Mammary glands, mammary tumors, and lungs were stored for further analyses. Serum was collected after centrifugation of clotted blood samples.

4.2.2 Animal diets

Semipurified modified AIN-93M diet was obtained from Research Diets Laboratory (New Brunswick, NJ) and used as the control diet. The test diets were prepared by Research Diets Laboratory by adding 0.3% α-tocopherol, δ-tocopherol, γ-tocopherol or γ-TmT to the AIN-93M diet. γ-TmT was supplied by the Cognis Corporation (Kankakee, IL) and contained 57% γ-tocopherol, 24% δ-tocopherol, 13% α-tocopherol and 1.5% β-tocopherol. γ-Tocopherol was purified from γ-TmT by silica gel chromatography to a purity of 97%, with no detectable α- and δ-tocopherol. δ-Tocopherol (containing 94% δ-tocopherol, 5.5% γ-tocopherol and 0.5% α-tocopherol) and α-tocopherol (containing 69.7% α-tocopherol, 2.6% γ-tocopherol and 0.2% δ-tocopherol) were purchased from Sigma-Aldrich (St. Louis, MO). The diets were stored at 4°C and the food was replenished with fresh pellets twice weekly.

4.3 Results

4.3.1 γ-Tocopherol delayed increased tumor latency in MMTV-ErbB2/neu transgenic mice.

We investigated the effects of 0.3% α-tocopherol, 0.3% δ-tocopherol, 0.3% γ-tocopherol, or 0.3% γ-TmT in the diet on mammary tumor development in MMTV-ErbB2/neu transgenic mice over the period of 55 weeks. The median tumor latency was 37 weeks in the control group, and 38, 37, 44 and 39 weeks in mice fed with a diet containing α-, δ-, γ-tocopherol and γ-TmT, respectively (Fig. 4.1). Only the diet containing γ-tocopherol significantly increased the median tumor latency ($p < 0.05$).

4.3.2 Dietary administration of tocopherols did not inhibit tumor growth or multiplicity in MMTV-ErbB2/neu transgenic mice.

The final mammary tumor weight in the control group was 1.17 ± 0.14 g, compared to α-tocopherol (0.88 ± 0.11 g), δ-tocopherol (1.02 ± 0.09 g), γ-tocopherol (0.84 ± 0.11 g), and γ-TmT (1.11 ± 0.07 g), which corresponds to 24%, 13%, 29%, and 5% inhibition, respectively (Fig. 4.2). The tumor multiplicity was 1.60 ± 0.30 in the control group, as compared to groups treated with α-tocopherol (1.18 ± 0.22), δ-tocopherol (1.32 ± 0.25), γ-tocopherol (1.28 ± 0.25), and γ-TmT (1.42 ± 0.28), which corresponds to 27%, 18%, 20%, and 11% inhibition, respectively (Fig. 4.2). At the conclusion of the 55 week study, we did not observe tumor inhibition by α-, δ-, γ-tocopherol or γ-TmT diets.

