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PREVENTION OF HORMONAL MAMMARY CARCINOGENESIS IN RATS BY DIETARY BERRIES AND ELLAGIC ACID

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ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Nutritional Sciences at the University of Kentucky

By
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Louisville, Kentucky

Director: Dr. Ramesh C.Gupta, Professor of Preventive Medicine
Lexington, Kentucky
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PREVENTION OF HORMONAL MAMMARY-CARCINOGENESIS IN RATS BY DIETARY BERRIES AND ELLAGIC ACID.

Breast cancer is the most frequently diagnosed cancer among women around the world. The hormone 17ß-estradiol (E_2) is strongly implicated as a causative agent in this cancer. Since estrogen acts as a complete carcinogen, agents that interfere with the carcinogenic actions of E_2 are required. Most agents effective against experimental mammary carcinogenesis have been employed as pure compounds disregarding the synergy that exists between several phytonutrients in a whole food. In these studies we have taken a unified approach, by employing a pure phytonutrient – ellagic acid and whole foods that contain the phytonutrient at various levels – berries, in the prevention of E_2-induced mammary cancer in ACI rats. We have also used a tiered approach by screening several phytochemicals *in vitro* and implementing these results in both short- and long-term studies. Initially, several phytochemicals were tested as pure compounds against oxidative DNA damage induced by 4-hydroxy estradiol and $CuCl_2$. Ellagic acid, was the most effective agent (>98% reduction). In a short-term *in vivo* study, both dietary blueberry and strawberry (5% w/w), were ineffective in reducing the baseline oxidative DNA damage in the livers of CD-1 mice. However, red raspberry (5% w/w) was highly effective (50% reduction) and ellagic acid (400 ppm) was moderately effective (25% reduction). Further both diets up-regulated hepatic DNA repair genes in a similar fashion. In a long-term estradiol-induced mammary carcinogenicity study in ACI rats, dietary berries (2.5% w/w) and ellagic acid (400 ppm) reduced both tumor volume and tumors

per animal to different extents (50-75%). One mechanism by which these dietary interventions inhibit mammary tumorigenesis may be via modulation of E_2 metabolism, especially at the early stages of carcinogenesis. At 6 weeks after E_2 treatment both berries and ellagic acid or berries alone significantly offset E_2-induced changes in CYP1B1 and CYP1A1 expressions respectively. In addition, no toxicity or adverse effects are observed when rodents were fed either berries (1 - 5%) or ellagic acid (400 ppm). These data taken collectively support the possibility of using natural foods such as berries as an adjuvant to current pharmacological therapies in the prevention and treatment of breast cancer.

Key words: Chemoprevention, Breast cancer, Berries, Ellagic acid, ACI rats.

Harini S. Aiyer

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This work is dedicated to all my teachers who inspired me towards the highest scholastic achievement possible

Ms. V.Sarala – *for igniting the light of interest.*

“Tuition Mami” Mrs. Jayalakshmi Santhanam – *for believing in me more than anyone ever did.*

Dr. Ramesh Gupta – *for taking a chance on a wild horse and making her worthy of the derby.*

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Chapter One: General introduction

Breast Cancer - Statistics

Breast cancer is the most prevalent cancer among women worldwide (Parkin *et al.*, 2005; Parkin & Fernandez, 2006). The global estimates of cancer are provided by the International Agency for Research on Cancer (http://www-dep.iarc.fr/globocan/database.htm). According to this, the global incidence of cancer is estimated to be almost 11 million and the mortality and prevalence figures are close to 7 and 25 million respectively (Parkin *et al.*, 2005). Breast cancer is the most frequent cancer contributing to 23% of all diagnosed cancers in women. Among the 1.15 million cases diagnosed globally, 31.3% (230,000) are in North America, which has the highest age-standardized incidence (99.4 per 100,000) (Table 1.1). Generally, it is seen that the incidence of breast cancer is higher in developed countries compared to the developing nations (Figure 1.1), which is attributed to combined influence of differences in lifestyle, hereditary factors and screening practices, etc,. (Althuis *et al.*, 2005). The mortality rate for breast cancer is the fifth highest ranking behind lung, stomach, liver and colon cancers. Breast cancer ranks as the most prevalent cancer among all cancers (17.9%) due to its good prognosis. The average survival rates for women with breast cancer are 73% and 57% for developed and developing nations respectively (Parkin *et al.*, 2005). The incidence and mortality of breast cancer in the United States are 213,000 and 41,000 respectively (ACS, 2007a). The 5-year survival rate after diagnosis of a localized breast cancer is 98%. Currently, there are over 2 million breast cancer survivors in the United States (ACS, 2007b; ACS, 2007a). However, this survival rate varies with age, stage of tumor at diagnosis, race/ethnicity and socioeconomic status of the patients (ACS, 2007b; ACS, 2007a). In Kentucky, over 3000 cases of breast cancer are expected to be diagnosed, with a 20% mortality rate (ACS, 2007b; ACS, 2007a). These global, national and regional statistics make breast cancer a primary public health concern for women.

Epidemiology

Epidemiology is the study of patterns, causes and control of a disease in a given population. Epidemiological studies are of 3 types – (1) Retrospective or case-control studies that look at the differences in risks between 2 populations that are with (cases) and without (controls) a particular disease; (2) prospective or cohort studies that involve study of outcomes of a particular disease in a given set of population observed over a period of time; and (3) cross-sectional studies that measure the prevalence of a disease in a particular population (Woodward, 2005).

Epidemiological studies of breast cancer indicate at least two distinguishable types of breast cancer. About 10% of all breast cancers diagnosed can be attributed to a familial or hereditary cause. Several genes have been linked firmly to the disease and are discussed in some detail below. However, about 90% of breast cancers diagnosed is termed as sporadic (Figure 1.2), which means that although there are several risk factors that elevate the risk of developing the disease, one major cause cannot be singled out. Sporadic cancer is thought to occur due to interactions between various risk factors.

The hereditary or familial breast cancer presents with germline mutations in certain genes that are passed on from one generation to another. Certain families or populations such as the Ashkenazi Jews, are at very high risk of breast cancer due to the high prevalence of these genetic mutations (Brinton *et al.*, 2002; Thompson & Easton, 2004). Among these mutations, the most common are the BRCA 1 and BRCA 2 mutations. These high-penetrance genes are mutated in about 65% of all familial breast cancers diagnosed (Studzinski & Harrison, 2002). The mutated gene produces an inactive protein that raises the risk of ever having breast cancer from about 3% at age 30 to 85% at age 70 (Studzinski & Harrison, 2002). Specific mutations that are prevalent in up to 2.5% of the Askenazi Jewish population have been identified (Studzinski & Harrison, 2002). The gene p53 is involved in cell-cycle arrest. Somatic mutations in this gene are present in 50% of all cancers and about 15-30% of breast cancers.

However, germline mutations in this gene are rare and associated with the Li-Fraumeni syndrome, which presents with early onset tumors in multiple organs including the breast. p53 mutations contributes to about 1% of all familial breast cancer cases (Figure 1.2). Another such syndrome is Cowden's syndrome with germline mutations in the PTEN gene. More recently, mutations in the cell-cycle check point gene-CHEK2 have been associated to familial breast cancer, not linked to BRCA 1 or BRCA 2 mutations (Vahteristo *et al.*, 2002; Oldenburg *et al.*, 2003; Thompson & Easton, 2004).

A great majority (90%) of breast cancer is considered sporadic since no single risk factor can be clearly attributed to causation and is thought to be caused by the interactions between multiple risk factors. Epidemiological studies have linked several risk factors in the etiology of breast cancer. These risk factors can be broadly classified into 3 categories (Table 1.2):

1. Non-modifiable risk factors such as age, gender, race/ethnicity, genetic polymorphisms, familial history, and previous breast history.

2. Modifiable or lifestyle risk factors that include diet, exercise, body weight, alcohol, and smoking.

3. Hormonal risk factors, including age at menarche and menopause, parity, breast feeding, and hormone-replacement therapy (HRT).

Non-modifiable risk factors.

Age is considered the foremost non-modifiable risk factor for any cancer. For women, the lifetime risk of developing cancer is slightly more than 1 in 3 (ACS, 2007b). Both the incidence and mortality due to breast cancer increase with age, with the median age of diagnosis being 61 years (ACS, 2007a). Women between the ages of 75 -79 have the highest incidence, while those from 20 to 24 have the lowest (ACS, 2007b). Women 50 and older have the highest rate of both invasive cancers and carcinomas *in-situ*. Age also affects survival and mortality trends after diagnosis. The rate of decline in mortality attributed to better treatment was only 2% for women older than 50 compared to 3.3% for

those under (ACS, 2007b). Conversely, the 5 year survival rate is 89% for women 40-74 compared to only 82% in those under 40. Female gender is a risk factor by default. However, up to 1% of breast cancer occurs in males which are usually linked to inherited traits (Studzinski & Harrison, 2002; ACS, 2007a).

It is known that African-American women have a lower incidence of breast cancer than white women, but they are more likely to die of the disease at every age (Bowen *et al.*, 2006; Smigal *et al.*, 2006). Other races and ethnicities have recorded a much lower incidence rates. African-American women also have a lower 5-year survival compared to Caucasians (76% versus 90%); this difference is attributed to biological differences in cancer types, later stage at diagnosis, poorer stage-specific survival, lack of disease awareness and socio-economic reasons (Eley *et al.*, 1994; Elledge *et al.*, 1994; Klauber-DeMore *et al.*, 2006).

Family history has been strongly linked to the incidence of breast cancer in women. It is known that a woman's risk increases linearly with the number of first-degree relatives diagnosed (McPherson *et al.*, 2000; ACS, 2007a). The reason for this linkage is manifold, including genetic mutations that occur at a very high rate in certain families, common environment, etc., (McPherson *et al.*, 2000; Mucci *et al.*, 2001). Mutations in the Ataxia Telangiectasia gene has been implicated in increased risk for breast cancer, although mutations in this gene by itself do not seem to significantly increase breast cancer risk (Ellisen & Haber, 1998). Other genes that are inherited as germline mutations and may confer a familial risk have been discussed earlier.

Other than the high-penetrance genes, genetic polymorphisms in several low penetrance genes confer a small to moderate risk to carriers. Although their risk-effect is low compared to high-penetrance genes, these variants are more common in the general population and hence they confer a much higher Population Attributable Risk (Nathanson & Weber, 2001). They include proto-oncogenes, tumor-suppressor genes and genes involved in cell-signaling, DNA repair, carcinogen metabolism, etc., (de Jong *et al.*, 2002). Such low penetrance

genes may play an important role in synergistically increasing the risk with an environmental risk factor such as smoking (Dunning *et al.*, 1999).

A woman's breast history plays an important role in the etiology of breast cancer. A benign proliferative change in the breast such as atypical hyperplasia is associated with a four-fold increase in cancer risk later in life (Colditz *et al.*, 1993)). Other changes in the mammary epithelium are associated with a slight non-significant increase in risk (McPherson *et al.*, 2000).

Modifiable risk factors.

Diet is a very important modifiable risk factor. Diet is a complex mixture of both carcinogens that enhance and protective factors that reduce risk. The role of diet in both cancer causation and prevention is discussed in detail later. Epidemiological studies have linked a high meat intake with increased breast cancer risk (Cho *et al.*, 2006). Indeed, chemicals present in meat such as heterocyclic aromatic amines (HAA) and polycyclic aromatic hydrocarbons (PAH), have been found to be carcinogenic in rodent models (Table 1.5) (Huggins *et al.*, 1961; Snyderwine *et al.*, 1998). In addition, DNA adducts presumably derived from these chemicals have been found in breast biopsies of women diagnosed with breast cancer (Li *et al.*, 1999), suggesting a causative link between these dietary carcinogens and breast cancer. In addition to chemical carcinogens, the role of dietary fat has been studied extensively in causation of breast cancer. Although, several epidemiological studies showed a positive correlation between high fat intake and breast cancer incidence (Hursting *et al.*, 1990; Cho *et al.*, 2006), larger analyses of data suggest that there may be no significant correlations (Smith-Warner *et al.*, 2001; Wakai *et al.*, 2005). However, recent concerns regarding recall-bias of actual dietary intakes have been reported (Prentice, 1996; Gonzalez, 2006a). If this is validated then re-analysis of previously reported data may yet again yield different results. Nevertheless, key risk factors, such as age at menarche, body weight and body fat content, are

influenced by diet and hence diet plays a key role in determining breast cancer risk (Marchant, 1982).

Obesity is associated with a two-fold increase in the risk for breast cancer in postmenopausal women (McPherson *et al.*, 2000). Both adult weight gain and increased waist to hip ratio are associated with increase in incidence risk (Brekelmans, 2003; ACS, 2007a). For women with a Body Mass Index (BMI) >25 (Normal- 18-25) the mortality risk from breast cancer are 1.3 to 2.1- times higher (ACS, 2007a). After menopause visceral fat stores are a major site for production of estrogens, which reflects in the increased risk (Simpson *et al.*, 1999; Lorincz & Sukumar, 2006).

Regular physical activity has been shown to reduce the risk of breast cancer among post-menopausal women (ACS, 2007a). This protective effect is additive when present along with parity and a normal BMI (Thune *et al.*, 1997). Although the mechanisms are not well defined the effects are thought to be induced by the effect of exercise on energy balance and hormones (Doll, 1996; Bentz *et al.*, 2005; McTiernan *et al.*, 2006).

Alcohol consumption increases breast cancer risk. The consumption of more than 24 g alcohol (two drinks a day) increases risk by 21% and there is a dose-dependant correlation between alcohol intake and breast cancer risk thereafter (Hamajima *et al.*, 2002). Since alcohol consumption and smoking often co-exist, it is seen that alcohol consumption can substantially confound the effect of smoking on breast cancer (Hamajima *et al.*, 2002). Alcohol is known to affect the metabolism of steroid hormones and thus increase breast cancer risk (Purohit, 2000; Singletary & Gapstur, 2001; Pierucci-Lagha *et al.*, 2006).

The correlation between both active and passive smoking and breast cancer remains inconclusive and highly debated. The report published by the Collaborative Group on Hormonal factors in Breast Cancer, which looked at 60,000 cases and 100,000 controls, suggests that there is no association

between smoking behaviors and breast cancer (Hamajima *et al.*, 2002), although there have been many smaller reports that contrast this (Chaturvedi, 2003). However, recently a study published by Reynolds and coworkers suggest that there are significant correlations between smoking and other lifestyle factors (Reynolds *et al.*, 2004). It was found that current-smokers tended to have a less healthy lifestyle than non-smokers and hence may be more susceptible to the effects of other risk factors. Associations between passive smoking and breast cancer risk are inconclusive (Lee & Hamling, 2006).

Ionizing radiation is also known to increase the risk of breast cancer. Studies among women exposed to nuclear radiation and also those exposed to excessive x-ray radiation especially at a young age (Sigdestad *et al.*, 2002), suggests that there is a correlation between radiation and breast cancer.

Hormonal risk factors

The mammary glands are under the constant influence of several hormones throughout the lifetime. Hence, hormonal factors play a major role in the causation of breast cancer. The mammary gland is highly responsive to hormonal influences. The developing mammary gland is under the endocrine influence of organs such as the pituitary, the ovary and the adrenals (Vonderhaar, 1988). In addition, the paracrine regulation by stromal cells is also involved (Cunha & Hom, 1996; Wiseman & Werb, 2002). The mammary gland development is, mostly but not exclusively, affected by 3 major hormones: estrogen, progesterone and prolactin. Estrogen and progesterone play an important role in the development of the mammary glands in non-parous women (Anderson, 2002). Prolactin, secreted by the anterior pituitary, plays a significant role in the development of the mammary gland during pregnancy and prior to lactation (Kelly *et al.*, 2002).

Estrogen is produced primarily by the ovaries in response to endocrine stimulus from the pituitary (Jones & DeCherney, 2003). The mammary gland is

constantly under the effect of estrogen. During each estrus cycle, the mammary ducts undergo proliferative changes during the follicular phase when the circulating levels of estrogen are higher (Schedin *et al.*, 2000). Estrogen exposure over lifetime is considered a significant risk factor for the development of breast cancer (Lippman *et al.*, 2001). This is supported by the increased incidence of breast cancer in women with early menarche and/or late menopause, resulting in a higher cumulative exposure to the ovarian hormone (Hsieh *et al.*, 1990). Also, women with serum estradiol levels in the highest tertile ($\geqslant$12 pmol/L) had a 2-fold higher risk of developing breast cancer than those in the lower tertile (<12 pmol/L) (Lippman *et al.*, 2001). Menarche and menopause are determined both genetically and by environmental factors (Graber *et al.*, 1995; Petridou *et al.*, 1996). The role of ovarian hormones in breast cancer risk is further substantiated by the fact that women who have either uni- or bi-lateral oophorectomy have a reduced risk of breast cancer, which directly implicates ovarian hormones in the development of breast cancer (Parazzini *et al.*, 1997).

Progesterone is another ovarian hormone that affects breast development. Progesterone is mainly secreted during the luteal phase of the estrus cycle. The link between exposure to endogenous progesterone and breast cancer has not been clearly defined, however, the exposure to endogenous progesterone would be proportional to the total number of menstrual cycles that a woman has in her lifetime. There is some indication that the average serum progesterone levels increases with age, but a correlation to breast cancer has not been established (Garcia-Closas *et al.*, 2002). Nevertheless, the link between exogenous progesterone and breast cancer risk has been explored with regard to HRT. Recent results from the Million Women Study, a study with the largest cohort as yet, done in the UK, shows a clear association between HRT use and increased breast cancer risk. They report that HRT increases the risk of both incident and fatal breast cancer risk and that this risk is higher for a combination therapy of estrogen and progesterone than for estrogen alone (Beral, 2003). This corroborates an earlier finding by the Collaborative Group on Hormonal Factors

in Breast Cancer (Lancet, 1997). Thus, progesterones may also be involved in the development of breast cancer.

Prolactin, secreted by the anterior pituitary has a significant role in the development of the mammary gland both before and during lactation (Topper & Freeman, 1980). The undifferentiated lobules in the non-parous mammary gland undergo both development and differentiation under the influence of prolactin (Horseman, 1999). The reduced risk of breast cancer caused by parity can be attributed to this differentiation. Breasts of parous women contain more differentiated lobules (Lob 2 and 3) compared to non-parous women which contain mostly undifferentiated structures (Russo *et al.*, 2001). Indeed, an early first full-term pregnancy is associated with a reduced risk of breast cancer as are multiple pregnancies (Rosner *et al.*, 1994). However, the protection decreases with increasing age of first pregnancy. It is also seen that lactation is associated with moderately reduced risk of breast cancer (Velie *et al.*, 2005) .

It is clear that sporadic breast cancer arises due to interplay between various risk factors. The hormonal milieu during a woman's lifetime affects both initiating as well as protective factors of breast carcinogenesis. To this end it is required to explore in detail the effect of hormones on breast cancer development and their interactions with other risk factors such as alcohol, body weight and diet.

Hormonal control of mammary gland development.

In order to understand the role of hormones in cancer causation, it is necessary to comprehend their role in the normal development of the mammary gland. The mammary gland is highly complex tissue composed of different cell types such as epithelial cells, stromal cells and adipocytes, that are both dependant on and responsive to endocrine control throughout development (Topper & Freeman, 1980). The role of cell-to-cell interactions, autocrine and paracrine controls of growth are of paramount importance in this tissue. Most studies on mammary gland development are based on rodent studies (Shyamala

et al., 2002). The development of mammary gland occurs discontinuously throughout lifetime, with major developmental stages being puberty, pregnancy and lactation (Vonderhaar, 1988; Shyamala *et al.*, 2002). At birth, the mammary gland largely consists of rudimentary ducts consisting of a layer each of luminal epithelial cells and myoepithelial cells surrounded primarily by connective tissue (Vonderhaar, 1988). Initial development of the mammary gland takes place during and after puberty (Vonderhaar, 1988). Puberty is marked by both a dichotomous and sympodial growth and branching of the rudimentary ducts (Russo *et al.*, 2001). There is also a corresponding change in the stromal cells, fat pads and other anatomical structures which contribute towards breast development. With the initiation of menstrual cycles, the cyclic release of the ovarian hormones imparts a gradual and constant growth in the mammary gland (Vonderhaar, 1988; Shyamala *et al.*, 2002). This proliferative process continues until the age of 35 when it reaches a plateau (Russo *et al.*, 2001). The two important ovarian hormones that affect breast development are estrogens and progestins. The active forms of estrogens are estrone, estradiol and estriol and of progestins are progesterone and 17-α hydroxy progesterone. The key effects of various hormones on mammary gland development are summarized in table 1.3.

Estrogens are known to cause proliferation of the mammary epithelial cells during each estrus cycle. After puberty, there is a gradual and extensive growth of the mammary ducts under the monthly influence of estrogen (Shyamala *et al.*, 2000). At the end of puberty, around the age of 15, a woman's breast consists primarily of undifferentiated lobules type 1 (Lob 1) (reviewed in Russo *et al.*, 2001). It is understood that many of these lobules do not undergo any differentiation in non-parous women, making these a prone target for transformation by carcinogens.

Much knowledge about the role of estrogen-receptors (ERs) in mammary gland development has come from rodent studies involving estrogen-receptor α null phenotype mice (αERKO) and others. Although a greater proportion of actively dividing cells in the terminal end buds (TEB) have been found to be ER-

negative (Zeps *et al.*, 1998; Russo *et al.*, 2001), there is a small proportion of ER-positive luminal cells that seem to control growth and proliferation of others in a paracrine fashion (Mueller *et al.*, 2002; Shyamala *et al.*, 2002). ERα is necessary for normal ductal development (Bocchinfuso *et al.*, 2000), its expression varies in the different cell types and is down regulated by estrogen in normal cells (Anderson, 2002; Shyamala *et al.*, 2002). Also, the presence of ERα in both stromal cells and epithelial cells are required for normal development, indicating the importance of cell-to-cell communication and paracrine controls involved in the development of mammary ductal structures (Mueller *et al.*, 2002).

Progesterone predominates the luteal phase of the menstrual cycle and plays an important role in mammary gland development. It primarily acts via 2 isoforms of its receptor PRA and PRB (Shyamala *et al.*, 2000). These isoforms are induced by estrogen (Shyamala *et al.*, 2002), suggesting that actions of both steroid hormones may be tightly coupled. It acts on cells already primed by estrogen exposure to cause some maturational growth during each cycle. While estrogen is considered important in the development of the ductal structure, progesterone seems to play a role in the development of the lobulo-alveolar structures, suggesting that it may play an important role in the differentiation of the mammary epithelial cells (Shyamala, 1999). The ratio of the two isoforms plays a major role in both normal mammary development as well as carcinogenesis (Osborne *et al.*, 2005).

Prolactin (PRL) is a peptide hormone secreted by the lactotrophs of the anterior pituitary (Barrett, 2003). It is a 23 kdA protein that acts via its transmembrane-receptor (PRLR) (Hennighausen *et al.*, 1997). Prolactin affects ductal side branching and TEB regression during normal mammary morphogenesis (Brisken *et al.*, 1999) and during pregnancy causes development of lobulo-alveolar structures involved in and for the post-partum milk production (Hennighausen *et al.*, 1997; Brisken *et al.*, 1999). Apart from its influence on the mammary gland, PRL also affects the ovary and the immune system (Hennighausen *et al.*, 1997).

Testosterone is the precursor hormone to estradiol and is converted to the product by the enzyme aromatase. Although, the direct effect of testosterone on mammary gland development is not clear, aromatase is a ubiquitous enzyme found in many tissues, especially tissues most responsive to estradiol indicating that testosterone plays an indirect role in mammary gland development (Hinshelwood & Mendelson, 2001). It has been suggested that growth hormone may indirectly affect ductal development during puberty via the stromal compartment (Kelly *et al.*, 2002; Wiseman & Werb, 2002).

In addition to the endocrine control, paracrine control by the stromal compartment plays a prominent role in mammary gland development (Cunha *et al.*, 2004) and is mediated through the action of growth factors such as EGF, TGF, IGF, CSF and MDGF etc., (Vonderhaar, 1988; Wiseman & Werb, 2002). It is considered that these paracrine and intracrine effects of the stromal compartment may play a critical role in breast carcinogenesis (Shekhar *et al.*, 2001; Wiseman & Werb, 2002).

Metabolomics of steroid hormones

To understand clearly the extent to which the human organism is dependant on steroid hormones, one has to only look at the expression of steroid receptors in the different organs. At least one type of ER can be found in every organ in the body (Balfe *et al.*, 2004). Also, several tissues in the body are capable of synthesizing steroids, especially estrogen through *de-novo* synthesis (Simpson, 2003). Hence, it is very important to know the metabolic pathways, which include synthesis, activation and detoxification pathways of steroid hormones to elucidate their role in a dysregulated state such as cancer. The following section will discuss the metabolomics of primarily estradiol and to smaller extent progesterone, the two steroids implicated in breast carcinogenesis.

The synthesis of 17ß-estradiol, the primary estrogen in the human body, begins with the enzyme aromatase, which converts androgen precursors to estrogens (Figure 1.3). In pre-menopausal women, this conversion occurs primarily in the ovary, but to varying extents in other organs (Simpson *et al.*, 1994). However, in men and post-menopausal women, this conversion is the primary source of estrogen for the various target tissues (Simpson, 2004). This is supported by the presence of aromatase expression in several tissues in humans and the highest aromatase expression among various mammalian species (Bulun *et al.*, 2005). The second pertinent enzyme, also expressed in all tissues is 17ß-hydroxysteroid dehydrogenase (17ßHSD) that interconverts estrone and estradiol. So far, 8 isozymes have been discovered and have specificities for conversion between estrone and estradiol. Types 1, 3, 5 and 7 catalyze the reduction of estrone to estradiol in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor and types 2, 4 , 8 in humans and 6 in rats catalyse the oxidative reaction using nicotinamide adenine dinucleotide (NADH) as cofactor (Reviewed by Luu-The, 2001). In many ways this is also a key enzyme as the presence of a particular isozyme decides the balance between the levels of a weak and a strong estrogen. Estrogen-sulpho transferases (EST) and estrogen sulphatases (STS) also take part in maintaining a mobile pool of estradiol by catalyzing either the forward or the reverse sulphonation reaction respectively (Sasano *et al.*, 2006).

Estradiol is further activated via hydroxylation at various positions by the phase I cytochrome P450 enzymes (Zhu & Conney, 1998). Of the different extra-hepatic P450s important in estrogen metabolism, CYP1A1 and 1B1 are present in the mammary and produce 2- and 4- hydroxy metabolites, known as catechols, respectively (Zhu & Conney, 1998; Liehr, 2000). These hydroxylated metabolites are further either glucuronidated (by UDP-glucuronosyl transferase), methylated (by catechol-O-methyl transferase) or glutathione conjutated (by glutathione-S-transferase), by the phase II enzymes (Zhu & Conney, 1998). Several of the enzymes involved in the synthesis and metabolism of estradiol are under its transcriptional control, via the ER pathway, indicating that estradiol can

affect its production and removal (Beischlag & Perdew, 2005; Sissung *et al.*, 2006). The recent identification of differences in progesterone receptors as well as metabolites, between the normal and tumor breast suggest that estradiol metabolism may not be singularly responsible for mammary tumorigenesis (Aupperlee *et al.*, 2005; Wiebe *et al.*, 2005).

Although the metabolism of steroids in the mammary is an important aspect of the development of mammary carcinogenesis, the involvement of other organ systems such as the liver, lung, kidney, adrenals, ovary and the pituitary, all of which as involved in either steroid production, removal or signaling must be taken in to consideration. It has to be stressed that changes in any of other organ systems can set forth a domino-effect that can ultimately affect the breast.

Role of oxidative DNA damage in breast cancer

Oxygen plays a key role in the metabolism of a cell; it is the final acceptor in the electron transport chain. Thus, cellular metabolism gives rise to a set of highly reactive molecules known as the reactive oxygen species (ROS), which include superoxide (O_2^-), singlet oxygen ($^{\bullet}O$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) (Waris & Ahsan, 2006). ROS can cause damage to cellular macromolecules such as DNA, RNA, proteins and lipids, altering their structure and hence function. The cell has particular defense mechanisms to protect itself against oxidative damage and the ultimate oxidative state of the cell is dependant on the production and removal of ROS. Oxidative stress is caused when there is a disruption in this balance. Both experimental and epidemiological evidence suggests that oxidative stress plays an important role in the development of many cancers including breast cancer. Several carcinogens are known to be pro-oxidant (Morris & Seifter, 1992). Also, depletion of antioxidant defense systems and induction of oxidative stress contributes to carcinogenesis in experimental animal models (Van Remmen *et al.*, 2003). Epidemiological risk factors such as smoking, alcohol consumption, hormonal exposure, high fat intake, etc., are known to indirectly increase oxidative stress (Ambrosone *et al.*, 2003).

The key effects of oxidative stress on a cell can be summarized as follows; damage to cellular macromolecules, alteration of cell signaling pathways, and causation and maintenance of neoplastic changes. Oxidative DNA damage includes oxidation of purine and pyrimidine bases, formation of abasic sites, strand breaks and microsatellite instability (Cavalieri *et al.*, 2000; Rizzati *et al.*, 2005). In case of proteins it results in altered function, and in case of lipids it may cause a chain of lipid-peroxidative events. Lipid peroxidation end products such and malondialdehyde (MDA) and 4-hydroxy-nonenol (4-HNE) can cause further DNA damage (Bartsch & Nair, 2005).

The NF-κB pathway plays a central role in oxidative stress-mediated changes in cellular signaling. It links several pathways that influence growth, stress response, and apoptosis, hence is key to the survival of both normal and cancer cells. ROS is an activator of NF-κB, which is found to be activated in several transformed cell lines, primary and invasive tumors (Wu & Kral, 2005; Biswas & Iglehart, 2006). Also, this pathway is associated with inflammation and this can be activated by several cytokines (Bubici *et al.*, 2006; Liu & Malik, 2006). The downstream effects include increased transcriptional activation, increased cell proliferation and evasion of apoptosis (Karin *et al.*, 2002).

The oxidative stress induced by catechol-estrogen metabolites have been implicated in estrogen-induced carcinogenesis (Yager, 2000). Either 17β-estradiol alone or a combination of a strong oxidant (menadione) and weak estrogen (ethinyl estradiol) induce renal cell carcinomas and increase the levels of 8-iso-prostaglandin $F_{2\alpha}$, an oxidative-stress biomarker (Bhat *et al.*, 2003). Further reports that catechol-estrogens can cause oxidative DNA damage *in vitro* in the presence of transition metal ions (Li *et al.*, 1994; Hiraku *et al.*, 2001; Aiyer *et al.*, 2002) designates a role for catechol-estrogens in inducing oxidative stress.

The oxidative status of the cell also dictates the progression stage of cancer. Although, hypoxia is known to play a major role in angiogenesis and neo-vascularisation of the tumor, ROS signaling may play a role in the differentiation of embryonic stem cells into a cardiovascular lineage which upon confrontation

with tumor tissue may participate in tumor-related angiogenesis (Sauer & Wartenberg, 2005). On the other hand, ROS has also been shown inhibitory to tumor-related angiogenesis, probably via antagonism of hypoxia-inducibe factor (HIF) pathway which is pro-angiogenic (Maxwell, 2005).

Multi-stage model of carcinogenesis

Carcinogenesis is a highly dynamic process by which normal cells are transformed in to neoplastic cells. This involves an inducting event by a physical, chemical or biological agent, subsequent transformation and clonal expansion into a tumor, followed by angiogenesis and metastasis of the tumor (Pitot & Dragan, 1991). The multi-stage model, which is a composite of many theories, explains the process of carcinogenesis in the most comprehensive manner. Although the development of cancer typically involves multiple steps, it can be broadly classified in to 3 systematic stages and the study of the mechanism of and interaction between these stages provides us with an opportunity to interrupt, control and reverse the carcinogenic process (Figure 1.4). An initiating event causes heritable genetic changes in the genome of a cell. This damage could be caused by endogenous agents such as free radicals or exogenous carcinogens. These genetic changes, which are irreversible, include gene mutations, chromosome rearrangement, gene amplification and aneuploidy (Barrett, 1993). Gene mutations can affect 2 key types of genes that control cell division and death, the proto-oncogenes and the tumor-suppressor genes. Proto-oncogenes are genes whose products allow for uncontrolled multiplication of a cell, while tumor suppressor gene products check growth and are involved in apoptosis. In a normal cell, the oncogenes are usually suppressed; a gain-of-function mutation or hypomethylation activates these genes (Ehrlich, 2002). On the other hand a loss-of-function mutation or hypermethylation is responsible for the suppression of the normally active tumor suppressor gene (Jones & Laird, 1999). It is believed that both of these events must occur simultaneously for a cell to gain a growth advantage (Knudson's two-hit hypothesis) (Knudson, 2001). Although the

two-hit hypothesis holds true for most cancers, it is often seen that multiple genes (3-10) are mutated in most adult malignancies (Barrett, 1993).

Studies in chemical carcinogenesis delineate the role for metabolic activation in the carcinogenicity of a given substance (Lijinsky, 1979).This further lead to the classification of carcinogens as being either genotoxic or epigenetic depending upon their action. Genotoxic carcinogens are those which either by themselves or through their metabolites can induce karyotic changes (Weisburger & Williams, 2000). Epigenetic carcinogens on the other hand cannot induce genomic damage; however act in a growth enhancing manner on the transformed cells (Pitot & Dragan, 1991).

In a comprehensive review, Hanahan and Weinberg (2000) discuss the alterations and molecular mechanisms that initiated cells must undergo in order to become malignant. To summarize, the promotional stage, usually defined as a reversible stage in classic chemical carcinogenesis may involve evasion of apoptosis and uninhibited cell growth in the presence of endogenous or exogenous growth factors. As the neoplasm proceeds, the cells acquire self sufficiency in growth and overcome inhibitory signals and immune surveillance, followed by angiogenesis and invasion of host tissue. To paraphrase the authors, tumor growth may be evolution gone awry.

It is necessary to apply the multi-stage model to breast carcinogenesis, as this malignancy can be caused by an endogenous carcinogen (estrogen); may involve activation of the carcinogen and other cellular and molecular changes best explained by the current model. Also, this model provides us with the opportunity to intervene at different stages to prevent, reverse or modify aberrant changes with preventive agents.

Estrogen as a complete carcinogen

Of the risk factors associated with breast cancer, estrogen exposure has the highest positive correlation to incidence. This is corroborated by 1) women

with high serum levels of estradiol have a higher risk, 2) age at menarche and menopause, which are correlates of cumulative estrogen exposure determine risk , 3) removal of the ovary before or after breast cancer incidence results in a more positive outcome, 4) high levels of tissue estradiol are found in breast tumor biopsies, pointing to either accumulation or *in-situ* synthesis, 5) treatment with anti-estrogens such as tamoxifen significantly reduces tumor recurrence .

Estrogen acts via 2 distinct pathways, both of which are equally important for its carcinogenic activity. First, estrogen causes genotoxicity via its metabolic pathway. Data for the support of this comes from numerous findings, extensively reviewed by Liehr (Liehr, 2000; Liehr, 2001) and can be summarized as follows. Pharmacological doses of estrogen induce renal-cell carcinomas in hamsters and mammary adenocarcinomas in ACI rats in the absence of other carcinogens (Li *et al.*, 1983; Shull *et al.*, 1997). Estradiol by itself can induce chromosomal aberrations in cell culture similar to those seen in estrogen induced tumors (Liehr, 2000; Li *et al.*, 2002a). Catechol estrogens, which are active metabolites of estrogen, can induce DNA damage causing both stable and unstable adducts (Cavalieri *et al.*, 2000; Liehr, 2000). Further, enzymes that convert estrogen to its catechol metabolites, such as CYP1B1, are found in high levels in breast tumors and microsomes from breast tumor tissues are known to metabolise estrogen to potentially harmful catechols (Liehr & Ricci, 1996; Oyama *et al.*, 2005). In addition, polymorphisms that increase the metabolic activity of phase I enzymes that activate and lower the activity of phase II enzymes that detoxify estrogen, are known to increase breast cancer risk (Thompson & Ambrosone, 2000). These facts evidence the importance of estrogen metabolism in the causation of cancer.

The other important pathway crucial for estrogen carcinogenicity is its role as a growth factor via steroid receptor signaling. The 2 types of estrogen receptors- ERα and ERß have been extensively studied. Of these, ERα is considered a diagnostic marker and an indicator of response to anti-estrogen therapy, in breast cancer (Balfe *et al.*, 2004). The mechanisms by which these

receptors regulate cellular pathways in normal and malignant cells are numerous. However, these can be broadly classified into direct transcriptional activation after ligand binding and protein-protien interaction with other transcription modulators, with or without ligand binding (Platet *et al.*, 2004). Estrogen is proliferative primarily through its action on ERα, which is also required for normal development and differentiation of the mammary gland (Dickson & Stancel, 2000). The role of ERß as an inhibitor of this proliferative stimulus has been explored leading to the conclusion the ERß may play a protective role in breast cancer (Balfe *et al.*, 2004; Paruthiyil *et al.*, 2004). On the other hand, Russo and colleagues have generated invasive characteristics in an ERα-/ERß+ cell line by estrogen treatment (Russo *et al.*, 2002). Further, it has been shown *in vitro* that estrogen may inhibit invasiveness and the correlation that ERα+ tumors are less invasive suggest that estrogen may play a protective role in breast cancer (Platet *et al.*, 2004). Also, the protein interactions of ERs have broad specificities and are further controlled by intarcrine and paracrine signaling molecules (Cunha *et al.*, 2000; Simard & Gingras, 2001; Wiseman, 2005; Clarke, 2006). These facts lead us to the conclusion that although the regulation of mammary tumorigenesis by the estrogen is a highly complex process and estrogen has a high potential to act as a complete carcinogen.

Role of diet in the causation and prevention of breast cancer

Diet is a complex mixture of both harmful and protective agents. The balance between these agents can modify a woman's risk for developing breast cancer. A typical Western diet is predominantly high is animal products and low in plant products (Cordain *et al.*, 2005). Particularly, the post-industrial revolution changes in both agricultural practices as well as food-processing methods have given rise to a diet that leads to several chronic diseases, including cancer (Cordain *et al.*, 2005).

Several epidemiological studies have explored the association between breast cancer risk and intake of foods such as red meat and high fat (Barrett-Connor & Friedlander, 1993; Cho *et al.*, 2003; Cho *et al.*, 2006). Rodent studies

have significantly implicated fats, a high percentage of calories from fat and several red-meat carcinogens in the development of mammary cancer (Welsch, 1992; Snyderwine *et al.*, 2002). Although for red-meat, epidemiological studies corroborate experimental studies; for dietary fat, such associations are not clear (Gonzalez, 2006b; Kim *et al.*, 2006). However, there is concern about biases and errors in methods used for recall such as the food frequency questionnaire etc., which modify the outcome of such studies (Prentice, 1996; Gonzalez, 2006b). Thus, re-analysis of the same data after adjusting for these may as yet yield different results. Also, increased red-meat intake is often associated with higher intake of saturated fats (from animal sources) resulting in a synergistic effect between the 2 risk factors. The method by which the food is cooked has a profound effect on its nutritional value. High-temperature cooking of meats leads to the pyrolysis and protein degradation products such as HAAs and polycyclic aromatic hydrocarbons (PAHs), that are carcinogenic (Ferguson, 2002; Sinha, 2002; Snyderwine *et al.*, 2002; Cross & Sinha, 2004).

The role of dietary factors in the prevention of various cancers has been studied for several decades (Doll, 1996). Studies clearly indicate the protective effects of fruits and vegetables in several cancers (Block *et al.*, 1992; Helzlsouer *et al.*, 1994; Freudenheim *et al.*, 1996). Furthermore, programs to promote consumption of atleast 5 servings of fruits and vegetables a day both in the United States and Europe strongly indicate the perceived protective effects of fruits and vegetables against cancer and other chronic diseases (USDA, Web resource; (Stanner, 2001).

The discovery of micronutrients and the effects of their deficiencies on health in the early part of the 20th century lead to the hypothesis that the addition of these micronutrients could potentially restore health (Underwood, 1998). Subsequently, research on micronutrients such as vitamins E, C, and A (ß-carotene and retinoic acid), and selenium played a very important role in shaping the next 2 decades in cancer prevention research (Shamberger, 1970; Cameron *et al.*, 1979; Niles, 2000; Hercberg, 2005). Historically, nutritional intervention in

prevention of disease started with inhibition of vitamin deficiency diseases via supplementation (Jukes, 1989). The concept of nutritional intervention in cancer was conceived primarily by Peto and colleagues in 1980s. The observational epidemiological studies until that time showed significant correlations between lifestyle factors such as smoking, dietary fat, dietary fiber and fruit and vegetable intake and disease risk. The idea that cancer could be prevented was introduced by Sporn in 1976 with chemoprevention being defined as “the stabilization, arrest and reversal of the progression of preneoplastic lesions” by either natural or synthetic agents (Sporn, 1976). Thus the concept of preventing cancer using dietary agents was ripe for application. The seminal paper by Peto *et al*., about the applicability of beta-carotene in human trials set forth prospective randomized intervention trials of various durations (Peto *et al.*, 1981).

The two major clinical trials α-Tocopherol ß-Carotene prevention trial (ATBC) in Finland and the ß-Carotene and Retinol efficacy trial (CARET) in the US were started in the mid 1980s (Table 1.4). These were done in smokers with an intervention dose of upto 30 mg ß-carotene and 25,000 IU of vitamin E compared to a recommended intake of 1.8 mg and 22 IU, respectively for non-smokers (RDI charts, USDA). Unexpectedly, the incidence of lung cancer in the high–dose intervention groups was higher than placebo (Blumberg & Block, 1994; Forman *et al.*, 2004). In a critical review, Block addresses several issues that may have lead to the discrepancy between the observational and clinical studies (Block, 1995). Certain important points worth noting are: first, most clinical “prevention” trials are actually intervention trials and studied the effect of mega-supplementation on already high-risk individuals; second, these trials mostly concentrated on the effect of a single agent, whereas most epidemiological study correlations are the result of interactions between several food constituents; third, these trials abandoned the effect of life-style factors before supplementation began, thereby discounting their effect on predisposition to a chronic illness such as cancer. Although, clinical trials are important tools to assess the efficacy of any intervention on a disease process, care needs to be

taken while building a hypothesis around a multi-factorial, chronic (involving several decades) disease such as cancer (Block, 1995).

There are about 25,000 known phytochemicals that have been identified in the various foods that are consumed (Forman *et al.*, 2004). In recent years due to the concerted efforts of several analytical, molecular and synthetic chemists, these are available in pure chemical forms to be tested in various experimental systems. However, most of these agents will never be tested in pre-clinical or clinical studies due to various complexities involved (Hercberg, 2005). Currently, experimental evidence suggests that different chemicals may potentiate each other in their anti-carcinogenic effects (Seeram *et al.*, 2005). Epidemiological evidence does not single out an agent that reduces the risk of cancer, although the protective nature of fruits and vegetables in cancer is irrefutable (Block *et al.*, 1992; Block, 1995). Thus, a piece-meal approach to cancer prevention that has lead to the failure of the 2 large intervention studies needs to be re-evaluated. As discussed by Block, cancer is neither an infectious disease caused by a single agent nor is it curable by a single pharmacological dose of one medication (Block, 1995). Any research on cancer prevention must take this into account in order to synthesize the right hypothesis as well as acquire effective results.

Estrogen-induced mammary tumors in ACI rats

Several *in vitro* experimental systems that simulate the mechanisms of cancer development are often used to study the effectiveness of an agent or drug. Since cancer is a whole-body process involving interactions between several organ systems, the study of the effects on isolated tissues in culture will not replicate the true effects of the drug (Clarke, 1996). Also, the study of therapeutic affects of different agents in human subjects raises both economic and ethical considerations (Clarke, 1996; Corpet & Pierre, 2005). Thus, the use of animal models for study of carcinogenesis is imperative and unavoidable. There are several advantages and disadvantages of using an animal model for studying a process as complex as cancer. The advantages include control of factors such as dose and duration of exposure, reduced interference from other

environmental exposures, lower biological variability due to a higher homogeneity in rodent populations as compared to humans, shorter lifespan of rodents, etc. The limitations of various rodent models of breast cancer have been extensively discussed by Kim *et al*., (2004). Although several rodent models of breast cancer are available, none of them typically simulate the human conditions (Clarke, 1996; Gusterson *et al.*, 1999; Kim *et al.*, 2004). Nevertheless, these models are very valuable for the purposes of testing intervention strategies and to understand the molecular mechanisms that lead to breast cancer.

Breast tumors undergo progression from the *in-situ* stage through invasive cancer to metastatic tumors (Clarke, 1996). Most animal models developed have tried to replicate this development from one stage to the other as closely as possible. The validity of most rodent tumor models has been derived based on the similarities, both histopathological and molecular, between tumors of rodent and human origin (Russo *et al.*, 1990; Thompson & Singh, 2000). Although, genetically engineered mouse models (transgenic and syngeneic) have illuminated to a large extent molecular mechanisms involved in breast tumorigenesis (Blackshear, 2001), their application in the treatment and prevention of sporadic human breast cancer is limited (Clarke, 1996; Kim *et al.*, 2004). Also, explant models are less predictive of validity for translational research (Gutmann *et al.*, 2006).

The use of carcinogen-induced mammary tumors in rats as a preclinical model has been popular for the past 4 decades. Table 1.5 highlights several carcinogen- induced mammary tumor models currently available. There is considerable heterogeneity in the incidence of mammary tumors in rats depending on the rat strain used, type of carcinogen, time and mode of carcinogen administration etc., (Huggins *et al.*, 1959; Thompson *et al.*, 1992; Shepel & Gould, 1999). Strikingly, the most common feature among all these models is that the disruption of the ovarian-endocrine axis by means of ovariectomy affects the ability of carcinogens to induce mammary tumors (Welsch, 1985; Shull *et al.*, 1997; Thompson *et al.*, 1998; Thordarson *et al.*,

2001). This suggests that the development of mammary tumors shares at least one common mechanism – ovarian hormone dependence.

The most commonly used carcinogen-induced mammary tumor models are listed in Table 1.5. Synthetic chemical-carcinogens such as 7,12, dimethylbenze[*a*]anthtacene (DMBA) or 1-nitroso-1-methyl urea (NMU) cause high incidence of mammary tumors in treated rats compared to less than 50% incidence with other carcinogens (Table 1.5). This makes the use of the DMBA- and NMU- induced model most popular for study of mammary tumors. A single intra-gastric dose of DMBA induces mammary tumors (Huggins *et al.*, 1961). However, not all induced tumors are adenocarcinomas and some tumors are known to spontaneously regress (Haslam & Bern, 1977; Thompson & Sporn, 2002). Both *in-situ* and invasive carcinomas are induced by a single intra-peritoneal or intra-venous dose of NMU (Gullino *et al.*, 1975; Thompson *et al.*, 1992). These tumors appear to have an ovarian-independent phenotype in that they can redevelop after initial regression following ovariaectomy (Thompson *et al.*, 1998; Thordarson *et al.*, 2001). H-ras mutations commonly seen in carcinogen-induced tumors are not present in humans (Sukumar *et al.*, 1983; Stanley, 1995; Gusterson *et al.*, 1999). Also, genomic instability such as anueploidy, which is a hallmark for human cancers, is rarely seen in chemical-carcinogen induced tumors (Li *et al.*, 2002a). Although such differences exist, chemical-carcinogen induced mammary tumors have been extensively studied and documented (Welsch, 1985; Russo *et al.*, 1990).

In contrast to this, the interest in estrogen-induced mammary tumors in A strain-Copenhagen-Irish hooded (ACI) rats, has waxed and waned since it was first studied. The rat strain is unique in that they develop mammary adenocarcinomas on exposure to estrogens. Initial interest in estrogen-induced mammary tumors was seen in the 1930s and 40s with description of strain differences in susceptibility to mammary tumors induced by different estrogens (Noble *et al.*, 1940; Dunning *et al.*, 1947; Dunning *et al.*, 1953; Noble & Cutts, 1959). Later, it was briefly revived by Shellabarger and coworkers in the late 70s

and early 80s (Stone *et al.*, 1979; Holtzman, 1988). Regardless of the type of estrogen used (diethylstilbesterol (DES), estrone (E1), ethinyl estradiol (EE), etc.,) ACI rats consistently develop mammary tumors, however they are resistant to chemical-carcinogen induced tumors (Dunning *et al.*, 1953; Cutts & Noble, 1964; Holtzman *et al.*, 1979). The model used in this thesis was first described by Shull and colleagues in 1997 and uses 27 mg of 17ß-estradiol (E2) in a silastic implant delivered subcutaneously for the induction of mammary tumors (Shull *et al.*, 1997). Another variation developed by Li and colleagues uses 2-3 mg of E2 in 20 mg cholesterol pellets to induce mammary tumors (Li *et al.*, 2002a; Li & Li, 2003). In both models, the incidence of mammary tumors in female ACI rats is 100% with a latency period of approximately 6 months. Although, the exact molecular pathways involved are currently being discovered, this model has a relative dearth of information compared with its DMBA-induced counterpart. These shortcomings aside, the model affords an apt system for testing preventive intervention.

Several key points support the use of this model to study breast cancer prevention. First, estrogen is clearly and undisputedly associated with the etiology of the disease in humans. Second, estrogen-induced tumors exhibit chromosomal instabilities, which are also often seen in human breast cancer (Li *et al.*, 2002a; Li *et al.*, 2004; Adamovic *et al.*, 2007). Further, E2- and DMBA-induced carcinogenesis involves genetically distinct mechanisms (Schaffer *et al.*, 2006). Although these rats are susceptible to estrogen-induced prolactinomas, the loci that control the pituitary and mammary tumor susceptibilities are genetically distinct (Gould *et al.*, 2004; Strecker *et al.*, 2005; Schaffer *et al.*, 2006). In addition, the chromosomes that are affected in estrogen-induced carcinogenesis are homologous to those that are affected in humans (Adamovic *et al.*, 2007). Finally, tumors display molecular markers such as an over-expression of cyclin D1 and c-myc, similar to breast cancer pathology in humans (Weroha *et al.*, 2006).

Although some intervention studies were done in DES-induced mammary tumor model, so far very few studies have looked at the effect of preventive intervention in the estradiol-induced model (Petrek *et al.*, 1985; Holtzman, 1988). Of the few, a study by Shull and colleagues looked at hypo-caloric feeding and prevention of mammary tumors (Harvell *et al.*, 2002). Other studies include prevention using Tamoxifen (Li *et al.*, 2002b), phenobarbital (Mesia-Vela *et al.*, 2006) and a short term *in vivo* assay using diallyl sulphide (Green *et al.*, 2005). Collectively, these facts make the ACI rat model an ideal preclinical model for exploring preventive intervention strategies which have a high applicability in the translational setting.

Berries in cancer prevention

Berries are ideal agents for the chemoprevention of cancer. Figure 1.5 shows the pictures of and table 1.6 shows the nutritive value and total anthocyanin content of some berries commonly consumed in the United States. It is evident that berries are a good source of several chemopreventive nutrients, including ß-carotene, selenium, vitamins A, C and E as well as phytonutrients such as lutien, ellagic acid and anthocyanins (Table 1.6).

Ellagic acid, a polyphenol present abundantly in many berries is a known chemopreventive agent. It has been shown to successfully reduce the incidence and progression of carcinogen-induced tumors in the skin, lung, esophagus, liver and colon, in rodents, when given orally (Reviewed by Stoner and Mukhtar, 1995). Several mechanisms such as antioxidant effect, modulation of detoxification enzymes, regulation of cell cycle pathways, DNA binding and DNA repair pathways have been attributed to this (Teel, 1986; Barch & Rundhaugen, 1994; Barch *et al.*, 1994; Ahn *et al.*, 1996; Chakraborty *et al.*, 2004; Han *et al.*, 2006). Among the different berries, black raspberries have the highest ellagic acid content and blueberries have the lowest (Table 1.6).

Anthocyanins are flavanoids present in many fruits and is the source of blue, red or purple color in plants (de Freitas & Mateus, 2006). Pro-anthocyanidins are polymers of anthocyanin molecules (Dixon *et al.*, 2005). Both are abundantly present in berries (Wu *et al.*, 2004). Structures of anthocyanins commonly found in berries are shown in Figure 1.6. Dark berries such as blueberries, blackberries and black raspberries have greater anthocyanin content than their lighter counterparts (Wu & Prior, 2005; Wu *et al.*, 2006).

Among the different berries, black raspberries have already been used in a pilot clinical trial for the prevention of Barrett's esophagus, a pre-disposing condition for esophageal malignancy (Kresty *et al.*, 2006). Bioavailability studies on black raspberries prove that both ellagic acid and cyanidins are bio-available, but excreted rapidly from the system (Stoner *et al.*, 2005; Tian *et al.*, 2006). Several other bioavailability studies performed show that anthocyanins are highly bioavailable, absorbed as such in the stomach or intestine and excreted with or without methylation in the urine of both rodents and humans (McGhie *et al.*, 2003; Talavera *et al.*, 2004; Tian *et al.*, 2006).

Since, 17β-estradiol is highly implicated in breast cancer etiology, targeting hormonal mechanisms is the best approach to prevention. Currently, Tamoxifen is the leading preventive therapy for breast cancer. However, treatment with Tamoxifen involves numerous adverse effects including increased incidence of endometrial cancer, cataracts, and thromboembolism (Cano & Hermenegildo, 2000; Morrow & Jordan, 2000). Although, Raloxifene was equivalent to Tamoxifen in prevention of invasive breast cancer with fewer adverse effects, thromboembolism, hot flashes and leg cramps are still possible side effects (Cranney & Adachi, 2005; Jordan, 2006). In addition, Raloxifene is poorly bioavailable and rapidly excreted, causing significantly reduced benefits in women with poor compliance (Jordan, 2006). Nevertheless, the search for better selective estrogen receptor modulators (SERMs) with fewer side effects is ongoing (Jordan, 2006). The chemical structure of both anthocyanins and ellagic acid is similar to estradiol (Figure 1.6). This similarity makes them ideal

candidates for being SERMs. In fact, both ellagic acid and berry anthocyanins show potent anti-estrogenic activities (Schmitt & Stopper, 2001; Papoutsi *et al.*, 2005; Larrosa *et al.*, 2006). Further, the neuroprotective effects of blueberries are thought to be mediated via antioxidant, anti-apoptotic and cell signaling mechanisms involving extracellular signal-related kinase (ERK) and protein kinase C (PKC) (Ramassamy, 2006). Incidentally, a pathway of estrogen-induced ERK activation involves PKC in neural cells (Setalo *et al.*, 2005). This implies that blueberries may act via estrogenic mechanisms to provide neuroprotective effects. These reports taken collectively vouch for the SERM effects of berries. Thus, berries could be used by themselves or as an augmentative therapy alongside other potent SERMs, in the prevention of breast cancer.

The failure of preventive trials with individual micronutrients has steered the scientific community towards appreciating the interaction between bio-active food components present in whole foods (Hampton, 2005; Meyskens & Szabo, 2005). Berries contain several such components (Table 1.6). Berries, such as blueberries and black raspberries show high anti-oxidant activity (Wang & Lin, 2000; Wada & Ou, 2002). Black raspberries are known to affect inflammatory cellular pathways such as COX-2, NF-κB involved in tumor progression (Chen *et al.*, 2006; Hecht *et al.*, 2006). Both ellagic acid and berry extracts inhibit *in vitro* proliferation of malignant cells through pro-apoptotic mechanisms (Seeram *et al.*, 2005; Han *et al.*, 2006; Seeram *et al.*, 2006). They also show anti-angiogenic effects by regulating vascular endothelial growth factor (VEGF) pathway, thus potentially affecting tumor metastasis (Losso *et al.*, 2004; Labrecque *et al.*, 2005; Huang *et al.*, 2006).

Since cancer is a multi-pathway disease, we need a multi-pronged approach for the prevention of this disease. The case for the use of berries in breast cancer prevention is strong because berries have been used for centuries without adverse side effects (other than allergic reactions), their tolerability studies are positive, their protective nutrients are highly bioavailable (Stoner *et al.*, 2005) and show anti-estrogenic, antioxidant, anti-inflammatory, anti-

angiogenic and pro-apoptotic activities that will be beneficial in cancer prevention. As discussed earlier, estrogen acts as a complete carcinogen via several molecular pathways, leading to breast tumor development. Berries are the perfect multi-pronged tool to prevent breast cancer as they mediate protection through multiple molecular pathways, several of which are also affected by estradiol.