4.4 Discussion and Summary

At the conclusion of the fifty-five week study, tumor weight and multiplicity were not significantly affected by any of the examined tocopherols, suggesting that tocopherols do not affect HER-2 driven mammary tumorigenesis. Treatment with α-, δ-tocopherol and γ-TmT did not delay tumor onset. However, there were modest effects by dietary administration of 0.3% γ-tocopherol in reducing tumor incidence, and further studies may be needed to determine if higher doses of tocopherols are protective against HER-2 breast cancer. There may be other reasons why tocopherols were not effective at preventing HER-2 breast cancer. Tocopherols may be more effective in preventing tumorigenesis in ER positive breast tumors, but not in HER-2 subtype of breast cancer. Alternatively, as the tumors become more aggressive, they become resistant to tocopherol treatment.
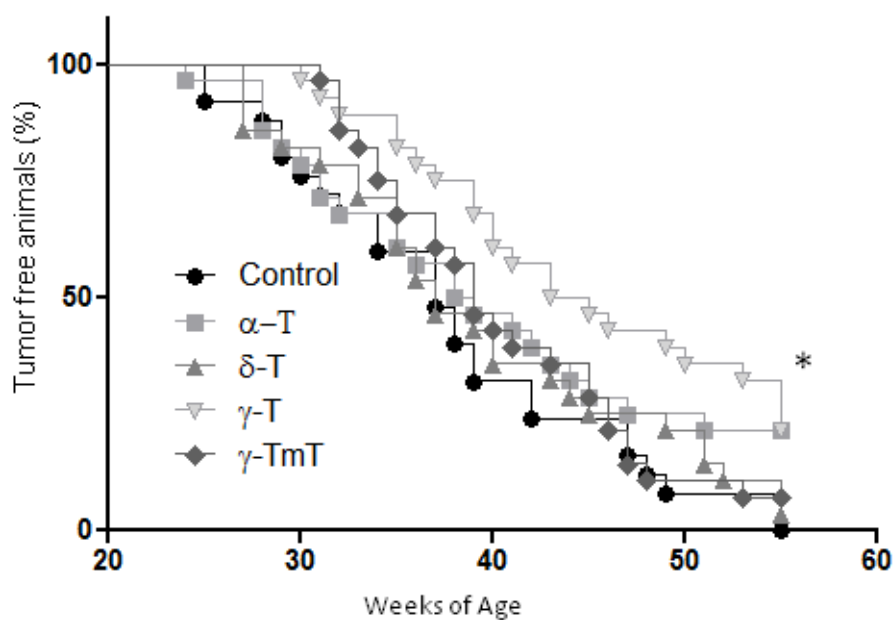
Figure 4.1 MMTV/ErbB2/neu transgenic mice were administered 0.3% α-, δ-, γ-tocopherol (T), or γ-TmT in the diet. Data are represented as mean ± S.E. (n=28 per group). Statistical significance was determined by ANOVA with Dunnett's adjustment, *p<0.05
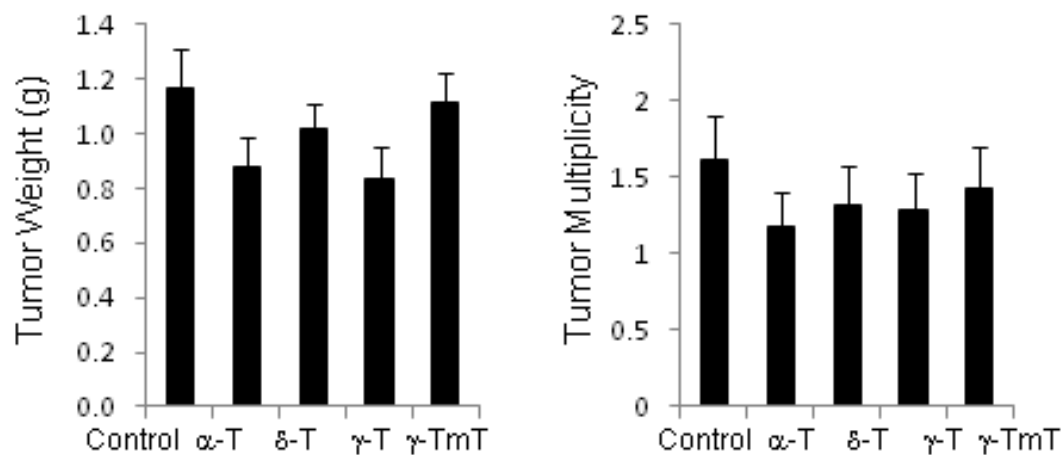
Figure 4.2 MMTV/ErbB2/neu transgenic mice were administered 0.3% α-, δ-, γ-tocopherol (T), or γ-TmT in the diet. Data are represented as mean ± S.E. (n=28 per group)

Overall Discussion

In 1947, the first stage of carcinogenesis was labeled initiation [382]. Initiation is caused by irreversible genetic changes, which predispose susceptible normal cells to malign evolution and immortality [382,383]. The initiated cell is not a neoplasic cell, but has taken its first step towards this state, after successive genotypical and phenotypical changes have occurred [383]. The clonal expansion of initiated cells results from a mitogenic process caused by an increase in the number of new cells and apoptosis inhibition, which prevents initiated cells from dying off [384].

The concept of promotion was introduced when chemical substances with low carcinogenic activity were discovered, which were still able to induce the development of cancer under experimental conditions [382]. These agents increase cell proliferation in susceptible tissues, contribute towards fixing mutations, enhance alterations in gene expression and cause changes in cellular growth control [385,386]. The promoter may be present for weeks, months and years and its effectiveness depends on its concentration in the target tissue [387]. Promotion is a reversible stage, after a promoter's disappearance a regression in cell proliferation can occur [387]. Not all cells exposed to promoters take part in the promotion stage, only cells which are stimulated to divide, that are undifferentiated, and have survived apoptosis, can contribute to instability between growth and cell death and lead to the appearance of a malign neoplasia [384].

The transformation from benign lesions into malign lesions is the last stage of carcinogenesis and is labeled progression [388]. In progression, a neoplastic phenotype is acquired through genetic and epigenetic mechanisms [389]. Progression is characterized by irreversibility and genetic instability associated with karyotypic change and this results in faster growth, invasion, metastasis and changes in the biochemical, cell transformation and morphological characteristics of cells [387,388]. Angiogenesis, as an epigenetic occurrence, is essential to neoplasic progression. The acquisition of an angiogenic phenotype precedes the

development of characteristics that contribute to malignancy and its inhibition delays neoplasic development [390].

Chronic inflammation is implicated in the development of a diverse range of human cancers [391]. Early in the neoplastic process, inflammatory cells are powerful tumor promoters, producing an attractive environment for tumor growth, facilitating genomic instability via DNA damage and promoting angiogenesis [392]. The inflammatory cells and the chemokines and cytokines that are produced influence the whole tumor organ regulating the growth, migration and differentiation of all cell types in the tumor microenvironment [392,393]. The pro-tumor actions of inflammatory cells include releasing growth and survival factors, promoting angiogenesis and lymphangiogenesis, stimulating DNA damage, remodeling the extracellular matrix to facilitate invasion and evading hose defense mechanisms [392]. Later in the tumorigenic process, neoplastic cells also divert inflammatory mechanisms such as selectin, MMP production and chemokine functions to favor neoplastic spread and metastasis [392,393]. As a result, inflammatory cells may facilitate epithelial cell invasion into the stromal and vasculature compartments, and lead to the metastasis of the tumor cells [394].