Hypothesis

I hypothesize that anthocyanins and ellagic acid, either individually or in combination, will have a protective effect against estrogen-induced breast cancer. To test this two different berries, one with a high-ellagic acid/high-anthocyanin content, and other with low-ellagic acid/high-anthocyanin content, and ellagic acid by itself will be provided via the diet to determine their potential for inhibit 17ß-estradiol induced mammary tumors in ACI rats. The results of these studies will have high translational value either to prevent or to augment existing preventive therapy for breast cancer. The following specific aims will be pursued to achieve my objectives

Specific Aims

1. To determine the *in vitro* antioxidant capacity of different polyphenols to protect against catechol estrogen-induced oxidative DNA damage.
2. To employ dietary berries in a short-term *in vivo* study to determine the protective biochemical effects.
3. To employ dietary berries of varying ellagic acid contents and ellagic acid, in an estrogen-induced mammary tumor model to study their chemopreventive potential.
4. To study the mechanisms by which dietary berries and ellagic acid cause prevention of cancer *in vivo.*

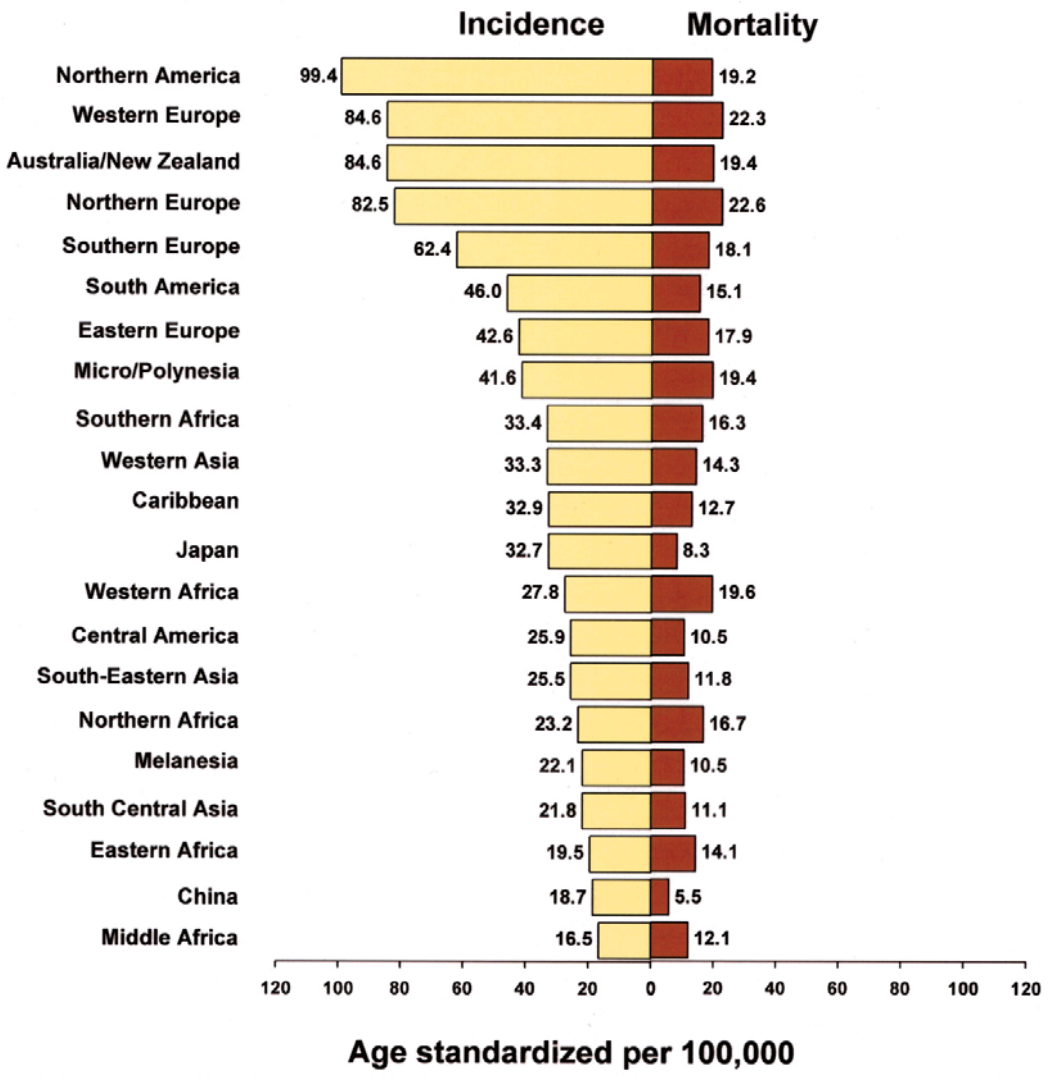


Figure 1.1. Age-standardized incidence and mortality rates for breast cancer per 100,000.

Source - Parkin, Bray , *et al.*, 2005.

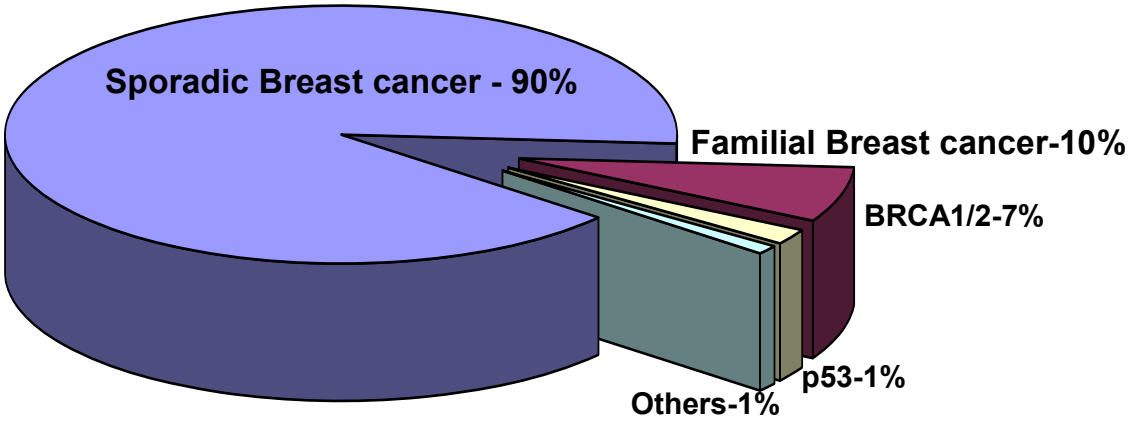


Figure 1.2. Epidemiology of Breast Cancer.

Adapted from Charpentier and Aldaz, Humana Press Inc., 2000.

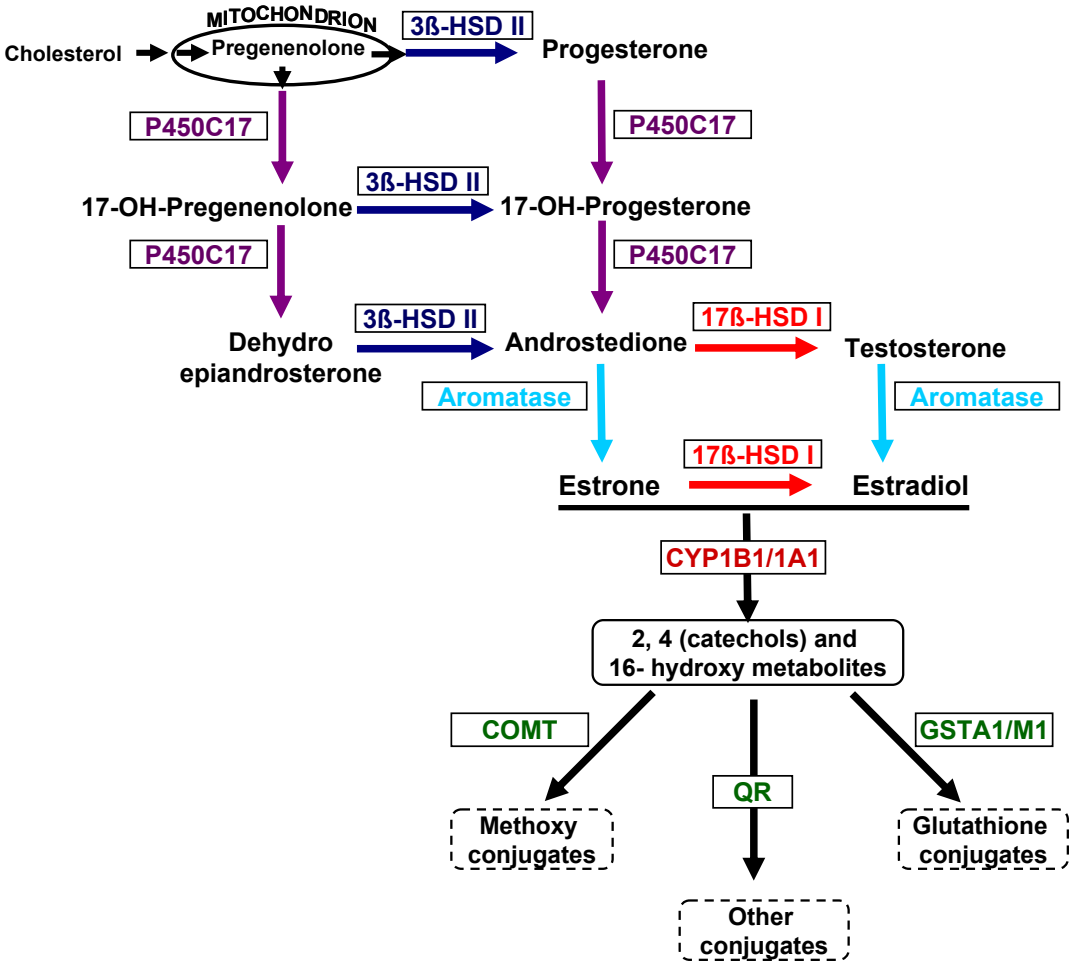


Figure 1.3. Metabolomics of Steroid hormones- schematic showing the synthetic and metabolic pathways of estradiol.

Adapted from Bulun et al., 2005.

Abbreviations: 3ß-HSD II- 3ß-hydroxysteroid dehydrogenase, type II; P450C17- 17α-hydroxylase; 17ßHSD I- 17ß-hydroxysteroid dehydrogenase, type I; CYP1A1/1B1- Cytochrome P450 1A1/1B1; COMT- Catechol-O-methyl transferase; GST-Glutathione-S-transferase; QR-NADP(H)-quinone reductase.

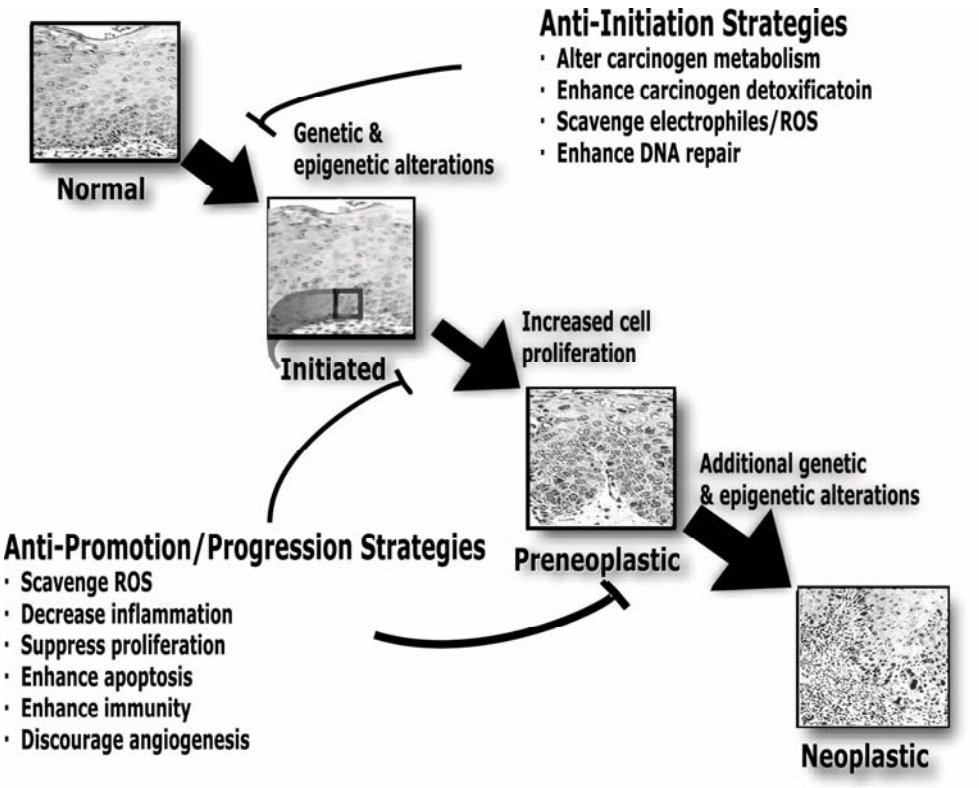


Figure 1.4. Multistage model of Carcinogenesis and intervention strategies at each stage.

Source- Forman *et al.,* 2004.

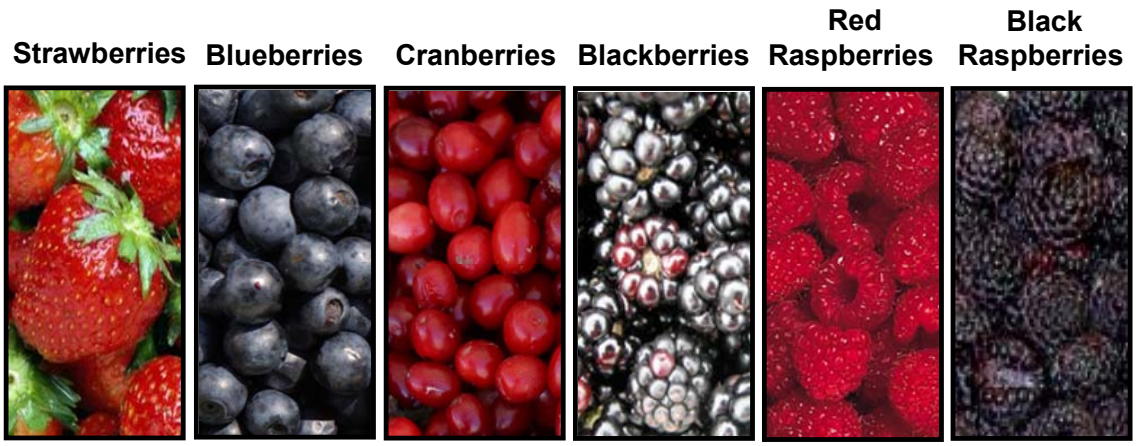
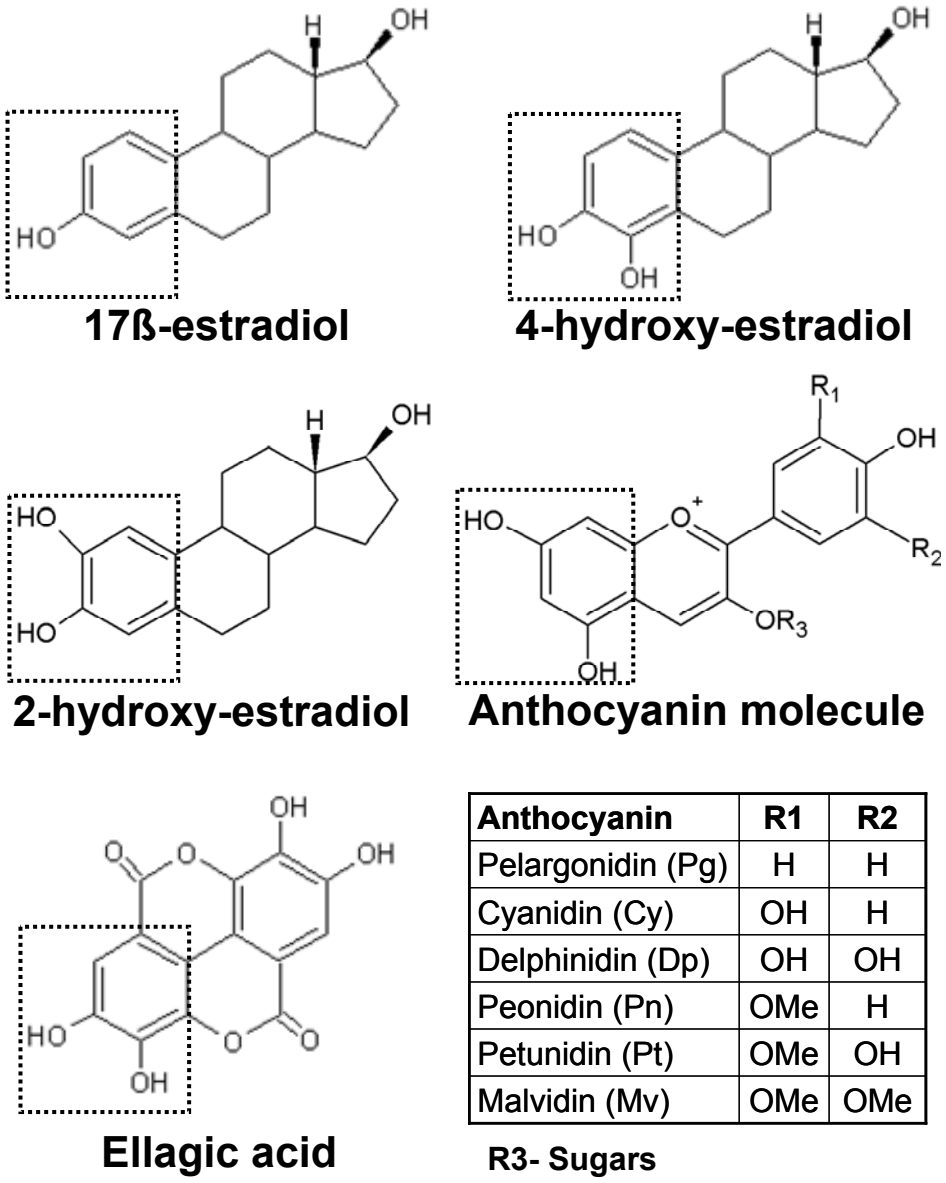


Figure 1.5. Pictures of berries commonly available in the United States.



Anthocyanin	R1	R2
Pelargonidin (Pg)	H	H
Cyanidin (Cy)	OH	H
Delphinidin (Dp)	OH	OH
Peonidin (Pn)	OMe	H
Petunidin (Pt)	OMe	OH
Malvidin (Mv)	OMe	OMe

R3- Sugars

Figure 1.6. Chemical structures of 17ß-estradiol, its metabolites and berry-phytochemicals. The structural similarities that may be responsible for possible estrogen-receptor binding of the various chemicals are highlighted. Table of different functional groups in the anthocyanin molecule adapted from Wu and Prior, 2005.

Table 1.1. Crude and age-standardized (World) rates of Breast Cancer in Northern America, per 100,000.

	Incidence			**Mortality**			**Prevalence**	
Country/Region	Cases	Crude Rate	ASR (W)	Deaths	Crude Rate	ASR(W)	1-year	5-year
Northern America	229,631	141.9	99.4	48239	29.8	19.2	230,990	1,058,170
Canada	19,540	124.0	84.3	53,05	33.7	21.1	19,590	89,439
United States of America	209,995	143.8	101.1	42,913	29.4	19.0	211,400	96,8731

Abbreviations: ASR (W) – Age-standardized rate (World)

Source- GLOBOCAN 2002, International Agency for Research on Cancer.

Table 1.2. List of risk factors for development of sporadic breast cancer.

Non-Modifiable Risk Factors	Modifiable Risk Factors	Hormonal Risk Factors
Age	Diet	Cumulative exposure to estrogen
Gender	Alcohol	Age at menarche and menopause
Genetics	Smoking	Parity
Family History	Body Weight	Lactation
Previous Breast Disease	Exercise	Hormone replacement therapy
	Radiation	

Table 1.3. Endocrine and paracrine control of mammary gland development

Hormone	Stage of development	Effect	Reference
Placental Lactogens	Pregnancy	Act Synergistically with prolactin	Forsyth, 1994
Human Chorionic Gonadotropin	Pregnancy	Terminal differentiation of mammary lobules	Russo and Russo, 1995
Estrogen (Mostly via ERα)	Post-natal (Primarily puberty and pre-parous period)	Ductal elongation and proliferation	Vonderhaar, 1988; Shyamala, 2002; Anderson, 2002
Progesterone	Post-natal	Lateral ductal branching and lobulo alveolar development	Shyamala, 2000
Prolactin	Post-natal	Ductal side branching and TEB regression	Brisken *et al.,* 1999
	Pregnancy	Lobulo alveolar development via JAK/STAT cell-signalling pathway	Hennighausen *et al,* 1997; Brisken *et al.,* 1999; Kelly, *et al.,* 2002
Androgens	Fetal (especially in male fetus)	Inhibition of epithelial growth and nipple attachment in male fetus by causing partial necrosis of mammary epithelium	Vonderhaar, 1988; Houdebine , 1985
Growth Hormone	Post-natal	Transformation of mammary fibroblasts in to preadipocytes which then secrete PGE2 that stimulates multiplication of mammary epithelial cells.	Houdebine, 1985; Kelly *et al.,* 2002.
Paracrine control	Mainly via the stromal compartment	Mediated by growth factors such as TGF, CSF-1, IGF-1, MDGF and EGF.	Voderhaar, 1988; Wiseman and Werb, 2002

Abbreviations: ERα-Estrogen receptor alpha; TEB- Terminal end bud; JAK-Janus kinase; STAT-Signal tranducers and activators of transcription; PGE2-Prostaglandin E2; TGF- Transforming growth factor; CSF- Colony stimulating factor; IGF- Insulin like growth factor; MDGF-Macrophage-derived growth factor; EGF- Epidermal growth factor.

Table 1.4. Lung cancer prevention trials evaluating ß-carotene supplementation

Trial	Agent(s)	Population	N	F/U	Risk effect
ATBC (Finland)	β-carotene(20 mg/d) Vitamin E (50 mg/d) (2 × 2 design)	Male smokers 50–69 yr (μ = 57 yr) 36 years of smoking	29133	5–8 yr μ = 6 yr	876 cases RR = 1.18 (1.03, 1.36)
CARET (United States)	β-carotene (30 mg/d) Retinyl P. (25,000 IU) (2 × 2 design)	Male and female Smokers (μ = 58 yr) 49 years of smoking	18314	4–7 yr μ = 4 yr	286 cases RR = 1.36 (1.07–1.73) β-Carotene suppl.
			14254		RR = 1.28 (1.04, 1.57) β-Carotene+ Retinyl P.
PHS I (United States)	β-carotene (50 mg/alternate d)	Male MDs age 40–84 11% smokers	22071	12 yr	82 cases in β-Carotene 88 cases in placebo RR = 0.98 (0.91- 1.06)
WHS (United States)	β-carotene (50 mg/alternate/d) of 8 groups	Females age 45 + 13% smokers	39876	2.1 yr	30 cases in β-carotene 21 in placebo

Abbreviations: ATBC- Alpha-Tocopherol β-Carotene Trial; CARET- Carotenoid and Retinol Efficacy Trial; PHS- Physicians' Health Study; RR- relative risk; WHS- Women's Health Study; F/U-years of follow-up during the trial; N- number randomized; μ- mean; Retinyl P- Retinyl Palmitate. All trials were designed as randomized controlled trials.

Source- Forman *et al.,* 2004

Table 1.5. Rat models of carcinogen-induced mammary tumors.

Rat strain	Carcinogen	Age at induction	Dose	Route	% Incidence	Latency *(weeks)*	Tumor type	Reference
SD	2-AAF	50 days	100 mg	PO	30%	8	Carcinoma	Huggins *et al.,* 1961
	3-MC		100 mg	PO	100%	7.3 ± 2	Carcinoma	
	DMBA		20mg	PO	100%	6.1 ± 1	Carcinoma	
	DMBA	48 days	1µM per gland	IM	100%	10 ± 2	Adenocarcinoma (62%) Fibroadenoma (6%) Fibrosarcoma (28%)	Cavalieri *et al.,* 1991
	DB[*a, l*]P				100%	11 ± 1	Adenocarcinoma (83%) Fibroadenoma (0%) Fibrosarcoma (16%)	
	B[*a*]P				45%	22 ± 1	Fibrosarcoma	
	NMU	50 days	50 mg/kg body weight	IP	100%	8	*In situ* and invasive carcinoma	Thordarson *et al.,* 2001
	PhIP	43 days	75 mg/kg body weight	PO -10 doses	24%	NR	Carcinoma	Snyderwine *et al.,* 1998
ACI	17ß-estradiol	49 days	2 or 3 mg	SC-P	100%	NR	*In situ* carcinoma	Li *et al*., 2002
		45 days	27 mg	SC-T	100%	21 ± 3		Shull *et al.,* 1997
		36-49 days	9 mg	SC-T	100%	NR		Ravoori *et al.,* 2007

Abbreviations: 2-AAF- 2-acetylaminofluorine; 3-MC- 3-methylcholantherene; DMBA- 7,12-dimethylbenz(*a)*antharcene; DB[*a,l*]P-dibenzo[*a,l*]pyrene; B[*a*]P-Benzo[*a*]pyrene; NMU- 1-methyl-1-nitrosourea; PhIP-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; SD- Sprague Dawley; ACI- August-Copenhagen-Irish-hooded; NR-Not reported.

Table 1.6. Nutrient composition of commonly available berries

Nutrient	**Units/** 100g fresh weight	**Strawberries** *Fragaria Ananassa*	**Blueberries** *Vaccinium Spp.*	**Cranberries** *Vaccinium macrocarpon*	**Blackberries** *Rubus Spp*	**Red raspberries** *Rubus idaeus*	**Black raspberries** *Rubus occidentalis*
Water	g	91	84	87	88	86	86[a]
Energy	Kcal	32	57	46	43	52	72[c]
Protein	g	0.70	0.7	0.4	1.4	1.2	1.4[c]
Total Fat	g	0.30	0.33	0.13	0.50	0.65	0.14[c]
Total dietary Fiber	g	2.0	2.4	4.6	5.3	6.5	1.7[c]
Calcium	mg	16	6	8	29	25	32[c]
Selenium	µg	4.4	0.1	0.1	0.4	0.2	NA
Vitamin A	IU	12	54	60	214	33	38[c]
Vitamin C	mg	59	10	13.3	21	26	2.4[c]
Vitamin E	mg	0.29	0.57	1.2	1.17	0.87	NA
β-Carotene	µg	7	32	36	128	12	NA
Lutein	µg	26	80	91	118	136	NA
Total anthocyanin	mg	21[a]	386[a]	140[a]	245[a]	92[a]	687[a]
Ellagic acid*	mg	6.3[b]	<1.0[b]	1.2[b]	15[b]	33.9[c]	53.7[c]
ORAC value	µmoles/ TE/g	21[d]	60[e]	95[e]	56[e]	21[d]	77[f]

Source: USDA National Nutrient Database except where mentioned.

a- Information from Wu *et al.,* 2006.

b- Information from Daniel *et al.,* 1989.

c- Information from Oregon Berry Commission.

d- Information from Kalt *et a*.l, 1999.

e- Information from Wu et al., 2004

f- Information from Wada and Ou, 2002.

Abbreviations: ORAC- Oxygen Radical Absorbance Capacity; NA- Information not available; TE-Tocopherol Equivalents.

Chapter Two: Effect of phytonutrients (pure compounds) and whole food (berries) on DNA damage and gene expression.

Introduction

Female hormone - 17β-estradiol (E_2) is associated with the etiology of breast cancer, which is the second leading cause of cancer-related death in American women (Russo *et al.*, 2000; ACS, 2007). It has been shown that E_2 and its metabolites can lead to mutations by increasing the rate of DNA damage as well as decreasing DNA repair (Russo *et al.*, 2000; Mailander *et al.*, 2006). Metabolites of E_2 such as 2- and 4-hydroxy estradiol ($4E_2$), can cause oxidative DNA damage in the presence of Cu^{2+} (Li *et al.*, 1994; Yager, 2000). Since oxidative DNA damage can ultimately lead to further downstream detrimental effects, effective inhibition of this damage may be a useful prevention strategy.

There are several methods available to assess DNA damage. Among these, ones that combine a chromatographic method with mass spectrometry have been used to measure numerous products at the same time (Dizdaroglu *et al.*, 2002). Also, ^{32}P-postlabeling in conjunction with thin layer chromatography (TLC), can be used to measure oxidative DNA damage of various DNA bases, including the benchmark oxidative lesion 8-oxo-2'-deoxyguonosine (8-oxodG) (Gupta & Arif, 2001; Gupta *et al.*, 2003; Ravoori *et al.*, 2006). Recently, we have discovered several polar DNA adducts by ^{32}P-postlabeling and low salt chromatography. Chromatographic comparison with oxidative DNA adducts formed by Fenton-type reaction (Cu^{2+}-H_2O_2) suggest that some of the polar tissue adducts may be oxidative adducts (Aiyer *et al.*, 2003; Gupta *et al.*, 2003). These adducts can be used as a biomarker for selection of antioxidant agents that can modulate DNA damage. Earlier studies from our laboratory have successfully used detection of DNA damage in conjunction with a cell-free system to rapidly screen for chemopreventive/antioxidant agents, thus expediting the process of agent selection (Smith & Gupta, 1999; Srinivasan *et al.*, 2002). In cancer prevention, a tiered preclinical approach involves screening of several potential preventive agents in cell-culture and rodents prior to their use in clinical

trials. We have been successful in using this tiered approach in reducing benzo-[*a*]-pyrene-induced DNA damage. Agents such as oltipraz and ellagic acid that were effective in an enzymatic cell-free system were effective in both cell-culture and *in vivo* (Smith & Gupta, 1999; Smith *et al.*, 2001a; Smith *et al.*, 2001b). This tiered approach can be used successfully in the selection of agents that would be effective in long-term studies without having the need for employing several compounds in expensive long-term *in vivo* studies.

There are several surrogate biomarkers available to assess the efficacy of dietary agents on a biological system. Among these, the liver due to its proximity and role in the first-pass mechanism represents a suitable surrogate tissue. In addition, due to its high metabolic activity, liver is constantly exposed to the oxidative by-products of cell metabolism, thus making it an ideal tissue to assess the modulation of baseline oxidative DNA damage by dietary agents.

A significant epidemiological association between fruit and vegetable intake and low cancer incidence has been reported (Block *et al.*, 1992). Flavonoids are low molecular weight compounds present ubiquitously in plants. They have a common 3-ring structure with various substituents which make them structurally diverse. Many of them have significant biological effects which include favorable metabolism of xenobiotics, antioxidant properties, and effects on cell-signaling that make them desirable candidates for cancer prevention (Reviewed by Middleton, Kandaswami and Theoharides, 2000).