A transient inflammatory signal may initiate an epigenetic change from non-transformed to cancer cells via a positive feedback loop [395]. microRNAs (miR-21 and miR-181b-1) with regulatory functions are directly activated by STAT3 and inhibits PTEN and CYLD tumor suppressors, which leads to the increased NF-κB activity required to maintain the transformed state [395]. This epigenetic switch leads to a positive feedback loop that links inflammation to cancer [395].

The innate and adaptive immune systems are both involved in the molecular mechanisms that underlie the pathogenesis of inflammation-associated cancer [392,396]. Highly reactive chemical compounds, such as superoxide, hydrogen peroxide and nitric oxide are released from activated phagocytic inflammatory cells of the innate immune system, and can cause oxidative or nitrosative damage to DNA in the epithelial cells, or react with other cellular components such as

phospholipids, initiating a free-radical chain reaction [397]. The result is that many host epithelial cells are damaged and killed. Epithelial cells that undergo DNA synthesis in the setting of these DNA-damaging agents are at an increased risk of mutation. Toxicological relevance of RNS include protein modification, DNA base deamination and the formation of N-nitrosamines, among the most potent mutagenic and carcinogenic compounds for humans [398]. Nitrite can cause structural modifications to a variety of endogenous and exogenous organic compounds such as polyunsaturated fatty acids, estrogens, tocopherol, catecholamines, furans, retinoids and dietary phenols [398].

At low levels of ROS and/or oxidative stress, the cells will be in homeostasis [135,399]. While at moderate levels of ROS and/or oxidative stress, epithelial cells may incure DNA damage and undergo transformation, proliferation or promotion phase of tumorigenesis [135,399]. A pro-oxidant chemopreventive agent may enhance ROS in transformed cells above the threshold required for anomalous proliferation and could potentially drive these cells to elimination via apoptosis or oncosis [135,399].

Under conditions of high oxidative stress, the abilities of cells to eliminate ROS become exhausted, and dietary sources of anti-oxidants are required. Fat soluble vitamin E is one of the most important antioxidants for protection of the hydrophobic lipid interior of membranes [400,401]. During inflammation, there is a redox imbalance that overproduces free radicals and decreases the level of endogenous antioxidants [402]. Pre-treatment with antioxidants may be promising as a protective means of lowering the reactivity of cascades, which form ROS [403]. Exogenous antioxidants may protect cells and tissues against ROS. The major steps in the TNF-mediated cytotoxicity cascade include G-protein-coupled receptor activation of phospholipases, generation of free radicals, and damage to nuclear DNA by endonucleases [403]. Protection by antioxidants may reduce TNF-mediated cytotoxicity via mechanisms by scavenging ROS and also by supporting the immune cell activity by regulating membrane fluidity [403].

In the event of unrelenting ROS/oxidative stress, the constitutive stimulation of redox-regulated transcription factors like NF-κB would promote a chronic inflammatory response that would lead to disease like arthritis, heart disease and cancer [404]. Antioxidants can block the constitutive activity of redox-regulated inflammatory mediators like NF-κB in cultured cells. Dietary fruits and vegetables are thought to be full of antioxidants that are presumed to promote health and prevent diseases like cancer [6]. Nevertheless, perhaps the real benefit of consuming fruits and vegetables lies not only in their nutrient content, but also in their mild toxicant/pro-oxidant constituents that continually prod the stress response in normal cells to protect against damage, while removing premalignant or damaged cells by apoptosis [135].

Dietary antioxidants may reduce cancer risk by altering levels of ROS or RNS, which are a driving force in cancer formation [405]. However, clinical trials with anti-oxidants did not find the health-promoting effects, and may even promote cancer in humans [406]. Recently, γ-tocopherol has been shown to be more effective at trapping reactive nitrogen species than α-tocopherol [193,224-228]. Previously, in a lung xenograft tumor model, δ-tocopherol, γ-tocopherol and γ-TmT administration reduced 8-oxo-dG and nitrotyrosine levels, whereas α-tocopherol did not [218]. In our NMU-induced breast cancer model, treatment with δ-tocopherol, γ-tocopherol and γ-TmT reduced 8-oxo-dG and nitrotyrosine levels in the mammary gland, whereas α-tocopherol did not. Interestingly, low levels of ROS and RNS markers were observed in mammary tumors and were not changed by tocopherols. Thus δ-tocopherol and γ-tocopherol may be more effective at reducing ROS or RNS during the prevention of mammary tumorigenesis.

ROS and/or oxidative stress can function as an extrinsic, reversible mediator for regulating stress responses in normal cells provides an attractive explanation for the prospective cytoprotective activity of certain pro-oxidant cancer chemopreventive agents [135]. One likely target of these agents in normal epithelial cells is Nrf2. Up-regulating Nrf2 activity by certain

cancer chemopreventive agents will confer cytoprotection through the induction anti-oxidant and phase II enzymes [128,407]. Numerous chemopreventive agents have been shown to activate the Nrf2/ARE pathway, and many of these inducers such as resveratrol [408], curcumin [407,409], and sulforaphane [410] can be found in the normal diet. Many tumor cell types also constitutively overexpress anti-oxidant enzymes like those regulated by Nrf2 [411,412], as well as mitochondrial anti-oxidant enzymes like manganese superoxide dismutase [412,413], which appear to suppress ROS-induced apoptosis in these cells. Together, these observations would suggest that transformed cells in the promotion phase of tumorigenesis are obligated to cope with higher levels of intrinsic ROS than their normal counterparts [399]. Nrf2 may be tumor suppressive by reducing oxidative stress, however, Nrf2 may be oncogenic by promoting cell survival under stress [414]. The role of Nrf2 may depend on the stage of tumorigenesis. In normal cells, Nrf2 activation may be cytoprotective by regulating genes encoding phase II enzymes to prevent cancer in humans; while at the malignant stage, Nrf2 may protect against the high endogenous levels of ROS and increase tumor survivability [414]. While at pre-malignant and early malignancy, the role of Nrf2 is still unclear [414].

A cancer chemopreventive agent may stimulate the activity and expression of Nrf2 to potentially block the transformation of normal cells [407]. Many of these same agents may encourage apoptosis in transformed cells to prevent the promotion and progression of malignant phenotype [135,136]. Thus, the activation of the Nrf2 pathway by chemopreventive agents should protect against inflammation and oxidative stress during the earlier stages of tumorigenesis [415].

Recently, it was shown that the expression of Nrf2 was suppressed in prostate tumors [237], and treatment with γ-TmT upregulated the expression of Nrf2 and detoxifying enzymes, and inhibited tumor development in TRAMP mice [75]. In our study with estrogen-treated ACI rats, the protein expression level of Nrf2 was increased in the mammary gland and liver. Protein levels of xenobiotic metabolizing phase II enzymes were increased in the liver by γ-TmT

treatment. The mRNA expression in the mammary gland and liver showed that phase II detoxifying enzymes were also induced by γ-TmT treatment, suggesting that γ-TmT increases the transcription of Nrf2-ARE-target genes and exhibits protective defense against estrogen-induced oxidative stress. In our NMU-induced breast tumorigenesis study, we showed that all tocopherol diets were able to increase protein levels of Nrf2 and subsequent down-stream phase II and antioxidant enzymes. Tocopherols are known direct antioxidants. Furthermore, all tocopherol treatment may induce Nrf2 protein levels and act as indirect anti-oxidants. The Nrf2 pathway may be stimulated via chemopreventive agents or under oxidative stress and nitrosative stress. Further *in vitro* studies need to clarify the role of individual tocopherols and γ-TmT in the activation of Nrf2 pathway. As Nrf2 is a target for chemoprevention, tocopherols may be effective agents in the prevention of breast cancer.

Estrogen signaling is strongly associated with cell proliferation and stimulates the PI3K/Akt pathway [71,370]. ERα was shown to physically associate with PPARγ and functionally interferes with PPARγ signaling in breast cancer [355], thus crosstalk between the nuclear receptors should be taken into account for their different chemopreventive activities by tocopherols. PPARγ is expressed in breast, prostate, and colon epithelium, and involved in lipid and glucose metabolism, cell proliferation and apoptosis, differentiation, and cell survival [241]. Specifically in breast cancer, stimulation of PPARγ increases the degradation of cell cycle genes (cyclin D1), interferes with estrogen receptor signaling, and NF-κB signaling cascades [243,245]. Thus, activation of PPARγ by tocopherols in breast tissue may have anti-estrogenic effects, inhibit cell cycle progression, and induce apoptosis to prevent breast cancer.

In our estrogen-induced hyperplasia model, PPARγ was increased at both the protein and mRNA level in the mammary gland of ACI rats when treated with γ-TmT while ERα expression was decreased. Furthermore, in our carcinogen-induced mammary tumors, levels of PPARγ were increased with δ-tocopherol and γ-tocopherol, but not α-tocopherol. This may indicate a possible

mechanism of action by δ-tocopherol and γ-tocopherol to induce PPARγ and increase apoptosis and reduce cell proliferation.

Vitamin E signaling could originate within different lipid environments of organelles [416] and is delivered consequently to subcellular distribution targets factors [417]. There are cytosolic proteins that bind to the hydrophobic domains of vitamin E and regulate trafficking and subcellular localization of vitamin E [416]. Sec14p-like proteins are prototype components of vitamin E regulation system that may play a key role in vitamin E signaling [200]. One member of this family, TAP/Sec14L2, is highly expressed in normal/benign breast, prostate and liver tissues as compared to lung, colon and kidney [418]. The expression of TAP/Sec14L2 was down-regulated in breast cancer cell lines, and 57% of 141 human invasive breast carcinomas had no or markedly reduced TAP/Sec14L2 expression [418]. TAP/Sec14L2 in combination with vitamin E may suppress mammary tumors [416]. Vitamin E and possible protein interactions for intracellular signaling have been reviewed and include kinases such as PKC, Akt/PKB, MAPK, cell cycle related kinases, down-stream components of the death domain and proteins of the endoplasmic reticulum stress signaling [198-200,263,264].