In this study, we have used a 2-tiered strategy to initially test more than 10 flavonoids in an *in vitro* test system and employ the most effective agent in a short-term *in vivo* study. The test agents selected were either well known flavonoids (ellagic acid, epigallocatechin gallate (EGCG), quercetin) or other less known compounds (naringenin, ferulic acid etc.,). Ascorbic acid and vitamin E were included as known antioxidants. The most efficacious agent in this tier was ellagic acid, which was tested in a short-term *in vivo* study as a dietary chemopreventive agent. Whole foods (berries) containing significant levels of ellagic acid were also provided via diet to compare the bioavailability and efficacy

of ellagic acid given as a pure compound and in whole food. The modulation of oxidative DNA damage in both systems was assessed by ^{32}P-postlabeling/TLC. In addition, the possible mechanisms by which these agents modulate DNA damage *in vivo* were explored by gene-expression analyses using microarray technology.

Materials and Methods

Chemicals. Ascorbic acid, biochanin and ferulic acid were purchased from Aldrich Chemical Company (Milwaukee, MI)., Ellagic acid, naringenin, resveratrol, silymarin, quercetin, α-tocopherol, acetone, dimethyl sulfoxide (DMSO) and salmon testes DNA (*st*-DNA) were purchased from Sigma Chemical Company (St. Louis, MO). Epigallocatechin gallate was purchased from LKT labs (St. Paul, MN). 4-hydroxy estradiol ($4E_2$) was purchased from Steraloids Inc. (Newport, RI). Chemicals involved in ^{32}P-postlabeling were purchased from sources described earlier (Gupta, 1996). All chemicals used were > 95% pure and were used without further purification. *Salmon testes (st)*-DNA was further purified before use, as described previously (Gupta, 1996).

Induction of DNA damage by 4E2/CuCl2. *Salmon testes*-DNA (300 µg/ml) in 10 mM Tris Hcl, pH 7.4, was pre-incubated with vehicle alone and the test agents dissolved in either DMSO or acetone (≤ 5% each) and $CuCl_2$ (100 µM) for 15 min at 37ºC. Redox-cycling was initiated by the addition of $4E_2$ (100 µM) in ethanol. After incubation at 37°C for 1h, DNA was purified by solvent-extraction and ethanol precipitation as described (Gupta, 1996; Ravoori *et al.*, 2006).

Animals and diet. Eight week-old female CD-1 mice were purchased from Harlan-Sprague Dawley (Indianapolis, IN). CD-1 mice were chosen as an exploratory model for estrogen-induced carcinogenesis as these animas are highly susceptible to estrogen-induced uterine cancer (Newbold and Liehr, 2000). Five groups (n=6) were fed *ad libitum,* either a control diet or diet supplemented

5% (w/w) with strawberries, blueberries, red raspberries or 400 ppm ellagic acid, according to the protocol (Figure 2.1). Three berries with low (blueberries; < 100 ppm), moderate (Strawberries; 500 ppm) and high (raspberries; 1500 ppm) ellagic acid content were chosen (Daniel *et al.*, 1989). The ellagic acid dose was selected based on a similar short-term study in rats (Ahn *et al.*, 1996). Organic blueberries, strawberries and non-organic raspberries were purchased as fresh produce locally (Lexington, KY). All berries were dehydrated in a food dehydrator, powdered, vacuum dried to remove remaining moisture, sealed airtight in zip-top bags and stored at -80°C until use. Ingredients for the diet were purchased individually from Dyets, Inc. (Bethlehem, PA). The control diet was slightly modified from the original composition for the AIN-93M diet (Reeves *et al.*, 1993) such that the carbohydrate calories were provided by corn starch and dextrose without the inclusion of sucrose (Table 2.1). The dried berries were added along with various ingredients at 5% (w/w) of the modified control diet and mixed in a Hobart mixer until homogenous. The corn starch component of the diet was adjusted in these diets such that all diets were isocaloric after supplementation with berries. Ellagic acid was added at 400 ppm without any adjustment to the diet and mixed as described. These diets were stored at 4°C until use. Animals and the diets were weighed weekly to assess differences in diet intake and weight gain. They were euthanized by CO_2-asphyxiation at the end of 3 weeks and liver was snap-frozen using liquid nitrogen in 2 aliquots, which were used for ^{32}P-labeling and microarray analysis respectively.

Analysis of polar oxidative DNA adducts by 32P-postlabeling/TLC.

DNA from liver was isolated as described (Gupta, 1996). Briefly, the tissue was homogenized in TE buffer (50 mM Tris-HCl/10 mM EDTA, pH 8.0) and the nuclear pellet was sequentially treated with RNases (RNase A-150 µg/ml, RNase T1-1 U/µl) and proteinase K (150 µg/ml), followed by solvent extraction and ethanol precipitation. The resultant DNA pellet was washed with 70% ethanol and dissolved in HPLC water. After shearing, the DNA concentration was measured spectrometrically considering $1A_{260}$= 40 µg. Fourteen µg was digested to 3'-monophosphates using micrococcal nuclease/spleen phospodiesterase

(Enzyme: DNA – 1:5, 5h, 37ºC). After removing 2 µg of digest for normal nucleotide analysis, 10 µg digest was enriched for novel oxidative adducts by treatment with nuclease P1 (E: S-1:2.5, 1h, 37ºC). Remaining 2 µg of the digest was enriched for 8-oxodG by polyethyleneimine (PEI)-cellulose TLC and 0.5-1µg was labeled as described (Gupta & Arif, 2001). The 5'-^{32}P-labeling of both enriched DNA adducts and normal nucleotides were done in parallel, using T4-polynucleotide kinase in a molar excess of [γ-^{32}P] ATP as described earlier (Gupta, 1996; Ravoori *et al.*, 2006). Labeled adducts were separated using 2 directional PEI-cellulose TLC using 50 mM sodium phosphate, pH 6.0 and 1 M formic acid in the D1 direction. D2 was perpendicular to D1 using a solvent containing 2 M urea, 2.8 M Ammonium hydroxide and 50% isopropanol. Adducts with decreasing polarities in tissue DNA were eluted by increasing the sodium phosphate concentrations (50 mM – 1,000 mM) in the presence of 1M formic acid (D1) but maintaining the same D2 solvent. Adducts and normal nucleotides were visualized using Packard *Instant Imager* and were counted individually. The enriched 8-oxodGp was labeled in parallel and chromatographed as described (Gupta & Arif, 2001). Adduct levels were calculated as relative adduct labeling (RAL) = (CPM adducted nucleotides/CPM normal nucleotides) X 1/dilution factor and are expressed as adducts/10^6 nucleotides (*in vitro* adducts) and adducts/10^9 nucleotides (*in vivo* adducts).

Gene expression analysis. RNA was isolated using the phenol: chloroform extraction followed by DNase treatment. cDNA probes were synthesized from poly A^+ RNA, using [α-^{32}P]- ATP (3000 Ci/mmol). These probes were then hybridized to Atlas™ nylon mouse stress array overnight. Following hybridization the membrane was exposed to x-ray film. The obtained autoradiographic images were then scanned with a MicroTek ScanMakerIII flat-bed scanner and then subjected to densitometric analysis using ArrayExplorer© to extract the gene intensities (Patriotis *et al.*, 2001). The data was normalized, using linear regression analysis. The gene expression profiles were estimated as $\log_2$ of the ratio of the gene intensities of the control diet vs. supplemented diet. The genes with significant down- or up-regulation were identified. These

analyses was performed in Dr.Margie Clapper's laboratory at the Fox Chase Cancer Center (Philadelphia, PA) and the data was presented under joint authorship at the 95th annual meeting of the American Association for Cancer Research (Aiyer *et al.*, 2003). Further this chapter was reviewed by Dr. Clapper and approved to be submitted (personal communication dated 3-31-07).

Statistics. The adduct levels for each test agent was compared to the level of its respective vehicle control using Dunnet's two-sided t-test. A Scheffe's t-test was used for comparing dose response studies. A p-value <0.05 was considered to be statistically significant. The results are expressed as mean ± SE.

Results

Modulation of 4E2/CuCl2-induced oxidative DNA adducts by phytonutrients. Analysis of DNA damage, induced by redox cycling of $4E_2$ in the presence of Cu^{2+}, revealed several unidentified polar adducts and 8-oxodG (Figure 2.2 A1-B4). These were chromatographically similar to adducts generated by treatment of DNA with H_2O_2 /$CuCl_2$ (Srinivasan *et al.*, 2001). Neither $4E_2$ nor Cu^{2+} by themselves increased the levels of these adducts from baseline (data not shown). The level of unidentified polar adducts and 8-oxodG in the untreated *st*-DNA were 9.73 ± 0.03 /10^6N and 11.5 ± 0.85 /10^6N, respectively, and this increased to 985 ± 54/10^6N and 1349 ± 189/10^6N after treatment with 100 μM each of $4E_2$ and $CuCl_2$.

All agents were tested initially at a final concentration of 300 μM, based on earlier studies (Smith & Gupta, 1999; Srinivasan *et al.*, 2002). In the initial screening, ellagic acid was the most effective showing >95% reduction of both unidentified oxidative adducts and 8-oxodG compared to the vehicle control (Fig 2.2 A3, B3; Figure 2.3, $p<0.05$). This agent also showed a dose-dependant modulation of DNA damage starting at a concentration of 30 μM (Figure 2.4, $p<0.005$). Other flavonoids such as resveratrol, quercetin and naringenin showed only moderate reduction (Figure 2.3), while some other agents such as silymarin,

and EGCG showed an increase in DNA damage. (Figure 2.3; $p<0.05$). Known antioxidants, namely vitamin E and ascorbic acid showed moderate reduction and pro-oxidant effects respectively (Figure 2.3). Based on these results, ellagic acid was selected for a short-term *in vivo* study.

Modulation of baseline oxidative-DNA damage by ellagic acid and berries. There was no significant difference in the diet intake or weight gain between the groups (Figure 2.5 A and B). No toxicity or weight loss was observed at doses tested. All groups represented qualitatively similar adduct pattern (Figure 2.6-Inset). The baseline levels of different subgroups of adducts in the liver of mice fed control diet were: P-1 – 3800 ± 1870; P-2- 2600 ± 1320; PL-1 – 180 ± 72 and L-1 – 2600 ± 1340 per 10^9 nucleotides. PL-2 adducts were too low to be quantified and 8-oxodG, PL-3 and L-2 adducts were not analyzed. In this study, red raspberry diet reduced all subgroups of adducts analyzed effectively, with a 50% reduction of P-1 adducts, 60% reduction of P-2 adducts, 50% reduction of PL-1 adducts and a 30% reduction of L-1 adducts (Figure 2.6). Ellagic acid showed similar effects albeit at a lower level with 30% reduction in P-1 adducts, 45% reduction in P-2 adducts, 50% reduction in PL-1 adducts and no effect on L-1 adducts. Blueberry diet only reduced the L-1 adducts (30%) and had no effect on other adduct subgroups. Strawberry diet was ineffective in altering any subgroup of adducts (Figure 2.6).

Modulation of gene expression by red raspberry and ellagic acid diets. Following the similar modulation of adduct patterns by both red raspberry and ellagic acid diets, limited gene expression analysis was done to determine effect of the intervention on genes involved in DNA repair and xenobiotic metabolism. Microarray analysis revealed that several genes were modulated in a similar fashion by both diets. In particular, genes involved with DNA repair such as – xeroderma pigmentosum group A complementing protein (XPA), DNA ligase III (DNL3), DNA excision repair protein ERCC1- were found to be over-expressed by 3 to 8 fold (Figure 2.7A and B). There was a significant similarity in the number of genes over- or under-expressed by both diets (Figure 2.7). Red

raspberry diet down-regulated genes such as Mitogen activated protein kinase 14 (MAPK14) and MAP kinase kinase (MAPKK), involved in key cell-signaling pathways, by >15 fold.

Discussion

The induction of oxidative DNA damage by $4E_2$ in the presence of Cu^{2+} is postulated to involve hydroxyl radicals (Hiraku *et al.*, 2001). The qualitative presentation of polar adducts generated using either $4E_2/CuCl_2$ or $H_2O_2/CuCl_2$ is similar (Srinivasan *et al.*, 2001; Aiyer *et al.*, 2002). Also, co-chromatography studies with oxidative DNA adducts from $H_2O_2/CuCl_2$ show that polar adducts generated from $4E_2/CuCl_2$ may be generated in part via oxidative mechanisms (Gupta *et al.*, 2003). Several studies indicate that H_2O_2/Cu^{2+} as well as Cu^{+}/Cu^{2+} redox cycling is involved in the generation of reactive oxygen species (ROS) by $4E_2$ (Oikawa *et al.*, 2001; Frelon *et al.*, 2003). The results of this study correlates well with earlier studies on reduction of 8-oxodG induced by $H_2O_2/CuCl_2$ by ellagic acid (Srinivasan *et al.*, 2002). The trend in induction of both unidentified oxidative adducts and 8-oxodG were similar, but the absolute levels of 8-oxodG was higher after $4E_2/CuCl_2$ treatment. Copper ions are known to be associated with purine bases in the DNA, thus imparting site specificity for oxidation of Guanine bases (Oikawa *et al.*, 2001). However, we are currently unable to speculate on the mechanism of induction of novel polar adducts as they are as yet unidentified. Nevertheless, we have used the non-enzymatic cell-free system effectively, to screen agents that reduce total oxidative DNA damage induced by $4E_2/CuCl_2$. This, in turn, has further application in selecting agents that may be effective in mammary cancer reduction since oxidative DNA damage induced by estradiol metabolites is linked to mammary cancer incidence (Bolton *et al.*, 2000; Cavalieri *et al.*, 2000; Anderson *et al.*, 2003).

Ellagic acid, a polyphenol present in berries and a touted antimutagenic agent, is very effective in reduction 8-oxodG, a known mutagenic lesion (Srinivasan *et al.*, 2002). In this study, ellagic acid showed a dose-dependant modulation of many oxidative DNA adducts and reduced the levels of unidentified

adducts even at 10 μM, whereas a much higher concentration of ellagic acid (100 μM) is required to inhibit 8-oxodG (Figure 2.4-Inset), suggesting that these 2 lesions may develop via separate mechanisms. Singlet oxygen is known to play a predominant role in the generation of 8-oxodG, whereas the hydroxyl radical causes a more promiscuous damage to all DNA bases (Hiraku *et al.*, 2001; Frelon *et al.*, 2003). Thus, ellagic acid may be more effective in protecting against hydroxyl radical induced DNA damage at lower concentrations. Further, it is reported that ellagic acid covalently binds with DNA, but with a higher affinity to poly (dAp X dTp) than poly (dGp X dCp) (Dixit & Gold, 1986; Teel, 1986). Such selective interactions with the DNA bases may also explain the differential effects at lower doses. All other flavanoids tested have shown antioxidant effects in several studies, however it appears that the prooxidant effects of some agents (silymarin, ascorbic acid, epigallocatechin gallate etc.,) is due to the presence of metal ions such as Cu^{+} (Anderson *et al.*, 1994; Toyokuni & Sagripanti, 1996; Duthie & Dobson, 1999; Srinivasan *et al.*, 2002; Furukawa *et al.*, 2003; Yen *et al.*, 2003). Although it is possible to provide a mechanistic explanation for the pro-oxidant effect of these agents, the selection of an agent to be employed in the next tier depended on its efficacy in the first tier. Also, though *in vitro* tests provide an easy and fast analytical system to assess the efficacy of chemopreventive agents with respect to a particular mechanistic aspect of cancer, no *in vitro* system can completely predict the effect *in vivo*. However, it is impossible to test every agent in an *in vivo* study. Based on these criteria, only ellagic acid was selected to be employed in a short-term *in vivo* study since it was the most efficacious in reducing *in vitro* oxidative damage caused by a catechol-estrogen metabolite.

Liver is a primary organ involved in the first-pass mechanism that is affected by both harmful and protective components of the diet. It is also a highly metabolic organ that is exposed to high levels of oxidative DNA damage resulting from normal metabolism. The ability of any dietary component to reduce the levels of this oxidative DNA damage at baseline would make it an ideal preventive agent in the presence of additional oxidative stress. To determine if

berries and ellagic acid would protect against oxidative stress, we tested their efficacy in reducing oxidative DNA damage in the liver of CD1 mice fed diet containing 5% (w/w) of different berries, or 400 ppm ellagic acid. The berries investigated have both different ellagic acid and total anthocyanin content – raspberries (1500 ppm ellagic acid; 10,000 ppm total anthocyanin), strawberries (500 ppm ellagic acid; 2,000 ppm total anthocyanins) and blueberries (<100 ppm ellagic acid; 38,000 ppm total anthocyanins) (Daniel *et al.*, 1989; Wu *et al.*, 2006). The dose of ellagic acid was selected based on earlier work by Stoner and colleagues who showed the 400 ppm of dietary ellagic acid, when fed to rats for 23 days, showed significant reduction in hepatic P450 content (Ahn *et al.*, 1996). Further, the same dose was also effective in reducing N-nitrosomethylbenzylamine (NMBA)-induced esophageal tumors (Mandal & Stoner, 1990). Both red raspberries and ellagic acid elicited similar effects in reducing the baseline oxidative DNA damage. Since raspberries have the highest ellagic acid content among the berries tested and showed similar effects as pure ellagic acid, we explored the possibility of a shared mechanism in reducing DNA damage. Gene-expression analyses suggested that this effect may posssibly be due to up-regulation of DNA repair genes (Figure 6). Also, both raspberries and ellagic acid modulate several genes in a similar fashion suggesting that the ellagic acid content of raspberries may play a role in their effectiveness. However, the concentration of ellagic acid available in the diet through raspberries is 5 fold lower (75 ppm Vs 400 ppm), which shows that ellagic acid which is present as ellagitannins in berries, may be more bioavailable from berries. Indeed 5% strawberries were more effective than 400 ppm ellagic acid reducing NMBA-induced esophageal tumors (Mandal & Stoner, 1990; Carlton *et al.*, 2001). Further, raspberries contain moderately high levels of anthocyanins, which are known antioxidants (Wang & Lin, 2000; Wu *et al.*, 2006). This may also account for their effectiveness. The results also show that P450s may be differentially modulated with certain enzymes such as CYP 2A4 and 2E1 being up-regulated and others such as 3A11 down-regulated. Several studies have shown hepato-protective effects of ellagic acid such as protection against metal-toxicity and carbon-tetrachloride induced liver fibrosis (Thresiamma & Kuttan,

1996; Ahmed *et al.*, 1999; Singh *et al.*, 1999). It is clear from our results that ellagic acid and raspberries are hepato-protective via similar mechanisms and are highly effective in reducing baseline oxidative DNA damage. Also, these agents show up-regulation of DNA repair genes. Since breast cancer involves both increase in DNA damage as well as decrease of DNA repair induced by estradiol and its metabolites (Malins *et al.*, 2006), ellagic acid and berries may provide protection in estrogen-induced mammary cancer.

This study shows that ellagic acid is highly effective in preventing oxidative DNA damage both *in vitro* and *in vivo*. Further, the prevention of oxidative damage induced by $4E_2$, which is a postulated carcinogenic metabolite in breast cancer, suggests that ellagic acid may be a good candidate for the prevention of mammary tumorigenesis. In addition, raspberry a natural source of ellagic acid has similar effects via similar mechanisms also making it a suitable candidate for nutritional intervention. This warrants the application of both ellagic acid as a pure compound and in whole food (berries) in further long-term studies to test their effectiveness against mammary carcinogenesis.

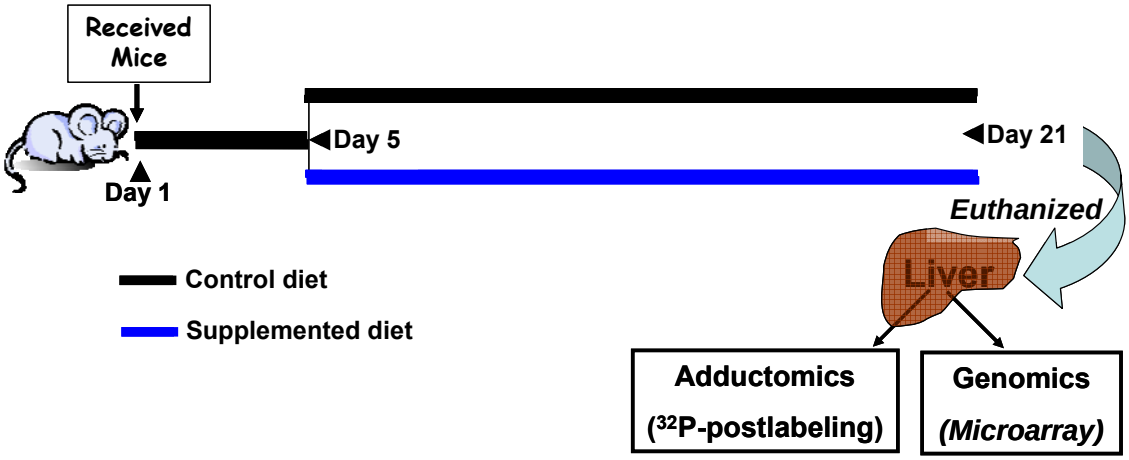


Figure 2.1. Experimental protocol- effect of berries and ellagic acid on DNA damage and gene expression in a short term *in vivo* study.

Five groups of 8 wk-old female CD-1 mice (n=6) were fed *ad libitum,* either a control diet (AIN-93M) or diet supplemented with 5% (w/w) strawberries, blueberries, raspberries or ellagic acid. Liver was analyzed for adduct levels and gene expression as described in Materials and Methods.

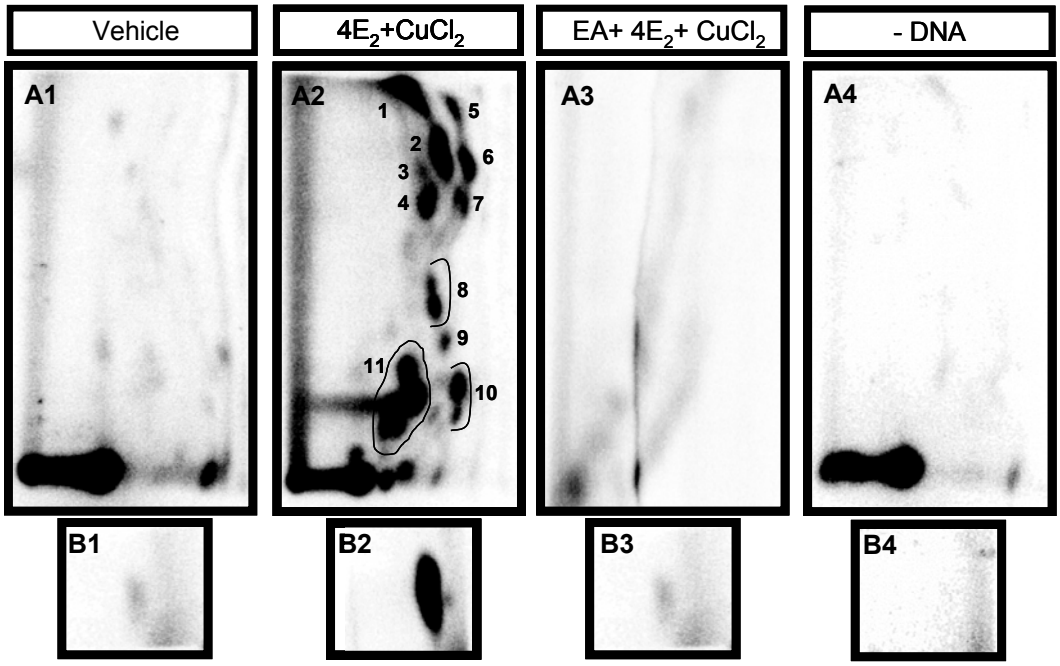


Figure 2.2. Representative ^{32}P-labeled DNA adduct maps of both uncharacterized polar oxidative adducts (A1-A3) and 8-oxodG (B1-B3) generated by redox cycling of $4E_2$ and $CuCl_2$.

The unidentified polar adducts (5 µg DNA) and 8-oxodG (0.5 µg) were ^{32}P-labeled and separated using two directional TLC. D1 (bottom to top) and D2 (left to right) solvents as described in materials and methods.

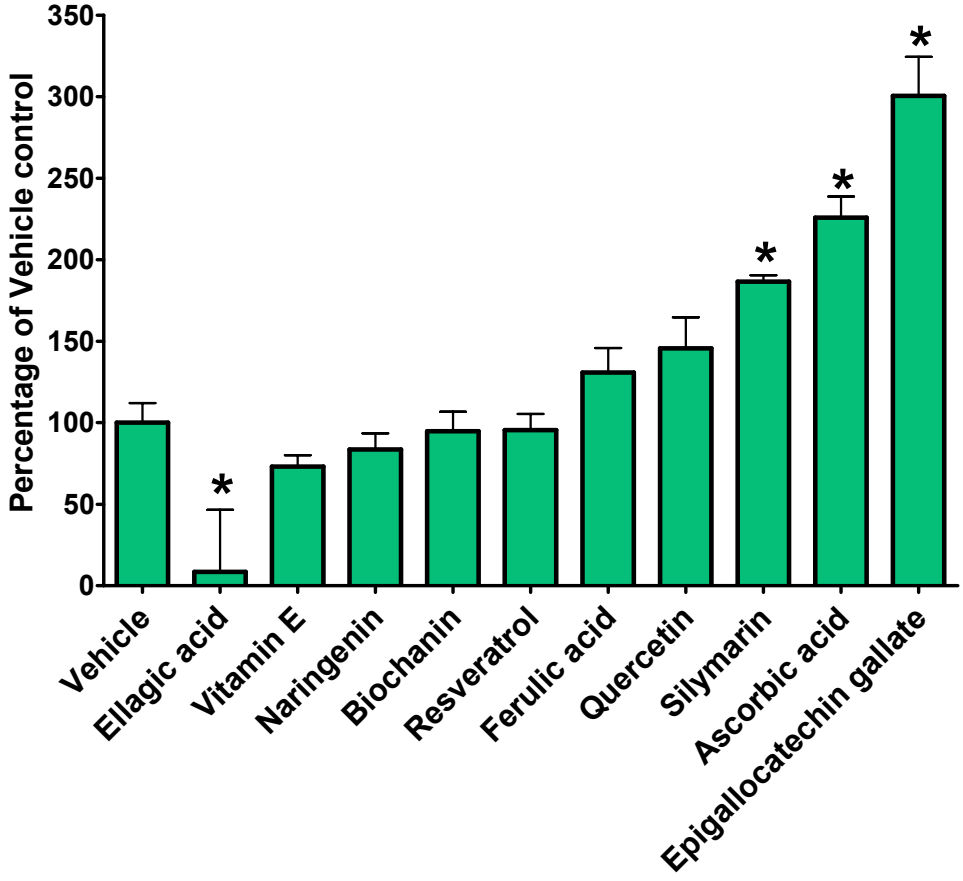


Figure 2.3. Modulation of oxidative DNA adducts by various phytochemicals.

The modulation of unidentified polar oxidative adducts is shown as a percentage of the vehicle control. The mean of 4 analytical replicates were compared by one-way ANOVA followed by Dunnett's post hoc test. A P-value <0.05 was considered significant and is denoted by an asterisk.

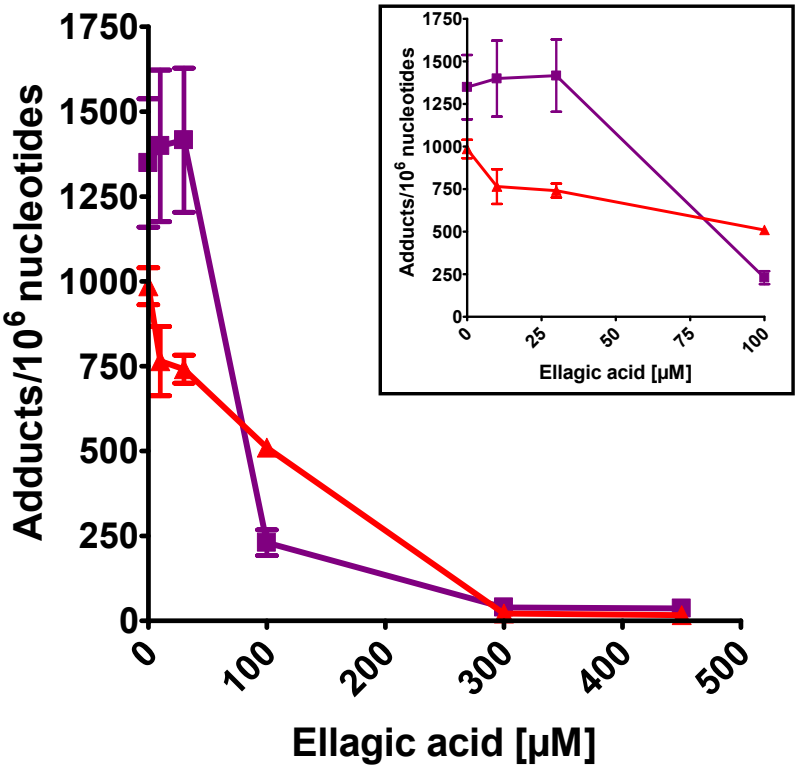


Figure 2.4. Effect of different concentrations of ellagic acid on oxidative DNA damage.

Both unidentified polar adducts (▲) and 8-xodG (■) were measured using ^{32}P-postlabeling/TLC and are represented as mean ± SE of 4 replicates. The inset shows the effects at lower concentrations. The test for linear trend was statistically significant with a p-value <0.0001.

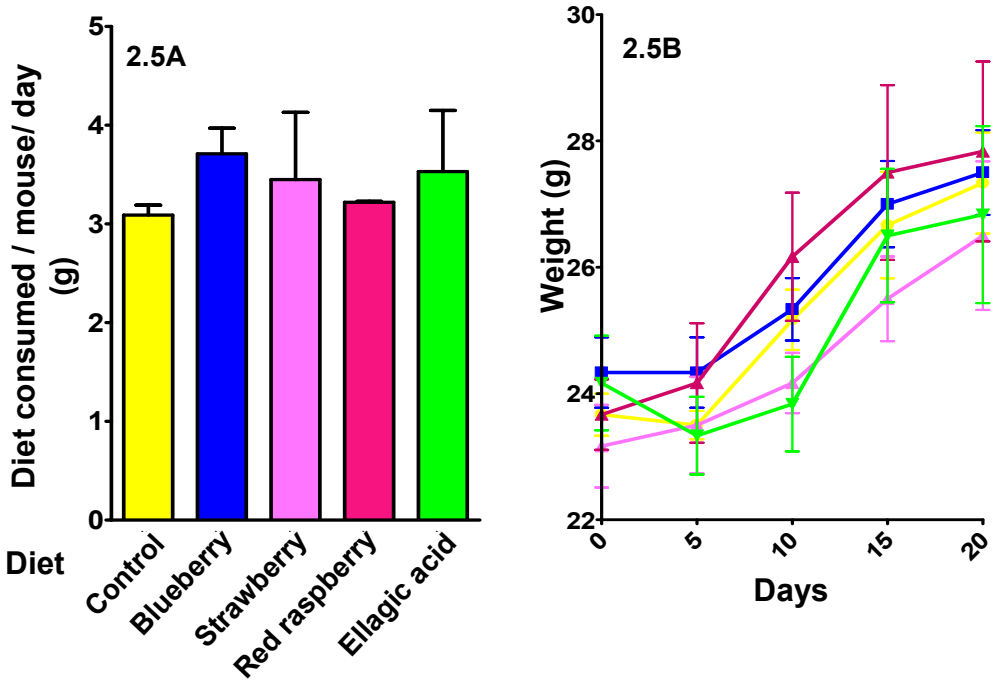


Figure 2.5. Comparison of diet consumption (A) and weight gain (B) between CD-1 mice fed different diets in a shot-term *in vivo* study.

The diet consumption and weight gains were recorded over a period of 5 weeks between CD-1 mice (n=6) fed control diet (●) or diet supplemented with either 5% w/w berries (blueberry-■; strawberry-♦; red raspberry - ▲) or 400 ppm ellagic acid (▼). Diet was weighed everyday and the consumption was calculated as described in Materials and Methods.

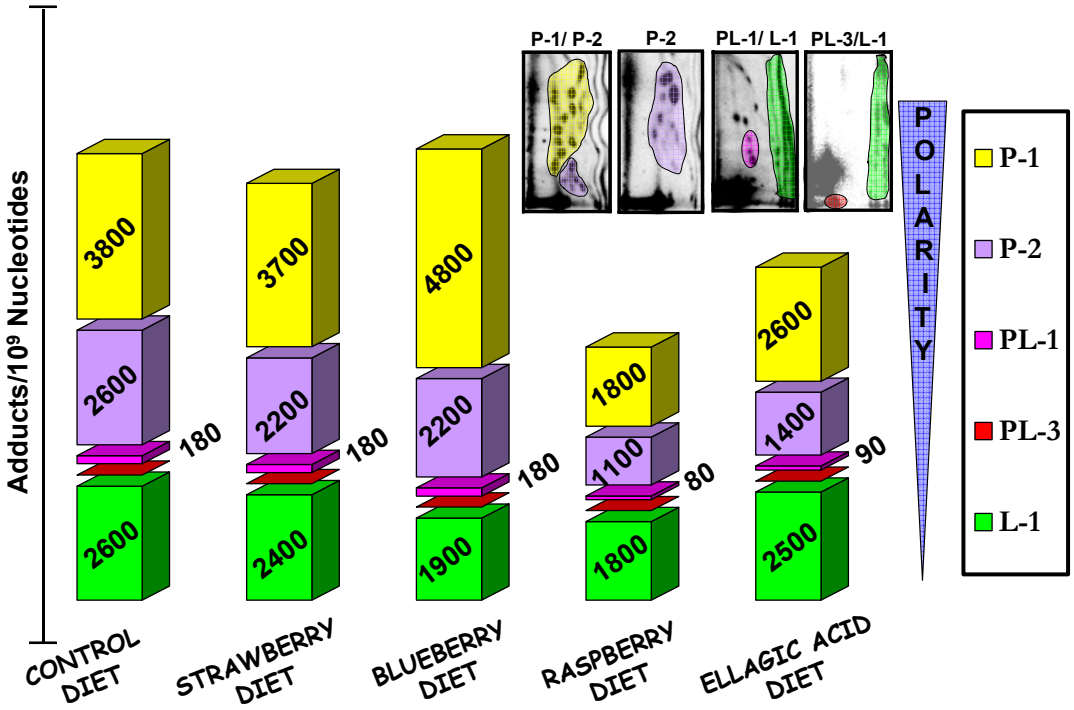


Figure 2.6. "Stackograms" exhibiting changes in the levels of various subgroups of liver DNA adducts in CD-1 mice fed either control or supplemented diet as indicated.

Adduct levels are mean of 6 individual values (SD 30%-50% of mean). Adducts whose levels are not marked were not measured. Inset - Representative ^{32}P-labeled TLC maps of subgroups of adduct. Each subgroup of adducts was visualized in the D1 using different salt concentration depending on their polarity as described in Materials and Methods.

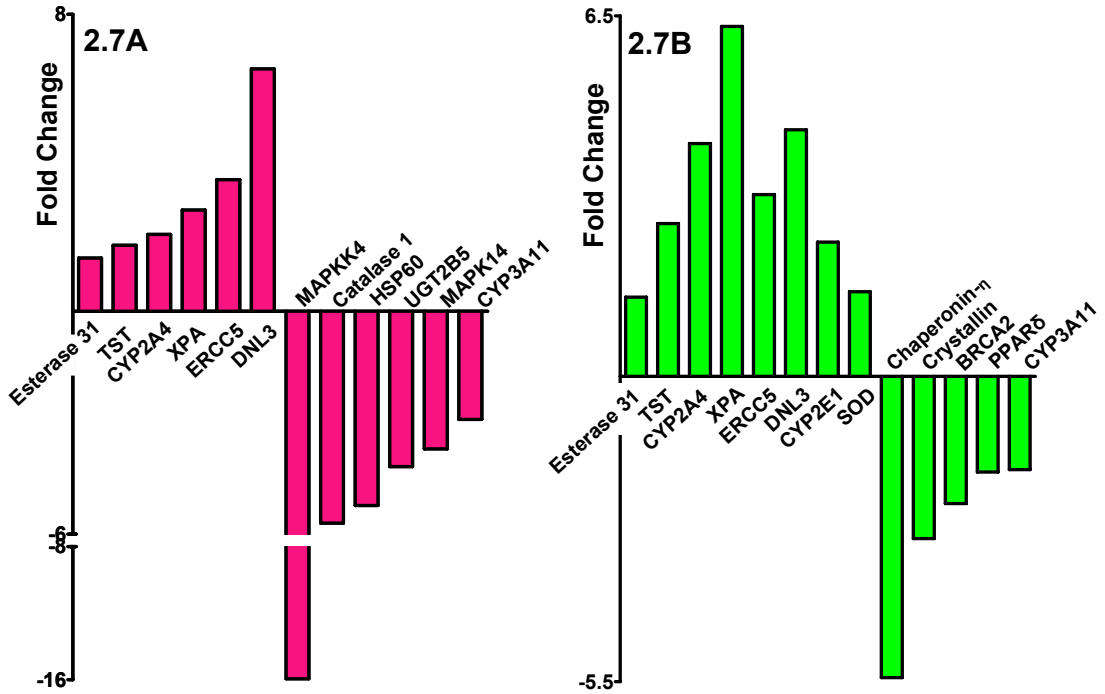


Figure 2.7. Genes with significantly altered expression in the liver of mice fed either 5% (w/w) raspberry- (A) or 400 ppm ellagic acid - supplemented diet (B). TST-Thiosulphate sulphur transferase; XPA- Xeroderma Pigmentosum group A complementing protein; ERCC5 - excision repair cross complementation group 5; DNL3 - DNA Ligase III; SOD –Superoxide Dismutase, extracellular; MAPK-Mitogen activated protein kinase; MAPKK-MAP Kinase kinase. Gene expression analyses were done in Dr. Margie Clapper's laboratory at the Fox Chase Cancer Center, Philadelphia, PA.

Table 2.1. Comparison of modified diet composition to AIN-93M diet.

Ingredients	**Percent of composition**	
	AIN-93M diet	Modified diet
Corn starch	46.6%	36.03%
Dextrose	15.5%	36.04%
Sucrose	10%	0%
Total CHO calories	***72.1%***	***72.07%***
Casien	14%	14%
Soy Bean Oil	4%	4%
Fiber	5%	5%
AIN93 Vitamin Mix	1%	1%
AIN93 Mineral mix	3.5%	3.5%
L- Cysteine	0.18%	0.18%
Choline Bitartrate	0.25%	0.25%

A modified version of the AIN-93M diet was fed to the mice. Diet ingredients were purchased individually from Dyets Inc., (Bethlehem, NJ) and mixed according to the given composition in a Hobart mixer until homogenous. The corn starch component was substituted for 5% berry diets such that the percentage of corn starch in these diets was 31.03%. No substitutions were done for the ellagic acid diet since this agent was added in insignificant quantities (0.04%). The diets were stored either at -80°C (>1 week) or 4°C (<1 week) until use. CHO- Carbohydrate. The composition of AIN-93M diet as described in Reeves *et al.,* 1993.

Chapter Three: Effect of dietary berries and ellagic acid on estrogen–induced mammary tumors in ACI rats.

Introduction

Breast cancer is the most commonly diagnosed cancer among women in the United States. Among the women diagnosed, over 53,000 women are expected to have ductal carcinoma *in-situ* (DCIS) (ACS, 2007a). DCIS remains one of the most commonly diagnosed breast cancers, with up to 25% recurrence as invasive carcinomas (Silverstein *et al.*, 1995). Prolonged exposure to physiological levels of 17ß-estradiol (E_2) is considered as a key risk factor for the development of sporadic breast cancer (Verheul *et al.*, 2000; Lippman *et al.*, 2001). Furthermore, associations between the use of hormone-replacement therapy and development of breast cancer in post-menopausal women (Verheul *et al.*, 2000; ACS, 2007b; ACS, 2007a) delineates a role for estrogens in human breast cancer.

Typically, in animal models, mammary tumors have been induced using carcinogenic doses of chemicals such as dimethylbenz[*a*]anthrazene, N-methyl-N-nitrosomethylurea, etc., (Cohen *et al.*, 1999; Kim *et al.*, 2004). Etiologically, exposure to any carcinogen in humans is usually chronic with DNA damage accumulating over a period of time, making exposure to bolus doses of carcinogens less relevant to the human scenario. Thus, there is a need for a relevant animal model to study breast cancer. August-Copenhagen Irish (ACI) rat strain is highly susceptible to estrogen-induced mammary tumors (Shull *et al.*, 1997; Li *et al.*, 2002b). These rats develop mammary tumors that bear close resemblance to human breast tumors in both histopathological and molecular aspects (Li *et al.*, 2002a; Weroha *et al.*, 2006). In addition, the etiology of disease development is also similar to the human scenario, with tumorigenesis occurring after chronic exposure to E_2 (Shull *et al.*, 1997; Li *et al.*, 2002b). Thus, the ACI rat model is highly relevant for chemopreventive intervention and translational research.

Translational research of cancer chemoprevention involves three major steps. First, epidemiological studies find an association between certain foods/diet and reduction in cancer incidence. Second, observational studies identify and employ the most potent components in these foods in various *in-vitro* and *in-vivo* assays to discover pathways, identify biomarkers and establish safety information. The last step is to employ these agents in clinical trials, and use established biomarkers to determine if the agent actually prevents or ameliorates cancer. Unfortunately, some of the agents identified and employed using this 3-step approach failed to show expected results in clinical trials (Hampton, 2005). Potential reasons for this failure are as follows: since most chemoprevention studies in animal models were performed using large doses of chemical carcinogens, mega-doses of chemopreventive agents were required for efficacy (Yuri *et al.*, 2003). Thus, the pharmacologic dose derived from such studies may result in adverse effects in humans. The concept of lower, more realistic doses of chemopreventive agents has been proposed after the high-dose adverse phenomenon observed in the CARET trials (Goodman *et al.*, 2004). Also, most observational studies fail to recognize the presence of and interactions between the different components found in whole foods (Meyskens & Szabo, 2005). The fractionation of a preventive food, green tea for example, usually yields one major component (epigallocatechin gallate) that is most effective and several other minor components that are somewhat less effective (Yang *et al.*, 2000). Nevertheless, the fact that there are several minor components that may play a role in the protective effect of the food is not commonly acknowledged (Meyskens & Szabo, 2005). These oversights may precipitate in the failure of an agent to elicit any positive response or even adverse effects in humans (Hampton, 2005).

Ellagic acid is a polyphenol formed by the dimerization of gallic acid in various plants (Maas *et al.*, 1991). It has been shown that ellagic acid may elicit cancer prevention by several mechanisms which include direct binding to DNA, attenuation of carcinogen metabolism via the P450 pathway, and down-regulation of cell-cycle activators and up-regulation of pro-apoptotic mechanisms

(Reviewed by Stoner & Mukhtar, 1995 and by Aggarwal & Shishodia, 2006). However, the bioavailability of ellagic acid as a pure compound after oral administration has been in question (Smart *et al.*, 1986; Teel & Martin, 1988). The effects of ellagic acid intervention on tumors other than those of the gastro-intestinal tract have largely been unexplored due to its suspected low bioavailability.

Ellagic acid in plants is present as ellagitannins (Larrosa *et al.*, 2005). It is released in the gut by the microflora and then absorbed as ellagic acid. Analysis of ellagic acid contents of various berries shows that while some commonly available berries such as black berries and raspberries are rich sources (1,500 ppm) others like blueberries (<100 ppm) are not (Daniel *et al.*, 1989b). Several studies by Stoner and colleagues have shown the protective effects of black raspberries on gastro-intestinal tumors induced by chemical carcinogens (Harris *et al.*, 2001; Kresty *et al.*, 2001; Stoner *et al.*, 2006). Although black raspberries and blueberries are good sources of anthocyanins, they differ significantly in their anthocyanin profile (Wu *et al.*, 2006). Black raspberry is a rich source of cyanidin-polymers whereas blueberry has a much wider range and is especially rich in delphinidin-polymers (Wu *et al.*, 2006). Blueberry has been much touted for its antioxidant properties, both historically and experimentally due to its anthocyanin content (Lau *et al.*, 2005; Yi *et al.*, 2005).

These facts formed the basis for the three-fold rationale of this study. First, we examined the efficacy of relatively low doses of natural chemopreventive agents, such as ellagic acid and berries, in reducing estrogen-mediated mammary tumors in ACI rats, so that maximum clinical relevance can be established from the results. Second, we provided both berries, a natural source of ellagic acid and pure ellagic acid in the diet to distinguish the effects of “whole food” versus “active ingredient” in its biological response. Finally, we chose berries which differed widely in their ellagic acid contents but had similar total anthocyanin levels to evaluate the role of each of the antioxidant components in yielding benefits.

In this chapter, the results from 3 independent studies involving supplementation with various berries and also involving 2 related animal models of estrogen-induced mammary carcinogenesis are presented.

Materials and Methods

Diets. Diets for Study 1 were ordered from Bio-Serv (Frenchtown, NJ) and diets for Studies 2 and 3 were ordered from Harlan-Teklad, Inc. (Madison, WI). The AIN-93M diet was supplemented with powdered berries (2.5% w/w) or ellagic acid (400 ppm). For Study 1, equal mixture of 5 different berries- red raspberry, black raspberry, blackberry, blueberry and strawberry- were used such that each berry constituted 0.5% (w/w) of the diet.

The black raspberries were procured as a freeze-dried powder from Van Drunen farms, (Momence, IL), through Dr. Gary Stoner of The Ohio State University (Columbus, OH). The processing of black raspberries was done as described (Harris *et al.*, 2001). All other berries were obtained as organic, fresh produce and processed in the laboratory. Blueberries were purchased from a local farm (Liberty, KY). Three different high bush cultivars of blueberry (V. corymbosum L) - Bluecrop, Berkeley and Bluejay - were harvested in the morning, stored overnight below 10ºC and transported the next morning to the laboratory for processing. Blackberries were purchased from Reed Valley orchards (Paris, KY). Red raspberries and strawberries were purchased from a local food co-op (Lexington, KY). All berries except black raspberries were processed similarly. Berries were rinsed with water and dehydrated using commercial food dehydrators (at 40° - 60°C). The dried berries were finely powdered using a kitchen blender (Sumeet Asia Kitchen Machine, Sumeet Research and Holdings Ltd., Chennai, India), sieved and lyophilized to remove residual moisture. All berries were then vacuum packed and stored at -20°C until use. The three different cultivars of blueberries were mixed in equal ratios prior to mixing in diet. Ellagic acid (>96% purity) was purchased from LKT labs (St. Paul, MN). The cornstarch and fiber components of the AIN-93M diet were replaced for the berry diets, based on the nutritional information available for each berry

(http://www.nal.usda.gov/fnic/foodcomp, Table 3.4). A proximate analysis was performed at Harlan-Teklad (Madison, WI) to ascertain that the diets were isocaloric. This included the measurement of protein (Kjeldahl method; factor NX6.38 for milk protien), fat (ether extraction), crude fiber, moisture (at 70°C) and ash (at 600°C). The carbohydrate levels were arrived at by subtraction of protein and fat values from the total calories. The daily feed intake by animals was assessed by subtracting the unused diet from the initial amount provided per cage divided by the number of rats in the cage.

Animal studies. Study 1 and Study 2: Female ACI rats were 7-8 weeks old when purchased and 10-11 weeks old when implanted with 3 cm silastic implants containing 27 mg 17ß-estradiol. Study 1 was conducted as a pilot study with number of animals as specified (Tale 3.1) and Study 2 was designed with enough number of animals in each group to achieve statistical significance (Table 3.2).

Study 3: Female ACI rats were 5-6 weeks old when purchased and 8-9 weeks old when implanted with 1.2 cm silastic implants containing 9 mg 17ß-estradiol (Table 3.3). This study was perfomed to avoid the mortality associated with the larger implant used in Studies 1 and 2.

Animal treatment and assessment of tumor indices. Female ACI rats were purchased from Harlan-Sprague-Dawley, Inc. (Indianapolis, IN), housed under ambient conditions and had access to food and water *ad libitum*. Animals were acclimated for 1 week on AIN-93M diet prior to randomizing them into different groups (Tables 3.1 -3.3). After feeding experimental diets for 2 weeks, animals then received one of the three E_2 implants - a 3 cm silastic implant containing 27 mg 17ß-estradiol or a 1.2-cm implant containing 9 mg 17ß-estradiol as described (Shull *et al.*, 1997; Ravoori *et al.*, 2007) or sham implants, depending on study design (Table 3.1-3.3). Briefly, silastic tubing (Allied Biomedical Inc., Ventura, CA) was cut in respective lengths and sealed at one end using a silicone adhesive (Factor II Inc., Lakeside, AZ). They were then filled

with 17ß-estradiol and the other end was sealed. These were then subcutaneously implanted in to rats under isofluorane anaesthesia (Ravoori *et al.*, 2007). Animals were weighed biweekly after estrogen implantation to track weight changes and disease progression. Starting at 12 weeks after estrogen implantation, animals were palpated weekly for tumor appearance. The frequency of palpation was increased to twice a week, upon appearance of the first tumor, to record tumor latency and incidence. The experiment was terminated after 24 weeks of estrogen treatment for Studies 1 and 2, and after 32 weeks for Study 3. At termination, animals were euthanized by CO_2 asphyxiation and examined grossly for the presence of mammary tumors. Each tumor was excised and measured in all 3 dimensions using calipers and the tumor volume was calculated using the standard formula for the volume of a spheroid - 2/3π r1*r2*r3, where r1, r2 and r3 represent the radii of the tumor. The tumor volume per animal is the sum of the volumes of all individual tumors. Representative tumors were analyzed for histopathology to confirm that they were mammary adenocarcinomas. The fixing, sectioning and H and E staining of the tissues were done at the Pathology Core Lab at the University of Louisviile (Louisville, KY), and the stained sections were examined by two trained pathologists – Dr. Srivani Ravoori and Dr. Sunati Sahoo to determine that they were mammary adenocarcinomas.

Analysis of 17β-estradiol levels. Trunk blood was collected from animals after euthanasia and the serum estradiol levels were measured by Roche E170 immunoassay analyzer using electrochemiluminescent detection. These analyses were done in the Pathology department at the University of Louisville Hospital under the supervision of Dr. James Miller and were paid for from grant support.

Statistical analysis. Experimental data were analyzed using the Statistical Analysis Software, SAS version 8. The longitudinal analysis of the data on body weights was carried out using the PROC MIXED procedure. A linear trend for weight change was established at p-value <0.0005. The differences in weight

gains or losses between different groups were assessed using the same procedure and, for this analysis, a p-value < 0.0001 was considered significant due to large number of weight comparisons at 13 biweekly time points. The tumor volume and multiplicity were compared using the General Linear Models (SAS procedure PROC GLM) and the Poisson Regression Model (SAS procedure PROC GENMOD) procedures, respectively, and a p-value <0.05 was considered significant. The difference in the mortality index was assessed using the non-parametric survival analysis techniques and the log-rank test. All statistical analyses except the non-parametric survival analysis and the log-rank test, were done by our collaborator Dr. Cidambi Srinivasan, Department of Statistics, University of Kentucky (Lexington, KY).

Results

Serum estrogen levels. Serum 17β-estradiol was analyzed only for Study 2. The levels were measured at 6 weeks and 25 weeks. At 6 weeks, the mean serum estrogen levels were significantly ($p<0.0001$) elevated (194 ± 20 pg/ml) in the treated versus control group (35 ± 9 pg/ml). The levels further increased somewhat after 24 weeks of treatment (236 ± 24 pg/ml) but the increase was insignificant. No significant change was seen in age-matched controls (44 ± 7 pg/ml). There was no effect of dietary supplementation on serum estradiol levels at both 6 and 25 weeks. The serum E_2 analysis for Study 3 were not performed but were assumed to be similar to values from a similar model established in our laboratory (65 ± 5 pg/ml at 12 weeks and 200 ± 44 pg/ml at 32 weeks) (Ravoori et al., 2007).

Effect of estrogen treatment and experimental diets on body weight. For all studies conducted, measurement of diet intake showed no significant difference between various groups, suggesting that berry supplementation had no effect on the diet intake. Furthermore, animals gained weight progressively irrespective of the implants; however, estrogen-treated animals gained more weight than their sham counterparts starting at 4 weeks after the treatment, irrespective of the diet, indicating that this weight gain was a direct result of the

estrogen treatment (Figures 3.1- 3.3). Sham-treated animals on control diet continued to gain weight until the end of the experiment in all studies (Figures 3.1-3.3). In Study 2, sham-treated animals receiving experimental diets also showed similar trends in weight gain, except that diet supplemented with black raspberries showed higher weight gain, starting as early as 2 weeks after the experimental diet, but the difference was significant only after 20 weeks of the dietary regimen ($p < 0.05$) (Figure 3.2A). However, both proximate analyses of the diets as well measurements of diet intake did not show a difference in caloric intake between the groups.

Effect of berry- and ellagic acid-supplemented diets on the disease-associated weight loss. In Study 2, at 22 and 24 weeks, the difference in weight between estradiol- versus sham-treated animals on same diet was significantly lower ($p<0.0001$) for all groups (compare figures 3.2 A and B). In contrast, none of the sham-treated groups lost weight until the end of the study suggesting that the weight loss was a disease-associated phenomenon in the estradiol-treated animals. Comparison of estradiol-treated groups on various diets revealed that animals fed control diet lost the most weight, followed by animals fed blueberry-, black raspberry- and ellagic acid-supplemented diets (Figure 3.2B). Ellagic acid-fed animals showed significant resistance to weight loss even towards the end of the study, i.e., from the 20^{th} ($p< 0.05$) to the 24^{th} week ($p<0.005$). Thus, there was an intervention-associated prevention of weight-loss in all estradiol-treated animals, with ellagic acid-supplemented diet showing the most-pronounced effect. Although similar trends were seen in Study 1, the effect did not achieve statistical significance due to small sample size.

Effect of estrogen treatment and experimental diets on the rate of mortality. In Study 2, the morbidity in estradiol-treated groups was defined by the loss of >7g a week. This was based on the weight gain comparison in sham-treated animals, whose weight gain was ≥ 3g per week. In addition, other parameters such as loss of mobility, balance, grooming, the presence of eye deposits and a dull hair coat were taken into account and scored subjectively on

a scale of 1 to 5 (1 being the best and 5 being the worst). Animals that did worse (score > 3) on 3 or more of these criteria and also had rapid weight loss were euthanized. This was taken as indicator of the mortality (Figure 3.4). Additionally, animals whose tumor size had reached >1.3 cm in diameter were also euthanized. These animals, however, were excluded from the mortality index because they did not meet the morbidity criteria. Estrogen-treated animals on control diet showed the highest morbidity and mortality rate starting at 18 weeks after the treatment. The survival rate in this group progressively declined, reaching <50% after 24 weeks, thus only 11 of 25 animals survived at the termination of the study. In contrast, all intervention groups were significantly different from the control diet (log rank test, p-value <0.005): Both ellagic acid- and blueberry-fed animals showed no morbidity and had >85% survival at 24 weeks. The group on black raspberry-supplemented diet initially showed a higher survival rate, but it declined rapidly and had 60% mortality at 24 weeks. Although the ellagic acid group showed no sign of morbidity, 3 out of 22 animals had to be euthanized before 24 weeks because of the large tumors. These data suggest that the disease progression, as measured by the incidence of morbidity, was significantly delayed by the intervention - by about 3 to 6 weeks compared with the control group.

In Study 3, there was no treatment associated mortality. This study employed the improved model of estrogen-induced mammary carcinogenesis first described by others in our laboratory (Ravoori et al., 2007, in press). This model significantly eliminates disease associated mortality at the cost of a slightly extended tumor latency period (32 versus 24 weeks). However, animals were euthanized before the final termination if the tumor size reached >1.3 cm in diameter. These animals are not included in the final comparison of tumor indices. It is notable to mention that, the control diet showed the highest animal loss before 32 weeks (5 out of 21) compared to diets supplemented with 2.5% blueberry (2/16), 1% black raspberry (1/16), 2.5% black raspberry (0/16) and 400 ppm ellagic acid (3/16), suggesting the preventive effects of supplementation on

tumor progression. The loss for animals supplemented with 1% blueberry was not different from the control (4/17).

Effect of experimental diets on tumor indices. Tumor incidence was 100% in all 17ß-estradiol-treated animals in all studies. The results for individual studies are described below.

Study 1. Tumors were first noticed at 150 days after estrogen treatment; however, this was not the first incidence as systematic palpation after 12 weeks was not done for this study. Also, though there was no mortality in this study, the tumor data for 4 animals from the ellagic acid group could not be obtained and are not included in the calculations. There was no difference in tumor latency or tumor incidence between the groups. On termination, the tumor multiplicity in the control diet group was 9.0 ± 1.6 and tumor volume was 4,231 ± 1,675 mm^3. Only tumors that were larger that 0.5 cm were analyzed for histology. In this particular study, a number of smaller tumors (1-2mm in diameter) were seen. Due to the peculiarity of this animal model, where gross examination of mammary tumors presents as a cluster of smaller tumors joining to form a bigger tumor, all nodules that appeared in the mammary regardless of their size or appearance were counted as mammary tumors. This may have lead to the over-estimation of the number of tumors in all groups, especially since lymph nodes often look like tumors on gross examination. Therefore, only tumor volume and to some extent tumor burden are reliable measurements of tumor indices in this pilot study. Blueberry diet reduced both tumor volume and volume/tumor by 70% ($p < 0.001$) and 84% ($P < 0.0001$) respectively. Mixed berries reduced tumor volume by 75% ($p < 0.01$) and volume/tumor by 60% ($p < 0.02$). Although, ellagic acid reduced tumor volume by > 40% and volume/tumor by >65%, it was statistically insignificant (Table 3.5, Figure 3.7).