p53 is a transcription factor that responds to DNA damage and upregulates gene involved in cell cycle arrest, DNA repair, and apoptosis. Following p53, PTEN is the most common tumor suppressor that is lost or inactivated in many human cancers, including breast cancer. One role of PTEN is antagonizing PI3K preventing induction of the pro-survival Akt pathway. In mice that over-express PTEN, there was reduced cellular proliferation and increased apoptosis in the mammary glands [365]. PTEN has been shown to regulate p53 expression and transcriptional activity by either (phosphatase-dependent) inhibiting PI3K/Akt-induction of Mdm2 nuclear translocation (phosphatase-dependent) or through direct interaction with p53 (phosphatase-independent) [366]. The cross-talk between PTEN-p53 has been shown to increase cell cycle arrest through modulation of cyclins and cyclin dependent inhibitors [367]. Protein levels of PTEN and p53 were increased with δ-tocopherol and γ-tocopherol, but not α-tocopherol. An

increase of these two important tumor suppressors may be an important part in the chemopreventive activity by tocopherol in mammary tumorigenesis. Furthermore, p53 is a major regulator of the cell cycle and contributes to the inhibition of cell growth.

Another molecule of interest is PKCα. Previous studies have shown that PKC is associated with the PI3K/Akt pathway [373] and increases motility, invasion and metastasis in tumor cells [374]. PKCα regulates tumor growth and activation of PKCα is correlated with a more aggressive phenotype in breast cancer [375]. The overexpression of PKCα in MCF-7 breast cancer cells leads to increased anchorage-independent growth, tumorigenicity and metastasis in mice [376]. Thus, the inhibition of PKCα by tocopherols indicates a decrease in cell proliferation, cell survival and aggressiveness. Besides controlling cell cycle regulation, cell proliferation and cell survival may be decreased when treated with tocopherols. In our NMU-induced mammary tumorigenesis model, δ-tocopherol and γ-tocopherol showed decreased protein levels in PKCα and p-Akt. This may indicate that a possible mechanism of action could be through the reduction of cell proliferation and cell survival to decrease tumorigenesis.

Estrogens have been implicated in breast cancer. Estrogen is a hormone that has numerous functions throughout the body such as promotion of female secondary sex characteristics, accelerate metabolism, increase fat stores, maintenance of vessel and skin, reduce bone resorption, increase bone formation, increase platelet adhesiveness and increase endometrial growth [419]. Vitamin E may have anti-estrogenic effects and has been shown to inhibit ER-positive cell proliferation and work as antagonists of estrogen signaling [194]. High doses of vitamin E may lead to a disturbance in the levels of estrogen and possibly bone homeostasis. In mice that are deficient in α-tocopherol transfer protein, indicating vitamin E deficiency resulted in higher bone mass as a result of a decrease in bone resorption [420]. *In vitro* assays indicated that α-tocopherol stimulated osteoclast fusion by inducing the expression of dendritic-cell-specific transmembrane protein, an essential molecule for osteoclast fusion [420]. Moreover,

wild-type mice that were fed α-tocopherol supplemented diet had a loss in bone mass [420]. This may indicate that serum α-tocopherol levels is a determinant of bone mass through its regulating of osteoclast fusion. As shown in Figures 2.1 and 3.3, individual (α-, δ-, γ-) tocopherol and γ-TmT reduced levels of estradiol below that of the control group in the *in vivo* studies. We utilized high doses of tocopherols and there could be toxic side effects by tocopherols in reducing estrogen levels. To confirm if tocopherols lower estrogen to deficient levels, future studies would include bone density analyses.

Our chemopreventive studies with individual (α-, δ-, γ-) tocopherol and γ-TmT suggest that ERα, PPARγ and the AKT pathway may be important target molecules in the inhibition of ER positive breast cancer. Furthermore, our studies implicate that δ- and γ-tocopherol, but not α-tocopherol can increase protein levels of PPARγ and inhibit tumor growth in a carcinogen-induced mammary carcinogenesis model. As all forms of tocopherols are anti-oxidants, there is still a major issue as to why that δ- and γ-tocopherol are more active than α-tocopherol in cancer prevention studies. Chemical structure of tocopherols may play a role. δ- And γ-tocopherol have an unmethylated 5-position on the chromanol ring which enables them to quench RNS. α-Tocopherol has a methylated 5-position on the chromanol ring which may lead to unstable nitrogen derivatives. Furthermore, α-tocopherol is readily transported back into the blood by the α-tocopherol transporter protein in the liver, while δ- and γ-tocopherol are less effectively transported into the blood and more readily metabolized. The metabolites still retain the chromanol ring structure, and are more water-soluble to travel and may trap ROS and RNS in the cytosol of cells. Thus a major factor between chemical structure and bioavailability may be a cause for the difference in activity of the individual tocopherols.

Many epidemiological studies utilizing α-tocopherol supplementation have resulted in disappointing results. To date, there have been no human studies using other forms of tocopherols. Thus, the purpose of this thesis is to distinguish differences between the different

forms of tocopherols. In our experiments, we utilize a dose of 0.3% individual tocopherol in the diet. This level equates to 3000 mg of individual tocopherol. The upper limit recommended by the FDA is 1000 mg/day of α-tocopherol. As such, we are administering a much higher dose. High doses of α-tocopherol have been shown to decrease serum levels of γ-tocopherol and possibly reduce the efficacy of cancer prevention activity. On the other hand, high doses of γ-tocopherol may also not be suitable since it has been shown to decrease the serum levels of α-tocopherol *in vivo*.