Study 2. The first palpable tumor was detected at 90 days after estradiol treatment without any intervention, with a mean tumor latency of 134 ± 6 days. The tumor development was marginally delayed in the intervention groups by 18,

20 and 21 days for animals fed blueberry-, black raspberry- and ellagic acid-supplemented diets, respectively (Figure 3.5). However, there was no significant difference in the mean tumor latency between the intervention groups and was 143 ± 5; 140 ± 6; 141 ± 6 for blueberry, black raspberry and ellagic acid supplemented diets respectively. On termination after 24 weeks the tumor multiplicity in the control diet group was 7.9 ± 2.4 and the tumor volume was 685 ± 206 mm^3 (Table 3.6). All tumors were confirmed to be mammary adenocarcinomas through histopathology (Figure 3.9). Blueberry diet resulted in a 40% reduction in tumor volume without any change in tumor multiplicity. Black raspberry diet resulted in a 70% reduction ($p<0.05$) in tumor volume and nearly 40% reduction in tumor multiplicity. Ellagic acid showed the highest reduction in tumor volume (>70%; $p<0.05$) and tumor multiplicity (>43%; $p<0.05$) (Table 3.6, Figure 3.8).

Study 3. In this study, two different doses of each berry were tested- the same dose (2.5% w/w) as used in Study 2 and a lower dose (1% w/w). The results are summarized in Table 3.7 and presented in figure 3.10. The first palpable tumor appeared 127 days after estrogen treatment in the control diet group, with a mean tumor latency of 154 ± 4 days. There was no difference in the appearance of the first palpable tumor between the groups (Figure 3.6). However, the mean tumor latency for each group was: 1% blueberry-152 ± 6; 2.5% blueberry-162 ± 6; 1% black raspberry-168 ± 6; 2.5% black raspberry-149 ± 6; 400 ppm ellagic acid 170 ± 5. At termination of the study, the mean tumor volume was 2804 ± 547 mm^3 in the control diet group and tumor multiplicity was 11.7 ± 1.4. None of the tumor indices measured was reduced by the 1% blueberry diet suggesting that blueberries may be ineffective at this dose (Table 3.7). At the 2.5% dose, blueberries reduced tumor volume by 45%, tumor multiplicity by >30% ($p < 0.05$) and volume/tumor by 43%. Black raspberry at both doses tested (1% and 2.5%) had similar effects on reducing tumor multiplicity (30% reduction; $p< 0.05$), however they varied in their effects on tumor volume, hence volume per tumor. The higher dose had a greater effect in reducing tumor volume (56% versus 33%). Ellagic acid reduced tumor volume,

multiplicity and volume per tumor by 45%, 37% ($p < 0.05$) and 47% respectively. The reductions in tumor volume and volume per tumor were not statistically significant due to high intra-group variability (Table 3.7).

Discussion

The application of preventive agents in randomized clinical trials involves three distinct steps: First, epidemiological studies reveal a correlation between the high intake of a particular food/diet and low prevalence of a certain disease. Second, data from both *in vitro* and *in vivo* studies usually in rodent models provide validation of these correlations. The initial step in this process is to analyze and fractionate the different components of the whole food, and find which of the component(s) are most effective. Although rodent studies are highly dependent upon pharmacological response, the studies generally use high doses of carcinogens, which in turn require high doses of chemopreventive agents to elicit biological response (Yuri *et al.*, 2003). The ACI rat model is highly amenable to translational prevention research due to its steady exposure to estrogen. However, only few intervention studies have been reported in ACI rats where mammary tumors are induced by E_2. Among these, Shull and co-workers examined the effect of hypo-caloric feeding on reduction of mammary gland tumors (Harvell *et al.*, 2002) and Li and his team examined the chemopreventive effect of tamoxifen (Li *et al.*, 2002b) and both studies reported effective inhibitions of mammary tumorigenesis. In our studies we have investigated the efficacy of both the whole food (berries) and one of its principal components (ellagic acid) in reducing estrogen-mammary carcinogenicity in the ACI rat model.

The third and final step in translational research is employing effective agents in clinical trials to ameliorate disease incidence in humans. The concept of “pharmacological intervention” versus “dietary supplementation” must be clearly differentiated. The translation of an epidemiological observation through experimental design cannot be effective unless the synergism between the different components in the food is acknowledged (Block, 1995; Meyskens and

Szabo, 2005). In this study we have addressed these issues by adopting a unified approach to chemoprevention.

Ellagic acid administered orally was initially shown to reduce tumors in both mouse-skin and mouse-lung tumor models (Lesca, 1983; Mukhtar *et al.*, 1984a; Mukhtar *et al.*, 1986). The various mechanisms attributed to these effects included direct binding of ellagic acid to the DNA and modulation of both phase I and phase II enzymes involved in the metabolism of carcinogens (Reviewed by Stoner and Mukhtar, 1995). Subsequent studies suggested that ellagic acid may not be highly bioavailable, since >50% of the material administered orally was excreted as such in feces and a large percentage of the material absorbed was removed by the kidney (Smart *et al.*, 1986; Teel & Martin, 1988). Thus, bio-concentration of ellagic acid may not be high enough to elicit a response in any organ site peripheral to the gut, where it has been shown to persist for at least 24 h after gavage (Teel & Martin, 1988). Also, there is evidence that ellagic acid strongly binds to all macromolecules in intestinal epithelial cells in tissue culture (Whitley *et al.*, 2003). The evidence taken collectively suggests that the gut may be the prime organ where ellagic acid is effective in eliciting anti-tumor effects. However, recently Stoner and colleagues have shown the presence of ellagic acid in the plasma of human subjects fed black raspberries orally (Stoner *et al.*, 2005). Also, it is reported that about 0.2 to 2% of the orally administered dose was found in peripheral organs after gavage (Smart *et al.*, 1986; Teel & Martin, 1988), implicating that if provided via diet over time, steady-state levels could be achieved.

Indeed, administered via diet at 400 ppm, ellagic acid was effective in reducing 2-acetylaminiofluorene-induced hepatocellular carcinomas in ACI/N rats (Tanaka *et al.*, 1988). However, when given at 8,000 ppm 4 weeks prior to 7,12-dimethylbenz[*a*]anthracene administration to Sprague-Dawley rats, ellagic acid achieved only a modest (20%) reduction in mammary tumor incidence (Singletary & Liao, 1989). Also, doses of 4,000 and 8,000 ppm ellagic acid failed to elicit significant response in azoxymethane-induced colon tumors (Rao *et al.*,

1991). Subsequently, Mandal and Stoner (Mandal & Stoner, 1990) demonstrated interplay between carcinogen and ellagic acid dose ratios, in a rat-esophageal tumor model. So far, ellagic acid has only been tested in animal models where tumors are induced by bolus doses of carcinogens. As discussed earlier, ACI rat model differs vastly from other chemical carcinogen-induced rodent tumor models in that it delivers a steady dose of the carcinogen over the entire duration of the tumorigenesis. Thus, the effects of bioavailability and bioconcentration in meeting the carcinogen challenge are altered. Further, it has been shown that both ellagic acid and several anthocyanins may act as a selective estrogen receptor mediators, which may partially account for their effect in our study (Schmitt & Stopper, 2001; Larrosa *et al.*, 2006).

Berries vary in their contents of ellagic acid from < 100 ppm (blueberry) to > 1500 ppm (black raspberry) (Daniel *et al.*, 1989a; Harris *et al.*, 2001). In Study 1, various berries were tested as a mixture to assess whether berries have some efficacy against estrogen-induced mammary tumors or not. Nevertheless, both the mixed berries as well as black raspberries provided a high-ellagic acid group to contrast with the effects of a low-ellagic acid group (blueberries only). Berries, apart from being good sources of ellagic acid are also rich sources of other phytochemicals such as anthocyanins, flavonoids such as quercetin, kaempferol, and vitamins and minerals (Harris *et al.*, 2001; Wada & Ou, 2002). Interesting to note is that each berry has a significantly different anthocyanin and total phenolics profile (Wada & Ou, 2002; Wu & Prior, 2005; Wu *et al.*, 2006). While, black raspberries are high in total anthocyanins, their anthocyanin source is primarily cyanidin-polymers, whereas blueberries are known to contain several different types of anthocyanins, including high levels of delphinidin-polymers (Wu & Prior, 2005; Wu *et al.*, 2006). Evidence suggests that several of these molecules may have partial estrogenic activities (Schmitt & Stopper, 2001; Larrosa *et al.*, 2006). It is not known yet how these will affect an estrogen-induced mammary carcinogenesis model. The possible interactions and outcomes are discussed in chapter 5 in some detail.

Ellagic acid is released from the ellagitannins by the action of the gut microflora (Larrosa *et al.*, 2005). It is not known whether a natural source of ellagic acid would result in a higher bio-availability due to synergistic effects of other natural components. The level of ellagic acid in the black raspberry diet or mixed berry diet is about 30 – 45 ppm (based on a 2.5% dietary dose), but it is highly effective in reducing tumor indices (Table 3.1-3.3). Pure ellagic acid, at about 10 times this dose elicits the same response. Thus, either ellagic acid is more bioavailable from ellagitannins in berries, or other components of berries such as anthocyanins as well as other flavanoids, work synergistically to offer better protection. There is support for the latter, since blueberry, a poor source of ellagic acid but rich in anthocyanins also elicits a moderate reduction in the estrogen mammary carcinogenicity as well a significant reduction in morbidity.

Disease progression can be understood as the decline in health of the animals as indicated by weight loss and increased morbidity score. It was theorized that the estradiol doses given to these animals may have been too high and the subsequent toxicity induced by the estradiol levels may have a confounding effect on the actual effectiveness of these diets. Li and co-workers in their initial work reported that animals implanted with 3 mg cholesterol pellet had a serum estradiol levels <145 pg/ml at 6 months. These animals did not show high mortality albeit a marginal weight loss (Li *et al.*, 2002b). In a recent report using the cholesterol pellet model, other investigators (Mesia-Vela *et al.*, 2006) reported that animals suffered significant weight loss even at 20 weeks. The serum estradiol levels in these animals were >300 pg/ml at 6 and 12 weeks. This suggests that a high serum estradiol level plays a significant role in inducing morbidity in the animals. It is clear from our results that although dietary interventions were highly effective in reducing weight loss and morbidity (Figure 3.4), the relatively low doses of dietary intervention may have been insufficient to completely protect from adverse effects of high circulating estradiol levels. Also, the varying effects of the two berries in preventing the morbidity may be related to the differences in their anthocyanin content as well as their anthocyanin profiles (Figure 3.4), (Wu *et al.*, 2006).

Using an improvised model our laboratory has found that reduced serum levels of estradiol delivered by shorter estradiol silastic implants can produce 100% tumor incidence at the expense of somewhat longer duration (7-8 months) (Ravoori *et al.*, 2007).This model was used in Study 3 and it was hypothesized that lower doses of the berries would be effective in reducing mammary tumor indices due to the presence of lower levels of circulating E_2 . However, the results from Study 3 fail to support this hypothesis (Study 3 versus Study 2) (Tables 3.2, 3.3). Although speculations about the estrogenic activities of berry components and their interactions with the cellular signaling pathways may partially explain the results, more investigation is necessary to ascertain the exact mechanisms.

In conclusion, these studies consistently show significant reduction of estrogen-mammary carcinogenicity by dietary berries and ellagic acid. They also reveal the *in-vivo* efficacy of berries and ellagic acid in reducing tumorigenesis in an organ site other than the gut.

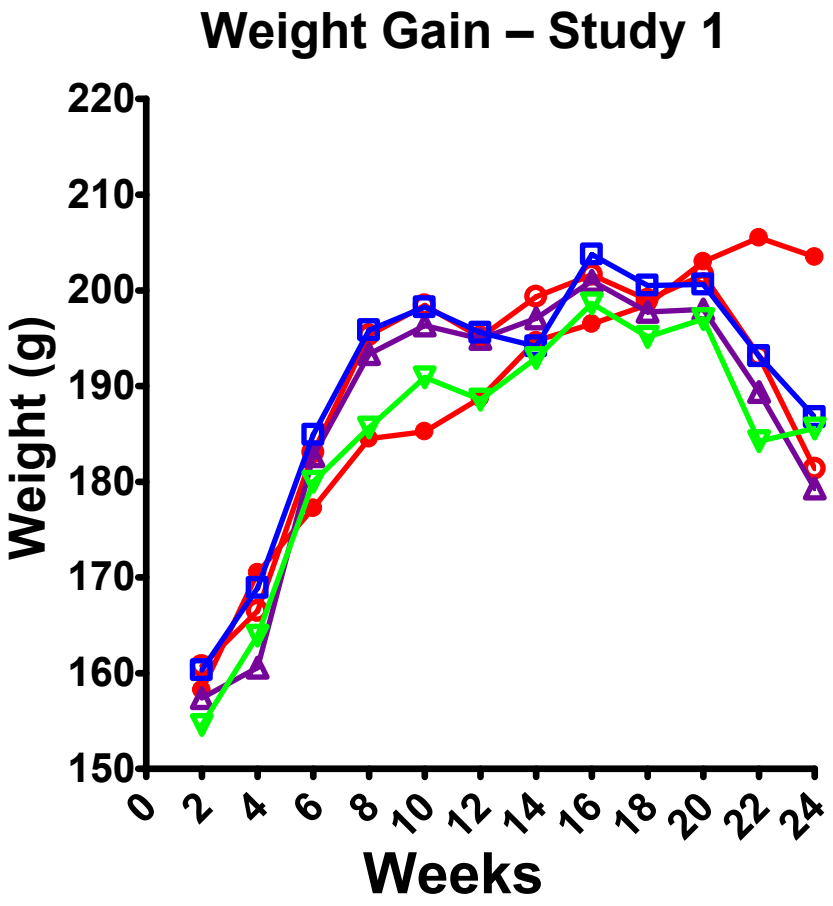


Figure 3.1. Comparison of weight gain in animals supplemented with dietary berries and ellagic acid and treated with either sham-implants or implants containing 27 mg E_2 in a pilot study.

Animals were weighed every fortnight until termination of study. Sham treated (closed symbol); estradiol treated (open symbols). Control diet ●, ⊖ ; Blueberry diet ⊟ ; Mixed berry diet ▲ ; Ellagic acid diet ▽ . The differences between the groups were not statistically significant due to low number of animals per group.

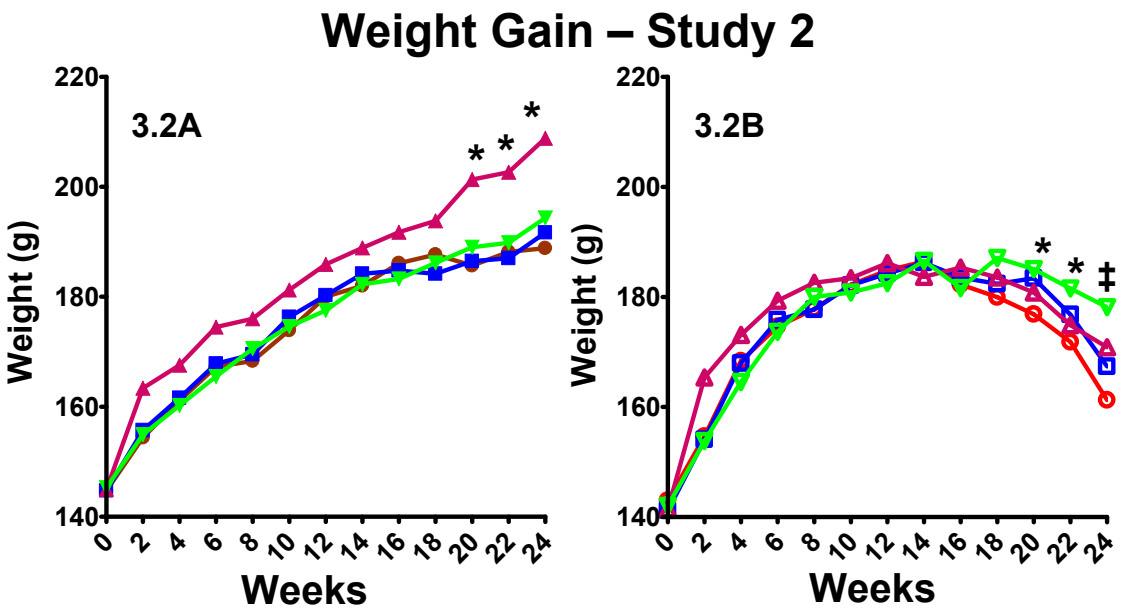


Figure 3.2. Comparison of weight gain in animals supplemented with berries and ellagic acid and treated with either sham-implants or implants containing 27 mg E_2. Animals were weighed every fortnight until termination of study.
3.2A -SH treated (closed symbols); 3.2B - Estradiol treated (open symbols).
Control diet , ; Blueberry diet , ; Black raspberry diet , ; Ellagic acid diet , . Statistically significant weight differences are indicated.
* - Statistically different from animals fed control diet ($p < 0.05$).
‡ - Statistically different from animals fed control diet ($p < 0.005$).

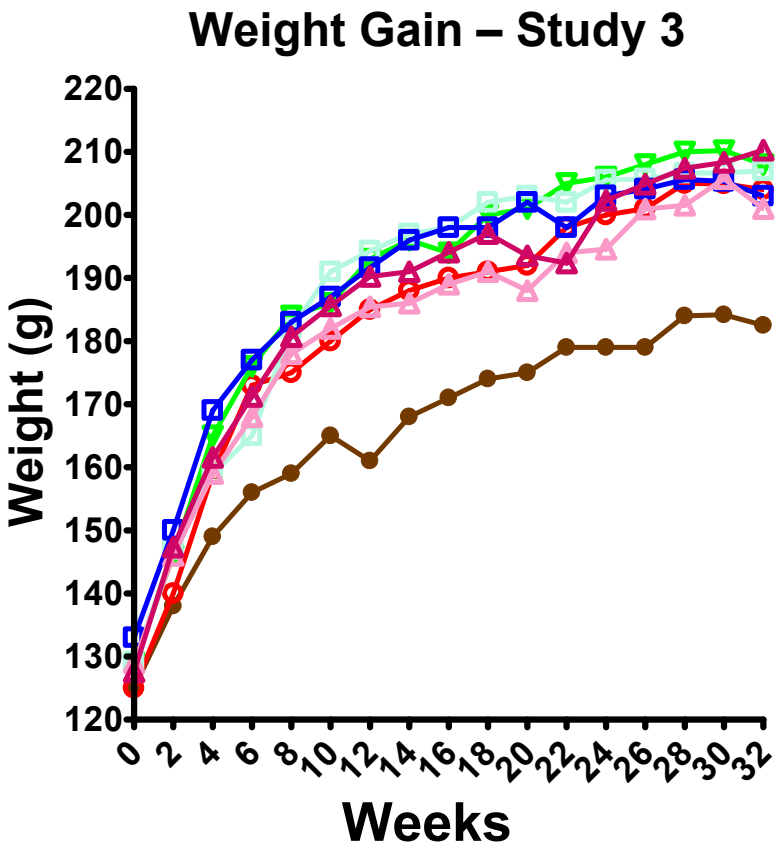


Figure 3.3. Comparison of weight gain in animals supplemented with dietary berries and ellagic acid and treated with either sham-implants or implants containing 9 mg E_2.

Animals were weighed every fortnight until termination of study.

Sham treated (closed symbols); E_2 treated (open symbols).

Control diet, ; Blueberry diet (1% w/w) ; Blueberry diet (2.5% w/w); Black raspberry diet (1% w/w) ; Black raspberry diet (2.5% w/w) ; Ellagic acid diet. The differences were not statistically significant between the estradiol treated groups at any time point. All estradiol treated groups were significantly different from the sham treated group starting at 8 weeks after treatment until the end of the study

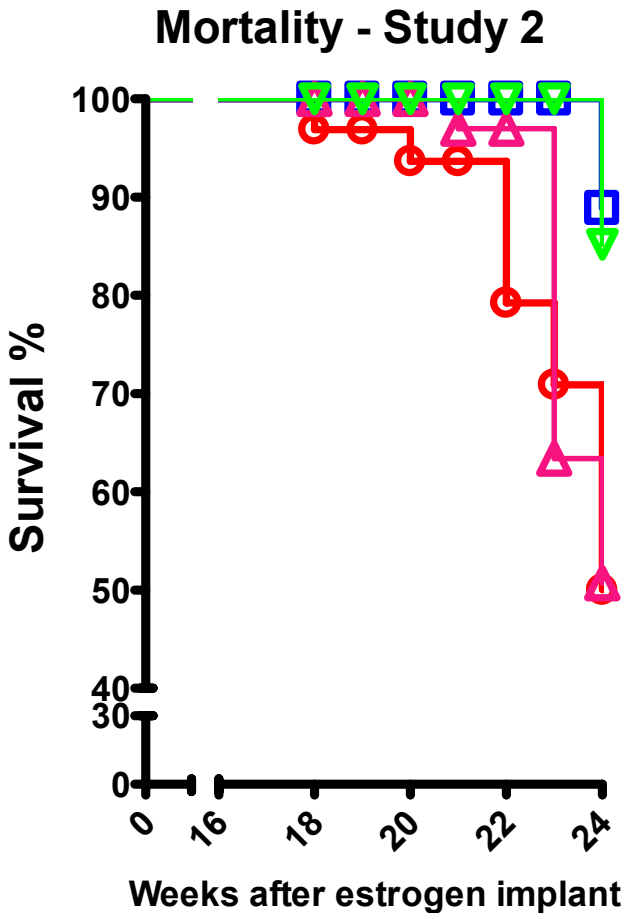


Figure 3.4. Kaplan-Meier survival curves for ACI rats with estradiol implants fed different diets and treated with silastic implants containing 27 mg E_2.
Control diet; Blueberry diet; Black raspberry diet; Ellagic acid diet .
All remaining animals were euthanized at 25 weeks.

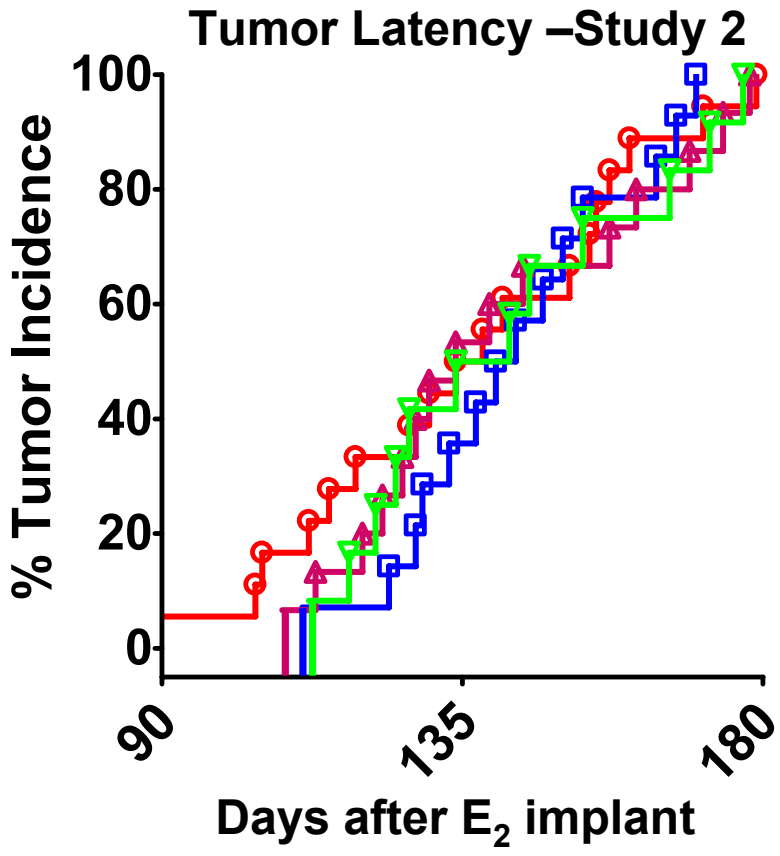


Figure 3.5. Effect of diets supplemented with dietary berries and ellagic acid on tumor latency in ACI rats treated with silastic implants containing 27 mg E_2. Control diet; Blueberry diet; Black raspberry diet; Ellagic acid diet.

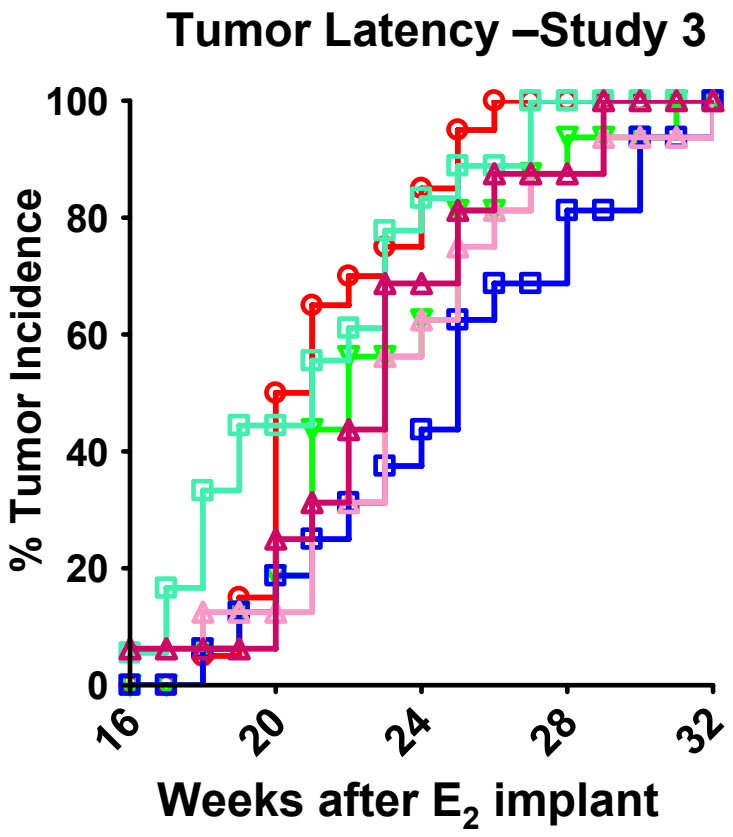


Figure 3.6. Effect of diets supplemented with berries and ellagic acid on tumor latency in ACI rats treated with silastic implants containing 9 mg E_2.
Control diet; Blueberry diet (1% w/w) ; Blueberry diet (2.5% w/w) ; Black raspberry diet (1% w/w); Black raspberry diet (2.5% w/w) ; Ellagic acid diet (400 ppm) .

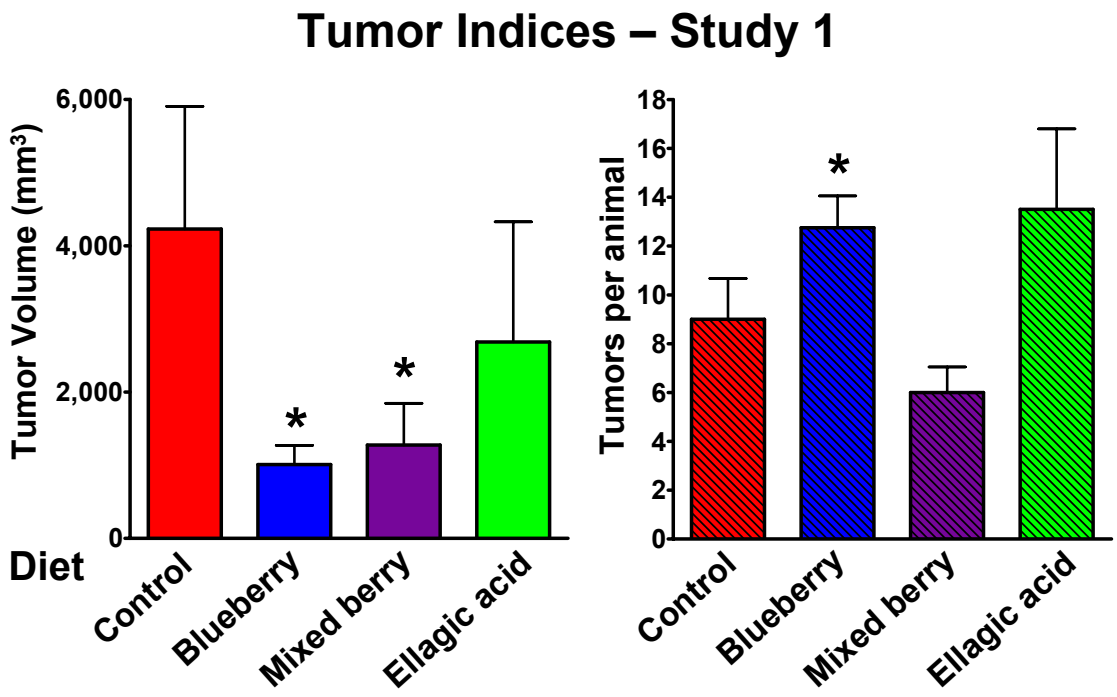


Figure 3.7. Effect of diets supplemented with dietary berries and ellagic acid on tumor indices of ACI rat mammary tumors induced by 27 mg E_2 (pilot study). The tumor multiplicity was compared with the GLM procedure and tumor volume was compared using the GENMOD procedure as described in methods. A p-value ≤ 0.05 was considered significant and is denoted by an asterisk.