The use of mixed tocopherols (γ-TmT) has a ratio of 58% γ-tocopherol, 24% δ-tocopherol, 13% α-tocopherol and 0.5% β-tocopherol. This ratio equates to 1740 mg of γ-tocopherol, 720 mg δ-tocopherol, 390 mg of α-tocopherol and 150 mg of β-tocopherol. The administration of 0.3% γ-TmT in the diet has slightly lower efficacy when compared to individual γ-tocopherol or δ-tocopherol, suggesting that there may be synergistic effects with a mixture of tocopherol. As γ-TmT is high availability and naturally occurs in the diet, it has a high potential for practical application. Thus, mixtures of tocopherols may have an advantage over pure tocopherols in cancer prevention. Our data will be valuable for future human intervention studies with mixtures of tocopherol in selecting the right dosage forms. Furthermore, we may help design better protocols for human breast cancer studies and determine which subtype of breast cancer tocopherols are most effective.

Conclusion

Breast cancer is a heterogeneous disease and is a continuous problem in the United States. Chemoprevention is a way to inhibit or reverse cancer initiation and promotion by utilizing vitamins, phytochemicals or other agents. Vitamin E is considered a chemopreventive agent and exists as four forms designated α, β, γ and δ. All forms are antioxidants, however, γ-tocopherol and δ-tocopherol are more effective at trapping reactive oxygen and nitrogen species because of the unmethylated carbon at 5-position on the chromanol ring. Furthermore, γ-tocopherol and δ-tocopherol are more readily prone to side chain degradation than α-tocopherol because the α-tocopherol transport protein preferentially transports α-tocopherol into the serum. The resulting hydrophilic metabolites of γ-tocopherol and δ-tocopherol have also exhibited protective biological activities. These differences in tocopherols may contribute to their different chemopreventive activities in breast cancer.

We have investigated the chemopreventive activities of γ-TmT and individual tocopherols (α-, δ-,γ-) in multiple mammary tumorigenesis models. Promising data was shown in the ACI mammary hyperplasia model and NMU-induced breast tumorigenesis model, but not in the MMTV/ErbB2/neu driven model. Nuclear receptors (ERα and PPARγ), cell cycle, and cell survival were major pathways that were regulated by δ-tocopherol and γ-tocopherol. Existing as a natural mixture of tocopherols, γ-TmT may be more practical for cancer prevention and have an advantage over pure tocopherols with synergistic activities. Overall, the results suggest that γ-TmT, δ-tocopherol and γ-tocopherol, but not α-tocopherol, should be considered as anticancer agents for hormone dependent breast cancer.

Future Works

In vivo studies:

In aim 1, we induced mammary hyperplasia in ACI rats with exogenous estrogen. The ACI model is a more biologically relevant model using estrogen as a carcinogen rather than a synthetic DNA damaging agent. Thus, estrogen-induced tumorigenesis in the ACI rat is a useful model in pre-clinical studies. Future experiments for this aim would include long-term studies. Using a slow release silastic implants of estradiol (9 mg), tumors would arise at 24-36 weeks after implantation. Individual tocopherol (α-, δ-, γ-) and γ-TmT would be administered on the same day as the estradiol implantation to study the chemopreventive effects against initiation of mammary tumorigenesis. To understand mammary carcinogenesis, it would be beneficial to examine changes during tumorigenesis development. Multiple time points of 1, 2, 6, 18 and 32 weeks may help determine changes during initiation and promotion of carcinogenesis. At the conclusion of these *in vivo* experiments, we will be able to determine changes in serum estrogen levels, serum inflammatory markers (PGE_2, LTB_4 and 8-isoprostane), changes in mammary hyperplasia and tumors (8-oxo-dG, nitrotyrosine, PCNA, c-Casp3, ERα, PPARγ, PTEN, p-Akt, Nrf2, COX-2 and iNOS) for each time point.

In aim 2, we utilized a NMU-induced mammary tumorigenesis model and treated the rats with dietary tocopherols one week after the carcinogen injection. This methodology examined if there were any inhibition by tocopherols in the promotion stage of tumorigenesis. Future studies with the NMU-induced model may include pre-treatment with dietary tocopherols since the carcinogen NMU exerts its DNA damaging effect within an hour of the i.p. injection [63]. Pre-treatment with tocopherols may give insight on blocking the initiation stage of tumorigenesis and may further inhibit tumor growth. Rather than sacrificing the animals after the development of tumors, it may be beneficial to pre-treat the animals with individual tocopherols and sacrifice the animals 1, 3, 5, 7 and 14 days after NMU-injection to catch early changes of tumorigenesis.

Tocopherols are well known anti-oxidants, and based on our current data, ROS and RNS markers where more evident in the mammary gland rather than the mammary tumor. By examining the earlier time points, there may be marked changes between treatment groups and help determine if tocopherols act by reducing ROS/RNS to reduce inflammation and DNA changes or by being a pro-oxidant and increase levels of ROS/RNS and induce apoptosis in damaged cells. Markers of ROS (8-oxo-dG), RNS (nitrotyrosine), inflammation (COX-2), cell proliferation (PCNA) and apoptosis (c-Casp3) would be examined in the mammary gland.