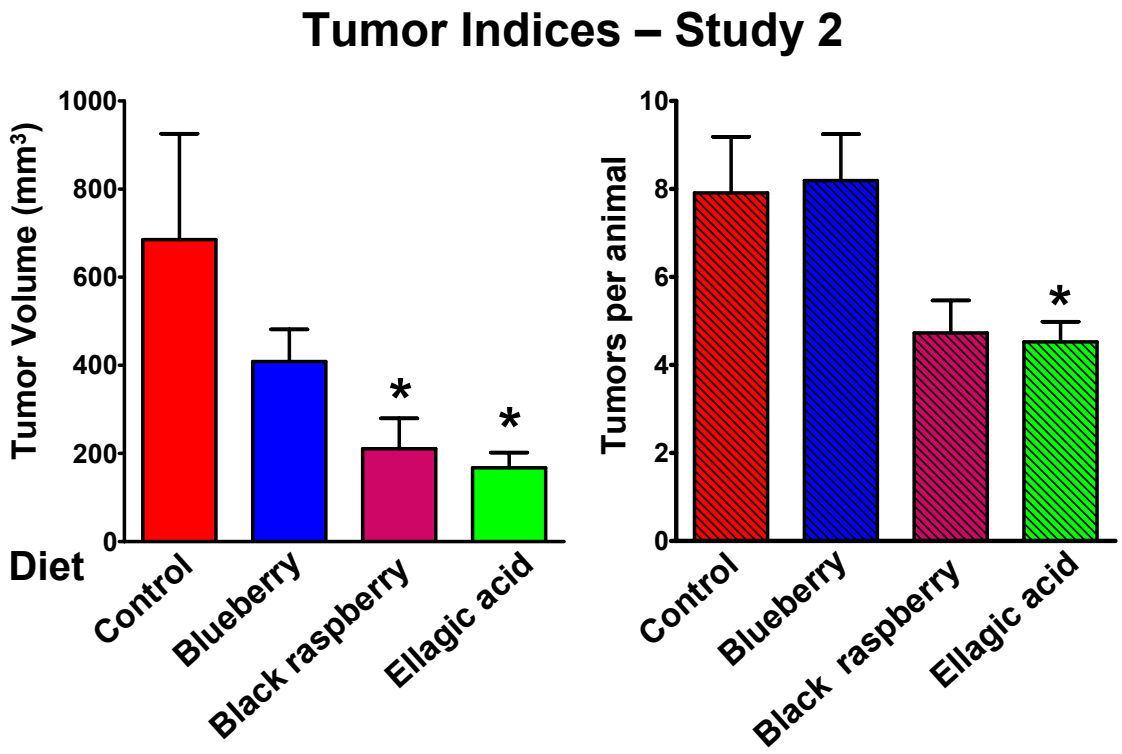


Figure 3.8. Effect of diets supplemented with dietary berries and ellagic acid on tumor indices of ACI rat mammary tumors induced by 27 mg E_2.

The tumor multiplicity was compared with the GLM procedure and tumor volume was compared using the GENMOD procedure as described in methods. A p-value ≤ 0.05 was considered significant and is denoted by an asterisk.

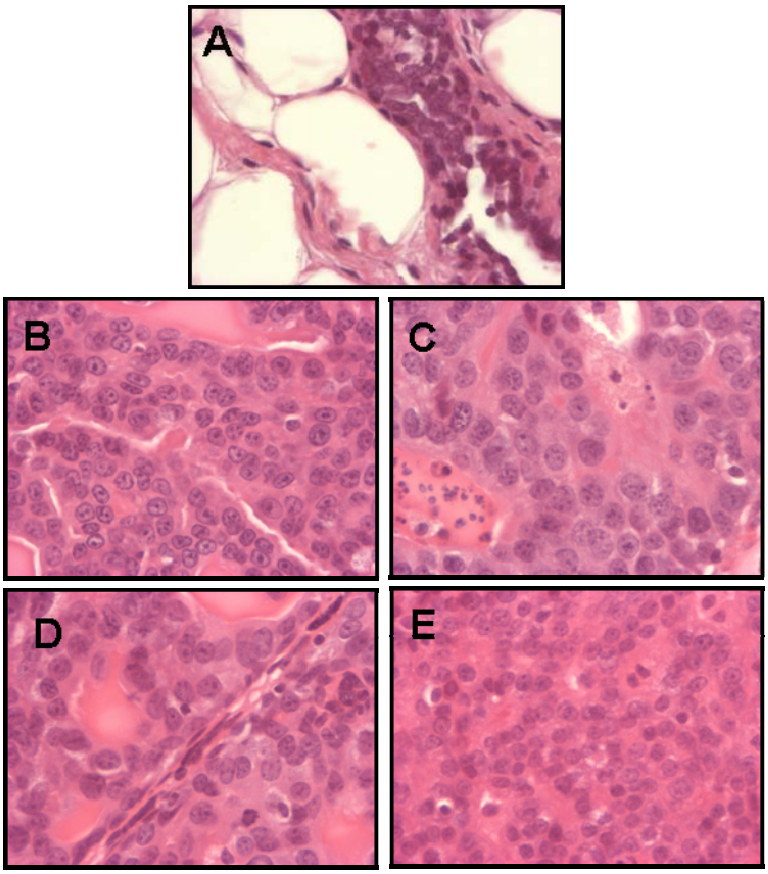


Figure 3.9. Representative H&E sections of sham-treated rat mammary glands (A) and mammary gland tumors (B-E) from Estradiol-treated rats.

Rats were fed Control Diet (A and B); Blueberry Diet (C); Black Raspberry Diet (D); or Ellagic Acid Diet (E) respectively. Shown at 400X magnification. The mammary tissues were processed in the Pathology Core Lab (University of Louisville, Louisville, KY) and confirmation of mammary adenocarcinomas by histopathologic exam was done by Drs. Srivani Ravoori and Sunati Sahoo.

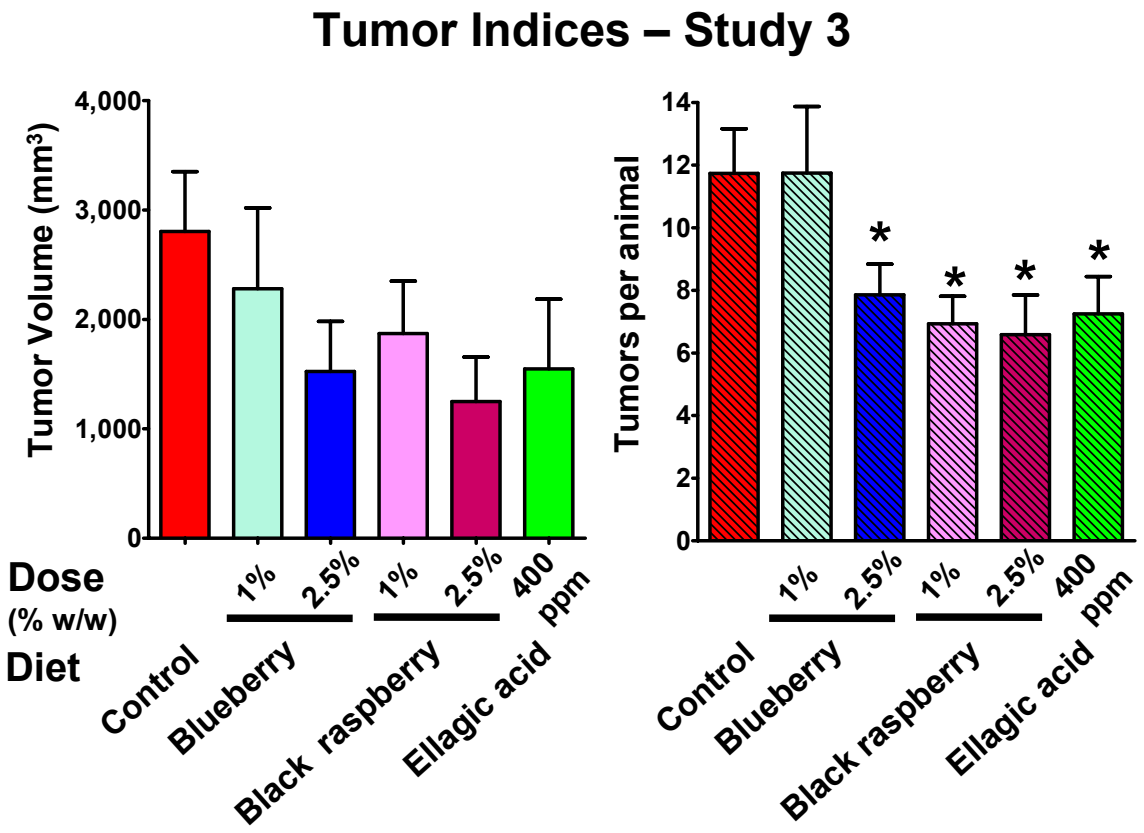


Figure 3.10. Effect of diets supplemented with dietary berries and ellagic acid on tumor indices of ACI rat mammary tumors induced by 9 mg E_2.

The tumor multiplicity was compared with the GLM procedure and tumor volume was compared using the GENMOD procedure as described in methods. A p-value ≤ 0.05 was considered significant and is denoted by an asterisk.

Table 3.1. Experimental protocol- Study 1 –A pilot study to asses the efficacy of berries and ellagic acid to inhibit mammary tumorigenesis in ACI rats induced by 27 mg E_2.

Diet	17ß-Estradiol (27 mg)	Number of animals
Control Diet - AIN 93M	-	2
	+	8
2.5% Blueberry	+	8
2.5% Mixed Berry	+	7
400 ppm Ellagic acid	+	4

Seven to eight week old animals were received and maintained initially on AIN-93M control diet for a week, followed by experimental diets for another 2 weeks before 17ß-estradiol implantation. Animals were maintained on experimental diets until the end of the study.

Table 3.2. Experimental protocol- Study 2 –A study to asses the efficacy of berries and ellagic acid to inhibit mammary tumorigenesis in ACI rats induced by 27 mg E_2.

Diet	17ß-Estradiol (27 mg)	Number of animals
Control Diet - AIN 93M	-	6
	+	25
2.5% Blueberry	-	6
	+	20
2.5% Black Raspberry	-	6
	+	19
400 ppm Ellagic acid	-	6
	+	22

Seven to eight week old animals were received and maintained initially on AIN-93M control diet for a week, followed by experimental diets for another 2 weeks before 17ß-estradiol implantation. Animals were maintained on experimental diets until the end of the study.

Table 3.3. Experimental protocol- Study 3 –A study to asses the efficacy of berries and ellagic acid to inhibit mammary tumorigenesis in ACI rats induced by 9 mg E_2.

Diet	17ß-Estradiol (9 mg)	Number of animals
Control Diet - AIN 93M	-	6
	+	15
1% Blueberry	+	12
2.5% Blueberry	+	14
1% Black Raspberry	+	15
2.5% Black Raspberry	+	12
400 ppm Ellagic acid	+	12

Five to six week old animals were received and maintained initially on AIN-93M control diet for a week, followed by experimental diets for another 2 weeks before 17ß-estradiol implantation. Animals were maintained on experimental diets until the end of the study.

Table 3.4. Composition of AIN-93M diet and diets supplemented with various levels of berries or ellagic acid.

	Type of Diet					
	AIN-93M	**1% BB**	**2.5% BB**	**1% BRB**	**2.5% BRB**	**EA**
Ingredient						
Casein	140.0	140.0	140.0	140.0	140.0	140.0
L-Cystine	1.8	1.8	1.8	1.8	1.8	1.8
Corn Starch	465.3	458.4	447.4	462.5	457.7	465.3
Maltodextrin	155.0	155.0	155.0	155.0	155.0	155.0
Sucrose	100.0	100.0	100.0	100.0	100.0	100.0
Soybean Oil	40.0	40.0	40.0	40.0	40.0	40.0
Cellulose (fiber)	50.0	47.3	43.3	43.2	33.0	50.0
AIN-93M Mineral Mix	35.0	35.0	35.0	35.0	35.0	35.0
AIN-93M Vitamin Mix	10.0	10.0	10.0	10.0	10.0	10.0
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
Blueberry powder	-	*10.0*	*25.0*			
Black raspberry Powder	-			*10.0*	*25.0*	
Ellagic acid	-					*0.4*
Starch equivalent from berries	-	6.9	9.6	2.8	7.6	-
Fiber equivalent from berries	-	2.7	6.7	6.8	17.0	-
Total starch	**465.3**	**465.3**	**465.3**	**465.3**	**465.3**	**465.3**
Total cellulose	**50.0**	**50.0**	**50.0**	**50.0**	**50.0**	**50.0**

The diets were ordered from Harlan-Teklad (Madison, WI). The cornstarch and fiber components of the AIN-93M diet were replaced for the berry diets, based on the nutritional information available for each berry (http://www.nal.usda.gov/fnic/foodcomp). A proximate analysis was performed at Harlan-Teklad (Madison, WI) to ascertain that the diets were isocaloric. BB- Blueberry; BRB- Black raspberry; EA- Ellagic acid.

Table 3.5. Study 1- Comparison of organ weights and tumor indices between ACI rats fed control diet or diet supplemented with blueberries, mixed berries or ellagic acid

Group	Animal Weight (g)	Liver (g)	Mammary (g)	Tumor Volume (mm^3)	Tumor Multiplicity	Volume/ tumor (mm^3)
Control diet (Sham) (n=2)	197 ± 11.5	6.1 ± 0.5	2.9 ± 0.8	NA	NA	NA
Control diet (n=8)	195 ± 2.4	5.9 ± 0.3	3.8 ± 0.3	4231 ± 1675	9 ± 1.6	505 ± 238
Blueberry diet (n =8)	194 ± 2.5	6.2 ± 0.2	4.8 ± 0.2	1278 ± 570 *p < 0.001*	12.7 ± 1.3 *p < 0.05*	81 ± 18.5 *p < 0.0001*
Mixed berry diet (n=7)	189 ± 3.3	5.8 ± 0.3	4.1 ± 0.4	1011 ± 262 *p < 0.01*	6 ± 1.0 *p < 0.2*	199 ± 78 *p < 0.02*
Ellagic acid diet (n= 4)	188 ± 3.1	6.3 ± 0.4	4.1 ± 0.7	2686 ± 1644 *p < 0.5*	13.5 ± 3.3 *p < 0.08*	167 ± 66 *p < 0.05*

Animals were euthanized after 24 weeks of estrogen treatment. Organ wet weights were measured after excision. Tumor volume was calculated as the volume of a spheroid (2/3 π r1*r2*r3). Values denote mean ± SEM. "n" designates only those animals that survived 24 weeks. All comparisons are between 17ß-estradiol treated animals on control diet and respective diets.

Table 3.6. Study 2- Comparison of organ weights and tumor indices between ACI rats fed control diet or diet supplemented with blueberries, black raspberries or ellagic acid

Group	*Animal Weight (g)*	*Liver (g)*	*Mammary (g)*	*Pituitary (g)*	*Tumor Volume (mm^3)*	*Tumor Multiplicity*	*Volume/ tumor (mm^3)*
Control diet (Sham) (n=6)	189 ± 6	5.2 ± 0.2	3.7 ± 0.2	Not measured	NA	NA	NA
Control diet *(n=11)*	169 ± 6.4	5.0 ± 0.3	3.84 ± 0.3	0.22 ± 0.03	685 ± 240	7.9 ± 1.3	115 ± 39
Blueberry diet *(n =16)*	159 ± 5.6	4.7 ± 0.2	3.89 ± 0.3	0.19 ± 0.01	409 ± 73 *p < 0.835*	8.2 ± 1.0 *p< 0.749*	45 ± 7 *p< 0.170*
Black raspberry diet *(n=11)*	162 ± 8.5	4.7 ± 0.3	4.41 ± 0.6	0.25 ± 0.02	211 ± 69 *p< 0.003*	4.7 ± 0.7 *p< 0.070*	38 ± 10 *p< 0.034*
Ellagic acid diet (n=19)	167 ± 4.3	4.9 ± 0.2	4.53 ± 0.3	0.19 ± 0.01	168 ± 34 *p< 0.001*	4.5 ± 0.5 *p< 0.027*	34 ± 7 *p< 0.009*

Animals were euthanized after 24 weeks of estrogen treatment. Organ wet weights were measured after excision. Tumor volume was calculated as the volume of a spheroid (2/3 π r1*r2*r3). Values denote mean ± SEM. "n" designates only those animals that survived 24 weeks. All comparisons are between 17ß-estradiol treated animals on control diet and respective diets.

Table 3.7. Study 3 -Comparison of organ weights and tumor indices between ACI rats fed control diet or diet supplemented with different doses of blueberries, black raspberries or ellagic acid

Group	*Animal Weight (g)*	*Liver (g)*	*Mammary (g)*	*Pituitary (mg)*	*Tumor Volume (mm^3)*	*Tumor Multiplicity*	*Volume/ tumor (mm^3)*
Control diet (sham) *(n=6)*	*182 ± 4*	4.6 ± 0.2	3.4 ± 0.2	9.6 ± 0.8	*NA*	NA	NA
Control diet *(n=17)*	*204 ± 2*	6.8 ± 0.2	4.8 ± 0.3	69 ± 4.5	*2804 ± 547*	11.7 ± 1.4	308 ± 83
1 % Blueberry diet *(n=12)*	*207 ± 3*	6.9 ± 0.2	5.4 ± 0.2	58 ± 7	*2280 ± 739*	11.2 ± 1.9	*208 ± 45*
2.5% Blueberry diet *(n=14)*	*203 ± 6*	6.6 ± 0.3	5.7 ± 0.4	68 ± 15	*1525 ± 457*	7.3 ± 0.9 *p< 0.05*	*176 ± 43*
1 % Black raspberry diet *(n=15)*	*201 ± 4*	6.5 ± 0.2	6.2 ± 0.4	59 ± 10	*1871 ± 479*	6.9 ± 0.8 *p< 0.008*	233 ± 46
2.5% Black raspberry diet *(n=16)*	*209 ± 6*	6.7 ± 0.3	6.8 ± 0.6	43 ± 5	*1241 ± 444*	6.9 ± 1.3 *p< 0.007*	182 ± 45
Ellagic acid diet (n=13)	208 ± 5	*6.5 ± 0.2*	5.5 ± 0.3	56 ± 5	1547 ± 639	7.2 ± 1.2 *p< 0.05*	*163 ± 56*

Animals were euthanized after 32 weeks of estrogen treatment. Organ wet weights were measured after excision. Tumor volume was calculated as the volume of a spheroid (2/3 π r1*r2*r3). Values denote mean ± SEM. "n" designates only those animals that were euthanized after 32 weeks. There was no mortality; however animals were euthanized before 32 weeks if their tumors were larger than 1.3 cm in diameter. All comparisons are between 17ß-estradiol treated animals on control diet and respective diets.

Chapter Four: Effect of berries and ellagic acid on estrogen metabolism during 17ß-estradiol- induced mammary tumorigenesis in the ACI rat.

Introduction

Estrogen is a known, yet unavoidable risk factor for breast cancer. Women, exposed to even physiological levels of this hormone chronically are at an increased risk to develop breast cancer (Lippman *et al.*, 2001). This risk is compounded by the presence or absence of several other factors. Although certain women are genetically predisposed to breast cancer due to heredity, they represent only a small fraction of women at risk (Brinton *et al.*, 2002; Thompson & Easton, 2004). On the other hand, a majority of the population is at a higher risk due to polymorphisms in low penetrance genes, especially those involved in estrogen metabolism (Nathanson & Weber, 2001). Estrogen metabolism occurs in several tissues of the human body to varying extents and to achieve different ends (Rieder *et al.*, 1998; Simpson, 2003). The primary organ involved in estrogen production is the ovary, under the control of anterior pituitary. However, the important role of estrogen in the maintenance of homeostasis can be garnered from the fact that several tissues are endowed with enzymes that can both produce and conjugate estradiol (Simpson *et al.*, 1994; Murray *et al.*, 2001). The breast, like several other tissues, is capable of producing estradiol via *de-novo* pathways and these pathways coupled with estrogen metabolism and signaling may play a major role in breast cancer. A simplified scheme for such interactions is shown in figure 4.1. Primary enzymes involved in *de-novo* estradiol synthesis are aromatase, which converts androgen precursors to estrone, and 17ß-hydroxysteroid dehydrogenase (17ßHSD), which converts estrone (E_1) to estradiol (E_2) (Milczarek & Klimek, 2005; Sasano *et al.*, 2006). Another, minor pathway is via estrogen sulphatase that converts sulfated estradiol/estrone to the original molecule (Sasano *et al.*, 2006). Research suggests that this *in-situ* sythesis of estradiol may play a major role in the development of breast cancer, especially in post-menopausal women (Chen, 1998; Simpson, 2003).

Aromatase is a target of current pharmacological therapy and the use of aromatase-inhibitors has been successful in the treatment of estrogen-receptor (ER) negative breast cancer (Tuxen *et al.*, 2007). Another enzyme, also crucially involved in E_2 biosynthesis is 17ßHSD. Eight isozymes, present in several tissues, have been identified so far and have both human and rodent homologues (Luu-The, 2001). The type 1 isozyme of 17ßHSD, which converts estrone to estradiol, is found in both normal and malignant breast (Miettinen *et al.*, 1996). The rodent homologue of this enzyme is 17ßHSD, type 7 (17HSD7), also known as the prolactin receptor associated protein (PRAP) (Duan *et al.*, 1996; Peltoketo *et al.*, 1999). This enzyme has high specificity for the conversion of E_1 to E_2 and is controlled by both prolactin and estrogen signaling pathways (Duan *et al.*, 1997).

There are several phase I and phase II enzymes involved in the metabolism of E_2, of particular importance in the breast are Cytochrome P450 1A1 (CYP1A1), CYP1B1, catechol-O-methyl transeferase (COMT), UDP-glucuronosyl transferase (UGT) and Glutathione-S-transferase (GST). The phase I enzyme, CYP1B1 has received wide attention due to its function in converting E_2 to 4-hyrdroxy estradiol ($4E_2$), a postulated potentially carcinogenic metabolite that causes oxidative DNA damage and induces renal-cell carcinomas in hamsters (Liehr, 2000; Hiraku *et al.*, 2001; Liehr, 2001). Also, breast tumors show high levels of both CYP1B1 and $4E_2$ (McFadyen *et al.*, 1999; Oyama *et al.*, 2005). Nevertheless, metabolites of CYP1A1 action such as 2-hydroxy estrone can produce stable DNA adducts and inhibition of CYP1A1 metabolism reduces the formation of estrogen-induced kidney tumors in hamsters, suggesting that this pathway may also play a definitive role in estrogen carcinogenesis (Liehr *et al.*, 1991). The hydroxy-metabolites of estradiol and estrone are conjugated for removal by several enzymes, including COMT, GST and UGT (Lakhani *et al.*, 2003; Abel *et al.*, 2004). The 2-hydroxy metabolites are better substrates for COMT (Liehr, 2000), suggesting that CYP1A1 and COMT expression may be coupled. Polymorphisms in both phase I and II genes have been associated with a risk of breast cancer, indicating the importance of these enzymes in the

production and removal of estradiol metabolites (Gallicchio *et al.*, 2006). The estrogen metabolism pathway interacts with the estrogen-signaling pathway. Hydroxy-metabolites of estradiol such as $4E_2$ and $2E_2$ bind to ERs with varying affinities (Zhu & Conney, 1998). Thus, enzymes such as CYP1A1 and CYP1B1 are regulated by ERs (Tsuchiya *et al.*, 2005; Sissung *et al.*, 2006). Progesterone receptor (PGR) is known to be up-regulated by estrogen via ER signaling, hence PGR expression is a downstream effect of ER activation (Mauvais-Jarvis *et al.*, 1986b). Thus, studying the expression of these genes provides some idea about control of estrogen metabolism in the mammary tissue.

Several epidemiological studies have shown that the high intake of fruits and vegetables may reduce the risk of breast cancer (Block *et al.*, 1992). Other than being rich sources of vitamins and minerals, fruits are also sources of phytochemicals (Forman *et al.*, 2004). Several phytochemicals share a similar chemical structure with endogenous steroids, thereby making them substrates for steroid receptors and as well as steroid metabolizing enzymes (Dixon *et al.*, 2005). Indeed, berry phytochemicals such as anthocyanins and ellagic acid show selective estrogen receptor modulating (SERM) activity in some studies (Schmitt & Stopper, 2001; Larrosa *et al.*, 2006). These phytochemicals are highly bioavailable in both humans and rodents and hence may play a significant role in modulating estrogen metabolism (Talavera *et al.*, 2004; Tian *et al.*, 2006). Data presented in the previous chapter show that both berries and ellagic acid can reduce estrogen-induced mammary tumorigenesis when provided via the diet. However, the exact mechanisms by which they provide protection are not known.

In order to determine this we examined the regulation of expression of key enzymes involved in estrogen metabolism and signaling, in the mammary tissue of ACI rats treated with E_2 by silastic implant during the course of mammary tumorigenesis. The effect of dietary berries and ellagic acid on these enzymes was also tested. Three time points – early (6 weeks), intermediate (18 weeks) and late (24 weeks) were chosen and the expression of 9 selective genes, 3 each involved in the phase I , phase II metabolism and estrogen signaling (Figure

4.1) were selected and their relative gene expression changes analyzed using quantitative real time PCR. The genes tested were: phase I metabolism- 17ßHSD7, CYP1A1, CYP1B1; phase II- COMT, GSTA1, GSTM1; steroid signaling – ERα, ERß, PGR. The results are presented herein.

Materials and methods

Animals, diet and treatment. Female ACI rats (7-8 weeks old) were purchased from Harlan-Sprague Dawley (Indianapolis, IN), housed under ambient conditions and fed AIN-93M diet and water *ad libitum*. After a week of acclimation, 18 animals each were randomized into different groups as per table 4.1. Two of the 5 groups received control diet and the other 3 received diets supplemented with 2.5% (w/w) blueberry; black raspberry or 400 ppm ellagic acid. After 2 weeks of pre-feeding, each group received either sham implants or E_2 implants as described (Shull et al., 1997, Table 4.1). The animals were maintained on their respective diets throughout the study period and 6 animals from each group were euthanized at 6, 18 and 24 weeks after E_2 treatment by carbon dioxide asphyxiation, and mammary tissue was collected and frozen for further analysis. Trunk blood was collected for measurement of serum estrogen levels and for future analysis of serum phytonutrient levels.

RNA isolation. RNA from whole mammary tissue was isolated using the Trizol® method (Invitrogen, Carlsbad, CA), with modifications. All procedures were done in a RNase free environment. Briefly, approximately 100 mg of mammary tissue was suspended in 2 ml of Trizol® at 4°C and homogenized with a hand-held polytron at maximum speed. This homogenate was then passed through a syringe with a 22.5 gauge needle (Beckton-Dickinson and Co., Franklin Lakes, NJ) to ensure complete dissociation of the mammary tissue. The 2 ml homogenate was then divided into 2 aliquots of 1 ml each, distributed into 1.5 ml tubes. After the addition of 200 µl chloroform the solution was vortexed briefly and centrifuged at 13,000Xg for 15 min at 4°C. The resultant aqueous supernatant was sequentially extracted with another 400 µl of chloroform and the aqueous phase was precipitated using ice-cold iso-propanol. The RNA pellet was

recovered by centrifuging at 13,000Xg for 15 min at 4°C, washed with 70% ethanol, dissolved in nuclease-free water and the aliquots were combined. The quality of the RNA was ascertained by gel electrophoresis and quantitated using NanoDrop® (NanoDrop Technologies, Wilmington, DE). The RNA was then diluted to 5 ng/µl concentration and stored at -80°C until use.

Design and standardization of concentration for PCR primers. Primers for quantitative real-time PCR were designed across exon boundary to avoid amplification of genomic DNA, using Primer express® 3.0 software (Applied Biosystems, Foster City, CA) and synthesized by Integrated DNA Technologies, Inc., (Coralville, IA). The sequences of the forward and reverse primers for each gene tested are listed in Table 4.2. The primers were tested at a concentration of 500 nM each initially to ascertain the presence of primer-dimers. If this was present, a dilution array containing varying concentrations of both forward and reverse primers was done and the combination at which there was uniform amplification and no byproduct was used further. For most genes tested 500 nM final concentration of each primer produced no primer-dimers, except for CYP1A1 for which 125 nM each produced a single uniform peak. Both sense and antisense primers were diluted such that 0.5 µl of the stock (10 µM or 2.5 µM for 1A1) provided 500 nM (or 125 nM for CYP1A1) of each primer at a final volume of 20 µl.

Real-time PCR efficiency studies. In order to ensure that all genes reverse-transcribed with the same efficiency, reverse-transcription efficiency studies were done initially. RNA of 3 animals from each group was pooled to generate the single test sample for each group, and cDNA was synthesized using the High capacity cDNA archive kit (Applied Biosystems, CA) in 10-fold serial dilutions such that the RNA concentrations were –1 µg/µl, 100 ng/µl, 10 ng/µl, 1 ng/µl, 100 pg/µl, 10 pg/µl and 1 pg/µl. The conditions for the reverse transcription were: 25°C for 10 min; 37°C for 2 hour; and 85°C for 5 seconds.