In aim 3, we utilized female MMTV/ErbB2/neu transgenic mice to examine a more aggressive subtype of breast cancer, HER2 positive. At 3 months of age when the animals reach maturity, we administered 0.3% individual (α-, δ-, γ-) tocopherol or γ-TmT. At the dose administered, we did not observe long-term protective effects by individual tocopherols or γ-TmT. Future studies may include a dose range (0.1%, 0.3%, and 0.5%) to determine if other doses of tocopherols are more protective for long-term studies. Furthermore, tocopherols may be more effective in the initiation and promotion stages of tumorigenesis. As such, it may be beneficial to administer dietary tocopherol treatment before the animal reaches physical maturity (for example at 1 or 2 months of age). In addition, earlier time points may be more beneficial to determine if there are molecular changes throughout the progression of HER2 positive breast tumorigenesis. Since mammary tumors develop between 24-55 weeks of age, time points of 20, 28 and 40 weeks of age will show mammary hyperplasia as well as early stages of tumor formation. HER2 positive breast cancer is often associated with inflammation. Mammary glands and tumors would be analyzed for HER2, p-HER2, COX-2, Nrf2, PCNA and c-Casp3.

In vitro studies:

Analysis of oxidative/nitrosative stress reduction by tocopherols in MCF-7 breast cancer cell line.

Intracellular ROS and RNS will be measured by using fluorescent probes dihydroethidium (DHE) (superoxide, O_2N_2), 2',7'-Dichlorofluorescin diacetate (DCFH-DA) (hydrogen peroxide, H_2O_2), Dihydrorhodamine 123 (DHR-123) (peroxynitrite, $ONOO_2$). DHE measures the generation of ROS by superoxide which leads to the release of membrane-impermeant ethidium cations that fluoresce on intercalating with nuclear DNA [421]. DCFH-DA measures reactive oxygen species formed by hydrogen peroxide. DCFH-DA diffuses through the cell membrane and is hydrolyzed by intracellular esterases and is rapidly oxidized to highly fluorescent dichlorofluorescein [422]. DHR-123 measures RNS produced by peroxynitrite, where it is oxidized and is highly fluorescent [423]. DHE, DCFH-DA, and DHR-123 will be measured in MCF-7 cells when treated without and with 17-β-estradiol. In addition, MCF-7 cells will be co-treated with tocopherols to determine whether tocopherols reduce oxidative and nitrosative stress after 17-β estradiol stimulation.

Furthermore, MCF-7 cells will be co-treated with 17-β-estradiol (10 pM) and individual tocopherols (1-50 μM) and measured for oxidative/nitrosative stress markers, 8-OHdG, γ-H2AX, and nitrotyrosine by flow cytometry or immunocytochemistry. The results from this study will provide information whether tocopherols may inhibit estrogen-mediated DNA damage and oxidative/nitrosative in ER positive cells.

Investigate the mechanism of action of tocopherols in activating Nrf2 in ER positive luminal type human breast cell line (MCF-7).

17-β-estradiol and catechol estrogens may induce oxidative stress which leads to DNA damage. Nrf2 induces phase II and antioxidant enzymes to reduce oxidative stress. We will treat MCF-7 cells with and without 17-β-estradiol and co-treat the cells with individual tocopherols (1-50 μM). Protein and RNA analysis of MCF-7 cells treated with individual tocopherols will help examine whether tocopherol treatment may enhance the expression of Nrf2 and Nrf2-driven genes, such as NQO1, GSTs, UGT, and HO-1. We will transfect ARE-luciferase reporter vector to

perform reporter gene assay to determine its activation by 17-β-estradiol and the effects of co-treatment with tocopherols in MCF-7 breast cancer cell line. We anticipate that δ- and γ-tocopherol, but not α-tocopherol, will activate the ARE-dependent luciferase activity. These results may help infer that there are different modes of actions between the individual forms of tocopherols.

We may utilize Nrf2 siRNA to verify that induction of phase II detoxifying and anti-oxidative enzymes (NQO1, GSTs, HO-1, and SOD) by tocopherols is mediated by Nrf2. This will help determine if tocopherols are dependent on Nrf2 to be effective in estrogen-receptor positive breast cancer. MCF-7 and MCF10A breast cancer cells lines will have a stable knockdown of Nrf2.

Investigate the mechanism of action of tocopherols in activating PPARγ in ER positive luminal type human breast cell line (MCF-7).

Based on preliminary data, δ- and γ-tocopherol are effective in increasing PPARγ level in MCF-7 cells, while α-tocopherol does not [170]. PPARγ signaling may play a role in ER-dependent breast cancer, since PPARγ and ERα physically and functionally interact and ERα negatively regulates peroxisome proliferator response element (PPRE)-mediated transcription activity [355]. In addition, PPARγ and ERα have opposite effects in regulating PI3K/Akt transduction [355]. To determine the effect of tocopherols on PPARγ, MCF-7 cells will be transfected with PPRE-luc and RXRs and treated with estrogen (10 pM) with and without co-treatment of tocopherols (1 to 50 μM). Luciferase activity will be measured. To determine whether there is crosstalk between PPARγ and ERα transduction pathways, co-immunoprecipitation assays will be performed. MCF-7 cells will be treated with estrogen and with or without tocopherols for 24 hrs. The cell lysates will be immunoprecipitated with anti-PPARγ. Western blotting will be followed using antibodies to ERα and p85 (subunit of PI3K).

It has been shown that PPARγ agonist induced PTEN expression via a PPARγ dependent mechanism [372]. As PTEN antagonizes the PI3K/Akt pathway, this may also be an important mechanism of action. We can utilize PPARγ siRNA in MCF-7 and MCF10A breast cancer cell lines to verify if the induction of PTEN by tocopherols is dependent on PPARγ. In addition we may utilize siRNA for ERα to determine which events are dependent on estrogen receptor and determine the role of the different signaling pathway in ER positive breast cancer.

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Appendix:

Table A.1 Antibodies used for Western blot and immunohistochemical analysis

Primary Ab	MW (kDa)	Source	Dilution for Western	Dilution for IHC	Company/ Cat #
8-oxo-dG		Mouse		1:100	JaICA/MOG-020P
Actin	42	Mouse	1:2000		Sigma/A1978
Akt	60	Rabbit	1:1000		Cell Signaling/9272
Akt-p (Ser473)	60	Rabbit	1:1000		Cell Signaling/9271
BCL-2	28	Rabbit	1:200		Santa Cruz/sc-783
c-Caspase 3 (Asp175)	17, 19	Rabbit	1:1000	1:200	Cell Signaling/9661
Catalase	60	Rabbit	1:2000		Epitomics/2058-1
CDK4	30	Mouse	1:1000		Cell Signaling/2906
CDK6	36	Mouse	1:1000		Cell Signaling/2906
Cox-2 (M19)	70-72	Goat		1:500	Santa Cruz/sc-1747
Cyclin D1	37	Rabbit	1:200		Santa Cruz/sc-718
ER α (F-10)	66	Mouse	1:200		Santa Cruz/sc-8002
ER-α	67	Mouse		1:200	ABR/MA3-310
ER β	55	Rabbit	1:500		ABR/PA1-311
Glutathion Peroxidase 1	22	Rabbit	1:1000		abcam/ab22604
Gstm1	26	Rabbit	1:2000		Epitomics/3353
p-H2A.X	15	Rabbit		1:500	Cell Signaling/9718
Heme Oxygenase	32	Rabbit	1:2000		Epitomics/2322
Keap1	69	Rabbit	1:200		Santa Cruz/sc-33569
Myc-c (9E10)	43-55, 67	Mouse	1:200		Santa Cruz/sc-40
Nitrotyrosine	215, 66, 13	Mouse		1:100	Millipore/MAB5404
NQO1	31	Mouse	1:200		Santa Cruz/sc-271116
Nrf2	100	Rabbit		1:1000	Epitomics/2178

Nrf2	55,100	Rabbit	1:500		Santa Cruz/sc-722
p21(F-5)	21	Mouse	1:200		Santa Cruz/sc-6246
p27 (F-8)	27	Mouse	1:200		Santa Cruz/sc-1641
PARP (cleaved)	89	Rabbit	1:1000		Cell Signaling/9541
PCNA	36	Mouse	1:1000	1:1000	BD/610664
PKC a (H-7)	80	Mouse	1:200		Santa Cruz/sc-8393
PPAR γ (E-8)	67	Mouse	1:200		Santa Cruz/sc-7273
PPAR γ	53, 57	Rabbit		1:400	Cell Signaling/2435
PTEN	54	Rabbit	1:1000		Cell Signaling/9552
Superoxide Dismutase 1	17	Rabbit	1:2000		abcam/ab16831
Thioredoxin 1	12	Rabbit	1:1000		Cell Signaling/2429
UGT	55-60	Rabbit	1:1000		Cell Signaling/4371
XIAP	53	Rabbit	1:1000		Cell Signaling/2042

Table A.2 Primers used for quantitative PCR

Primer (gene)	Species	Cat No.
Catalase (Cat)	Rat	Rn00560930_m1
Cdkn1a	Rat	Rn00589996
Chemokine ligand 28 (Ccl28)	Rat	Rn00586715_m1
Comt	Rat	Rn00561037_m1
Cyp1A1	Rat	Rn00487218_m1
Cyp1B1	Rat	Rn00564055_m1
ER α	Rat	Rn01430445_m1
ER β (ESR2)	Rat	Rn00562610_m1
Gapdh	Rat	Rn99999916_s1
Glutamate cystein ligase (Gclm)	Rat	Rn00568900_m1
Glutathion peroxidase 1 (Gpx1)	Rat	Rn00577994_g1
GSTM1	Rat	Rn00755117_m1
Heme oxygenase 1 (Hmox1)	Rat	Rn01536933_m1
KEAP1	Rat	Rn00589292_m1
LOC501110	Rat	Rn01757146_m1
NQO1	Rat	Rn00566528_m1
NRF2	Rat	Rn00756179_m1
PPARγ	Rat	Rn00440945_m1
Superoxide dismutase 1 (Sod1)	Rat	Rn00566938_m1
Thioredixin1 (Txn1)	Rat	Rn00587437_m1
UGT1A1	Rat	Rn00754947_m1