The PCR amplification was done in a final reaction volume of 20 µl containing 10 µl of 2X Power SYBR® Green PCR master mix (Applied Biosystems, CA); 0.5 µl each of forward and reverse primers specific for each gene (final concentration- 500 or 125 nM as mentioned) and 3µl of cDNA such that the equivalent RNA concentration was- 3µg, 300 ng, 30 ng, 3 ng, 300 pg, 30 pg and 3 pg. Quantitative PCR was performed using a 7500 Fast-Real Time PCR system (Applied biosystems, Foster City, CA) using the absolute quantification protocol and standard curves generated. The PCR conditions were: 50°C for 2 min; DNA polymerase activation at 95°C for 10 min; followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 min. The concentration range with a slope closest to -3.3 was selected and the highest concentration in this range was used for all further experiments. For all samples and genes tested, this concentration was found to be 1 ng/µl for reverse transcription and 3ng for the PCR reaction. Individual samples were analyzed henceforth using these standardized conditions.

Analysis of gene expression. Gene expression analysis was done using the relative-quantification ($\Delta\Delta$cT) method as described (Livak and Schmittgen, 2001). Each sample (refers to cDNA from individual animals) was analyzed in triplicate for each gene tested and the average of these values was taken as the cT value for that gene. ΔcT was calculated as the difference between cT of gene of interest and the house-keeping gene (ß-actin) ($\Delta cT = cT_{GOI} - cT_{ß\text{-}actin}$). One sample (sham treated) was chosen as the calibrator and $\Delta\Delta$cT of all other samples was calculated using the formula $\Delta\Delta cT = \Delta cT_{sample} - \Delta cT_{calibrator}$. The fold change ($2^{-\Delta\Delta \mathbf{cT}}$) in gene expression was calculated for all genes and samples**.** At each time point, for all genes tested, the calibrator sample was the same (typically a sample from the sham treated group), such that the results are represented as relative fold change, which represents the biological variation within a specific group and absolute fold change, which is the relative fold change of E_2-treated groups divided by the relative fold change of sham-treated group on control diet. The absolute fold change represents the

actual up- or down-regulation of genes by the E_2 treatment both in the presence and absence of intervention, and is always 1 for the sham treatment.

Statistical analysis. Relative fold changes in each group were compared using one-way analysis of variance (ANOVA), followed by a Tukey's multiple comparison post test. A p-value <0.05 was considered significant. All statistical analyses were performed using the Graphpad Prism ® software (Graphpad Software, San Diego, CA).

Results

Reverse-transcription efficiency studies. The reverse-transcription (RT) efficiency studies were performed in order to confirm that all transcripts reverse-transcribed at the same efficiency. The cDNA for each of the test samples was made in 10-fold serial dilutions of individual RNA and each dilution was used in PCR as described in materials and methods. In quantitative real-time PCR, every cycle involves the doubling of the number of transcripts and hence a cycle-threshold (cT) value of 3.3 approximately indicates a 10-fold difference. Since, the reaction kinetics can differ based on a number of factors including efficiency of the enzyme, total number of transcripts, etc, it is important to assess the RNA concentration at which all transcripts tested, amplify with the same efficiency. In testing a series of 10-fold serial dilutions, a negative slope close to 3.3 would indicate equivalent amplification. For most house-keeping genes tested this range was 100 ng/µl to 1 pg/µl for the E_2-treated group, however for the sham-treated group this range was 1 ng/µl to 1 pg/µl. For other genes tested, there was no amplification in the lower pg/µl range. Thus, 1 ng/µl of RNA was reverse-transcribed and 3ng cDNA equivalent was used for PCR amplification. The validity of this concentration was further confirmed when a standard curve with a negative slope of 3.3 was observed when using 10-fold serial dilutions of cDNA prepared using 1 ng/µl RNA .

Changes in phase I enzymes at various time-points after estrogen treatment. At 6 weeks after estrogen treatment, all phase I enzymes tested were

significantly affected in the E_2-treated groups compared with sham-treatment. The levels of 17ßHSD7 was elevated by 4.8 fold ($p<0.001$) in E_2-treated animals maintained on control diet (E2-CD). However, this overexpression was partially offset, i.e., only 1.8, 2.2 and 2.5 fold in blueberry, black raspberry and ellagic acid groups respectively (Figure 4.2; Table 4.3). This anti-estrogenic effect was not present at 18 and 24, weeks (Figure 4.5 and 4.8). The enzyme that showed the highest up-regulation at 6 weeks was CYP1A1 with a 48 fold increase after estrogen treatment ($p<0.0001$) and stayed elevated by 15 and 8 fold at 18 and 24 weeks respectively ($p<0.001$) (Figure 4.11; Tables 4.3 and 4.5). Both blueberry and black raspberry diets significantly offset this elevation to 21 ($p<0.01$) and 12 ($p<0.001$) fold, respectively after 6 weeks of E_2 treatment. This protection continued at 18 weeks for blueberry diet but not for black raspberry (Figure 4.5). Ellagic acid diet showed effects similar to control diet at all time points. At 24 weeks, the elevations in CYP1A1 were only slightly offset, with black raspberry showing the greatest reduction ($p<0.05$) (Figure 4.8; Table 4.5). CYP1B1 was significantly down regulated by estrogen treatment at all time points tested ($p<0.001$) (Figures 4.11). All diets at 6 weeks, suppressed CYP1B1 expression by up to 5 fold compared with E_2-treated animals on control diet and by up to 11 fold compared to sham-treated animals (Figure 4.2; Table 4.3). These changes were not seen at other time points.

Changes in phase II enzymes at various time-points after estrogen treatment. In general, COMT and GSTA1 were up-regulated by estrogen treatment. At all time points, COMT increased by up to 2 fold ($p<0.05$) after estrogen treatment for the control diet and this was attenuated by dietary intervention significantly only at 18 weeks (Figure 4.6; Table 4.4). At 6 and 18 weeks, GSTA1 was up-regulated by estrogen treatment and dietary intervention did not have any significant effect on this increase. At all time points, GSTM1 was, however, found to be down-regulated after estrogen treatment by up to 3 fold ($p<0.05$) with no effect of intervention.

Changes in steroid receptors various time-points after estrogen treatment. The estrogen receptor α (ERα) was significantly down-regulated ($p<0.001$) by estrogen treatment throughout the study period (Figure 4.13; Tables 4.3 to 4.5). Neither berries nor ellagic acid affected this change (Figure 4.13). On the other hand, ERß was unaffected by estrogen treatment (Figure 4.13). Progesterone receptor was significantly up-regulated at both 6 and 18 weeks after estrogen treatment (Figure 4.8, 4.9 and 4.13).

General trends in gene-expression modulation. In general, it was seen that estrogen treatment had a uniform effect on the regulation of several estrogen-metabolism genes tested and the greatest difference in most genes was seen at the earliest time point- 6 weeks after treatment (Figure 4.11 to 4.13). At 18 weeks, there were fewer genes that were differentially modulated and the effect was lower. At 24 weeks for most genes tested, all E_2 treated groups, regardless of supplementation, appeared to have similar levels of expression.

On one hand, CYP1A1 is highly over-expressed beginning at 6 weeks after treatment and the expression changes are maintained to a lesser extent at both 18 and 24 weeks. This up-regulation is countered effectively by both the berry diets, especially at 6 weeks, but not by ellagic acid. On the other hand, CYP1B1 expression is down-regulated after estrogen treatment. The trend of down-regulation is the highest at 6 weeks and subsequently reaching a plateau at 18 and 24 weeks (Figure 4.11). There is a similar response for 17ßHSD7, which suggests that the early gene regulation after estrogen treatment can be seen at 6 weeks, when the E_2 levels are increasing and that this expression changes reach a plateau, possibly due to acclimatization of the tissues to high circulating E_2. The effects of supplementation can also be seen at this early stage, with most of the differences between control diet and supplemented diet seen at 6 weeks. The only exception to this was COMT, for which the difference in modulation was seen at 18 weeks. The genes for which there are no considerable differences between groups are ERß and GSTM1, with the latter being down-regulated after estrogen treatment with no change between control

and supplemented diet. Although GSTA1 is over-expressed after estrogen treatment at 6 weeks, the change is statistically insignificant (Figure 4.5), but is significant at 18 weeks. PGR is over-expressed at 6 and to lesser extent, at 18 weeks, but not at 24 weeks. However, there was no effect of the supplemented diets on these changes. These trends signify that these agents may act differentially through multiple signaling pathways.

Discussion

The results presented show the modulation of selected genes involved in phase I, phase II estrogen metabolism, and receptor signaling at different time points after E_2 treatment, and the effect of diets supplemented with blueberry, black raspberry, and ellagic acid. Since several variables are involved, the effect of estrogen treatment *per se* on the different genes, the modulation of these at different time points, the effect of supplemented diets on estrogen-induced modulation at specific times, and the possible reasons for the same will be discussed in that order.

There are very few published reports describing the expression of these genes after chronic administration of E_2 in these animals. A recent study reported that diethyl stilbesterol (DES), another potent estrogen, when administered intra-peritoneally for four days continuously at a bolus dose of 50 mg/kg body weight, does not modify the expression of either phase I or phase II enzymes in the ACI rat mammary on the fifth day (Green *et al.*, 2007). However, this differs considerably from the model in this study, which involves systemic delivery of constant levels of estradiol (Shull *et al.*, 1997; Ravoori *et al.*, 2007). Kauffman and coworkers have studied the activities of phase II enzymes after chronic E_2 treatment in ACI rat liver and brain (Sanchez *et al.*, 2003; Stakhiv *et al.*, 2006). They report that E_2 increases the protien and activity levels of hepatic GST and NADPH-quinone reductase in rat treated for 6 weeks with a 2 mg of E_2 delivered via a cholesterol pellet (Sanchez *et al.*, 2003). E_2 has a similar effect also on the brain enzymes (Stakhiv *et al.*, 2006). The primary consideration in the interpretation of the results of this study is that, other variables such as effect of

E_2 on other organ systems (pituitary, liver etc.,) have not been considered and the mammary is taken as a unitary tissue. However, these variables and their possible effect on estrogen-induced mammary carcinogenesis and its prevention are discussed in Chapter 5.

Another important detail that must be taken into account while interpreting results in the current study is the proliferation of mammary epithelial cells in response to E_2 treatment. At 6 weeks after treatment, the E_2-treated mammary largely consists of proliferating cells of epithelial origin; however, sham-treated tissue consists of a much higher percentage of stromal cells. As reported from our laboratory the proliferative index markers PCNA and Ki-67 are significantly increased after 90 days of E_2 treatment and the representative tissue sections also indicate differences in the predominant cell types (Ravoori *et al.,* 2007 in press; Chapter 3, Figure 3.9). Thus, the differences in the cell composition between untreated and treated rats may potentially confound the results as these analyses were done from total tissue RNA.

The serum level of estradiol was 35 pg/ml in sham-treated and 194 pg/ml in estradiol-treated animals, 6 weeks after treatment (Chapter 3). This 5.5 fold increase in the estradiol level is reflected both in the increased proliferation of the mammary tissue as well as the significant down-regulation of ERα and concomitant up-regulation of PGR. The levels of ERß were not affected. E_2 down-regulates ERα and up-regulates PGR in the epithelial cells of a normal mammary gland (Mauvais-Jarvis *et al.*, 1986b; Shyamala *et al.*, 2002). Induction of mammary tumors in ERKO/Wnt-1 mice by E_2 shows that ER is not necessary for E_2-induced carcinogenesis (Devanesan *et al.*, 2001). In cultured human epithelial cells, synthetic progesterones antagonize the proliferative action of E_2 via PGR (Mauvais-Jarvis *et al.*, 1986a; Poulin *et al.*, 1989). Although, the up-regulation of PGR occurs at 6 weeks and to a lesser extent 18 weeks, but is absent at 24 weeks, the down-regulation of ERα continues till 24 weeks, suggesting three things- i) the mammary tumors in the ACI rats may arise in an ER-independent fashion; ii) that epithelial cells are acclimated to high circulating

estradiol levels and hence do not show consistent up-regulation of PGR, which is a downstream effect of estradiol treatment more apparent at an early time point; and iii) that these tumors may not be responsive to the negative-regulation, if there is any, by progesterone .

Although most transcriptional regulation of estradiol is attributed to its action via the estrogen receptor, it can also act in an ER-independent fashion (Coleman & Smith, 2001). The transcription of CYP1A1 and 1B1 are regulated by both the ER and the aryl hydrocarbon receptor (AhR) (Hollenberg, 2002; Tsuchiya *et al.*, 2005; Sissung *et al.*, 2006). However, there is a disjunction between these 2 pathways, depending on the clonality of the breast cancer cell line, with the 2 pathways acting in a mutually exclusive manner (Angus *et al.*, 1999). Both CYP1A1 and 1B1 can be induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) depending on the ER status of the cell (Angus *et al.*, 1999). This suggests that ER may not directly control the expression of these enzymes. Moreover, the expression of these enzymes has been attributed to specific cell types, i.e., the epithelial cells versus the stromal fibroblasts. It is reported that CYP1B1 expression is constitutively higher in the rat mammary stroma; whereas CYP1A1 can be induced beyond basal levels only in the epithelial cells by PAHs and TCDD (Christou *et al.*, 1995). Thus, in these animals where the epithelial-cell proliferation in response to estradiol treatment has occurred, the levels of total tissue CYP1B1 in the untreated animals may reflect the stromal compartment and are thus higher than E_2 treatment. Contrarily, CYP1A1, that is predominantly present in the epithelial cells is up-regulated by over 40-fold, suggesting that this up-regulation is reflective of both the cell population and the treatment.

These phase I enzymes are predominantly responsible for the conversion of estradiol to genotoxic metabolites (Russo & Russo, 2004). It has been reported that ACI rat mammary is highly susceptible to mutations after intra-mammillary administration of estrogen-3-4-quinone (Mailander *et al.*, 2006). Also DNA damage caused by different catechol estrogens has been extensively reported by Cavalieri and coworkers (Cavalieri *et al.*, 2006). However, all these

reports have looked at short term, bolus dose of moderately toxic doses of these agents (Li *et al.*, 2004; Mailander *et al.*, 2006). However, the status of these metabolites in E_2- induced mammary cancer is not known and the metabolites themselves do not cause mammary tumors in ACI rats (Turan *et al.*, 2004). Both CYP1A1 and CYP1B1 can convert E_2 to $4E_2$, although CYP1B1 predominates (Liehr, 2000; Cribb *et al.*, 2006). Nevertheless, 15-20% of the E_2 metabolite produced by CYP1A1 is $4E_2$ (Liehr, 2000). Thus, there is a possibility to generate genotoxic metabolites in the presence of excess E_2 and high expression of CYP1A1, since CYP1A1 partially catalyses the production of $4E_2$. Further, in comparison to CYP1A1, COMT and GST, enzymes whose substrates are CYP1A1 products, are only up-regulated by 2- to 4-fold indicating an imbalance.

Another interesting finding is the up-regulation of 17ßHSD7 by estradiol. This enzyme has high specificity for the conversion of estrone to estradiol in the mammary, suggesting that estradiol may influence *in-situ* estrogen synthesis. However, 17HSD7 expression is affected by both E_2 and prolactin in the rat corpus luteum (Duan *et al.*, 1997; Risk *et al.*, 2005), and E_2 induces pituitary prolactinomas in this model (Shull *et al.*, 1997). Thus, either E_2 may directly influence the expression of 17ßHSD7 or this may be a downstream effect of increased prolactin secretion. This enzyme is involved in the conversion of estrone to estradiol, however, the expression of aromatase which forms estrone from androgen precursors, is almost undetectable in the mammary tissue of the ACI rat (data not shown), which raises questions about the role that 17ßHSD7 plays in *in-situ* estrogen synthesis.

It has been shown in MCF-7 cells and rat uterine lieyomieomas that physiological levels of E_2 down-regulates COMT expression via ER pathways (Xie *et al.*, 1999; Al-Hendy & Salama, 2006). In this study, there is a slight over-expression of COMT at all time points. The results with respect to GSTA1 are corroborated by published reports of increase in GST activity in ACI rat liver and brain after 6 weeks of E_2 treatment (Sanchez *et al.*, 2003; Stakhiv *et al.*, 2006). However, these studies look at GST activities and thus do not differentiate

between GST isozymes. Nevertheless, the induction of P450 enzymes by their substrates is well documented (Hollenberg, 2002; Hayes *et al.*, 2005). Thus, the over-expression of both COMT and GSTA1 may be in response to the presence of catechol-estrogen metabolites. It has been shown that the conjugation of E_2-metabolites can be isoform specific (Abel *et al.*, 2004). Also, the 2 families of cytosolic GSTs Alpha (A) and Mu (M) heterodimerise and have similar substrate specificities for xenobiotic metabolism (Hayes *et al.*, 2005). These factors may influence the differential regulation of GSTA1 and GSTM1 by E_2.

In this study, neither berries nor ellagic acid show a differential expression of the steroid receptors, suggesting that the berry phytochemicals act by alternate mechanisms to bring about their specific effects. The berries show a significant effect with regard to the regulation of CYP1A1. CYP1A1 expression is controlled by AhR/ARNT signaling and whether berries mediate their effect by affecting this signaling pathway is not clear. However, the significant reduction in the CYP1A1 expression may translate to lower levels of harmful estradiol metabolites, as discussed above. Further, proof that berries may affect metabolite formation is evident from the CYP1B1 data, berries and ellagic acid down-regulate CYP1B1 expression much more than estradiol, suggesting that there may be a net reduction in harmful estradiol metabolites in the mammary epithelial cells of animals fed berries and ellagic acid. This is substantiated by the effect of both berries and ellagic acid on COMT expression at 18 weeks. The significant reduction in the COMT expression may be due to the constant suppression in the production of catechol-estrogen metabolites by sustained down-regulation of CYP1A1 and to a lesser extent of CYP1B1. Ellagic acid does not alter CYP1A1 expression, suggesting that it differs from other berry phytochemicals (anthocyanins) in its mechanism of action. Several reports suggest that dietary ellagic acid does not alter the expression of hepatic CYP1A1 (Barch *et al.*, 1994; Ahn *et al.*, 1996). However, it inhibits CYP1A1 activity both *in vitro* and *in vivo* (Barch *et al.*, 1994). Further, ellagic acid also increases the expression of hepatic quinone reductase, which is involved in the removal of harmful estrogen-metabolites via an antioxidant response element (Barch &

Rundhaugen, 1994). This enzyme is down-regulated after E_2-treatment in ACI rat mammary (Montano *et al.*, 2006). It is not known whether berries or ellagic acid up-regulate its expression in the mammary. Berries and ellagic acid also down-regulate 17ßHSD7, which may further reduce *in-situ* E_2 formation.

Collectively, these data suggest that CYP1A1 may play a major role in the generation of harmful catechol-estrogen metabolites in E_2-induced mammary tumorigenesis and that intervention by berries significantly reduce the formation of these metabolites and thus lead to prevention of mammary tumors. Ellagic acid acts via a different mechanism to reduce the levels of these metabolites. These results have to be further confirmed by analyzing the levels of various E_2-metabolites in the mammary tissue. Also, the differential effects of both E_2 and the chemopreventive agents on the various cell types must be delineated to understand cell-type specific actions of these agents.

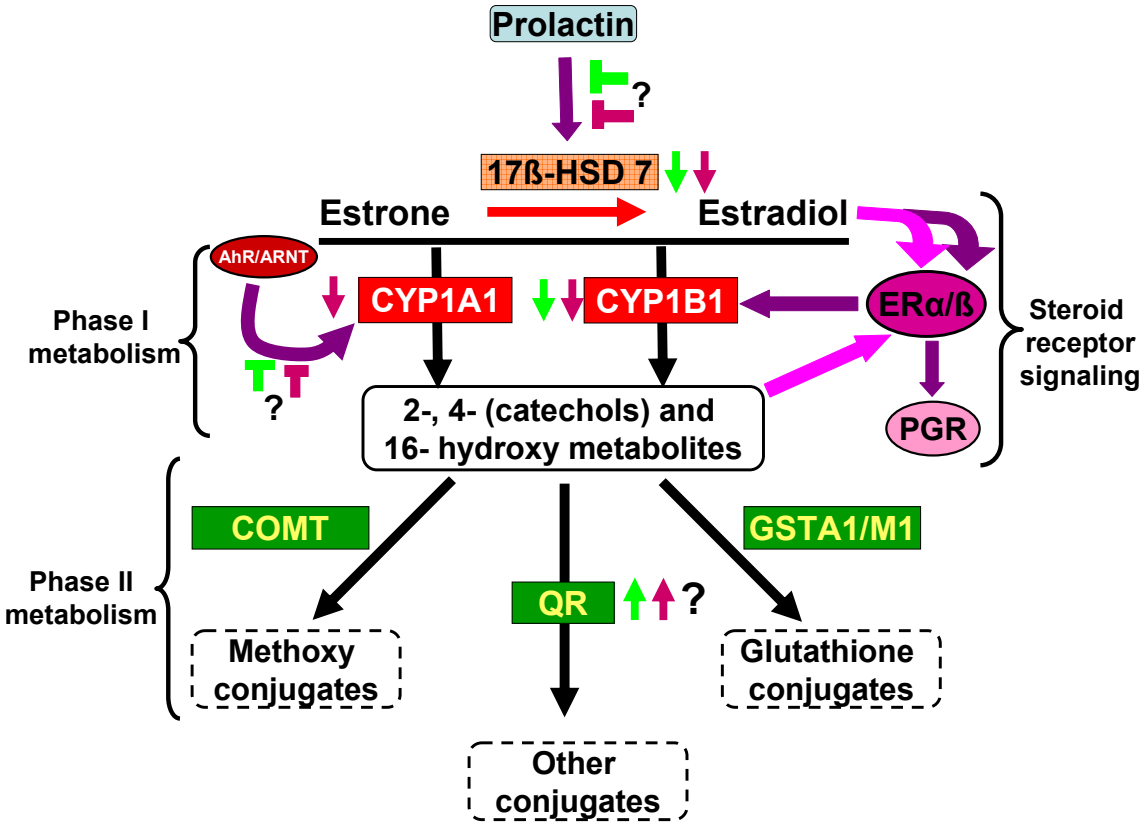


Figure 4.1. A simplified schematic representation of *in-situ* estrogen metabolism and steroid receptor signaling in the ACI rat mammary.

Pink arrows indicate binding and purple arrows indicate transcriptional regulation by nuclear factors. Up regulation is represented by ↑,↑ and down regulation is represented by ↓,↓ for ellagic acid and berries respectively. Regulations that need to investigated are marked with a "?". 17ß-HSD7 – 17ß-hydroxy steroid dehydrogenase, type 7; CYP1A1/1B1- Cytochrome P450 1A1/1B1; COMT- Catecho-O-methyl transferase; GSTA1/M1- Glutathione-S-transeferase.

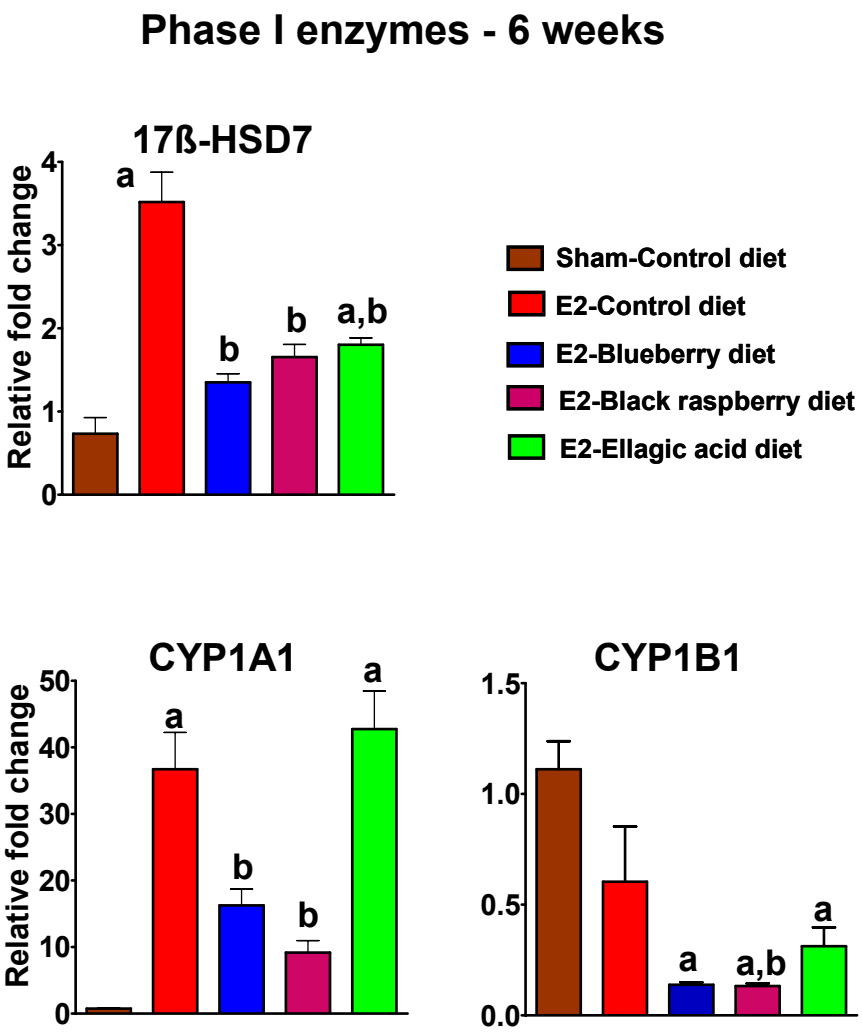


Figure 4.2. Effect of diets supplemented with indicated agents on the expression of genes involved in phase I estrogen metabolism, 6 weeks after estrogen treatment.

The relative fold change is expressed as mean ± SEM of n=6 per group. The results were compared using one way-ANOVA, followed by Tukey's test and the significant differences ($p<0.05$) are denoted as follows: a - significantly different from sham treated on control diet; b - significantly different from E2 treated on control diet. 17ßHSD- 17ß hydroxy streroid dehydrogenase, type 7.

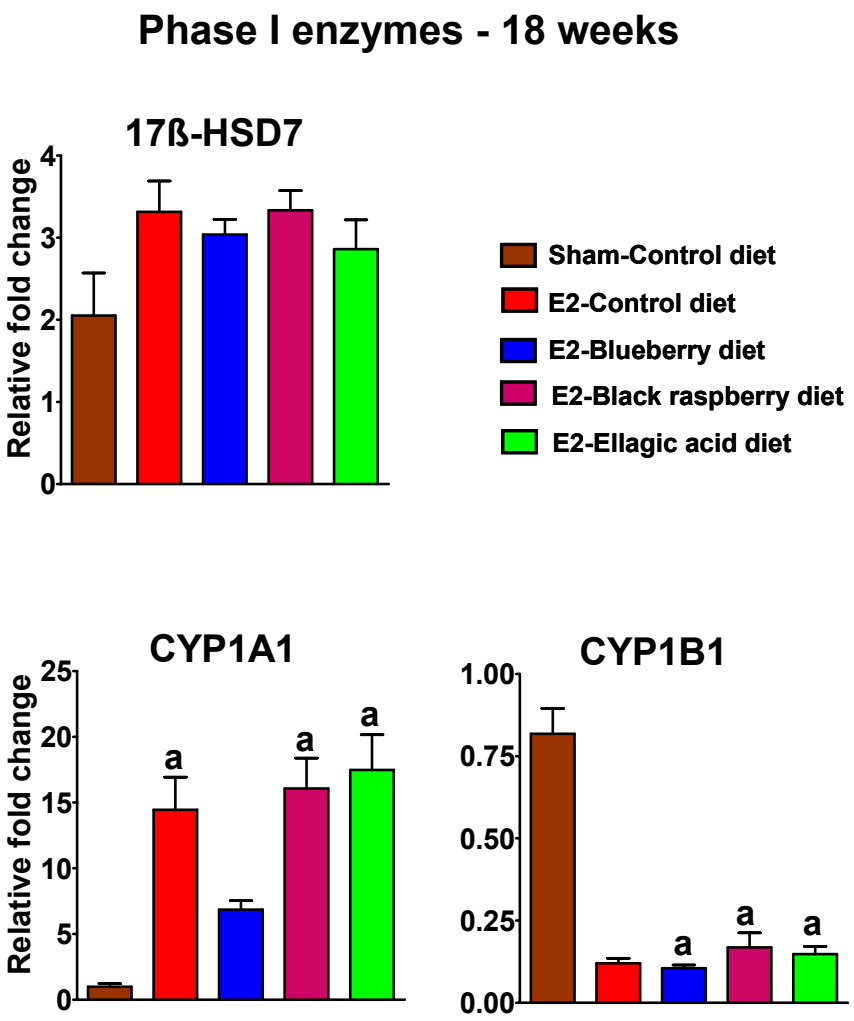


Figure 4.3. Effect of diets supplemented with indicated agents on the expression of genes involved in phase I estrogen metabolism, 18 weeks after estrogen treatment.

The relative fold change is expressed as mean ± SEM of n=6 per group. The results were compared using one way-ANOVA, followed by Tukey's test and the significant differences ($p<0.05$) are denoted as follows: a - significantly different from sham treated on control diet; b - significantly different from E_2 treated on control diet. 17ßHSD- 17ß hydroxy streroid dehydrogenase, type 7.

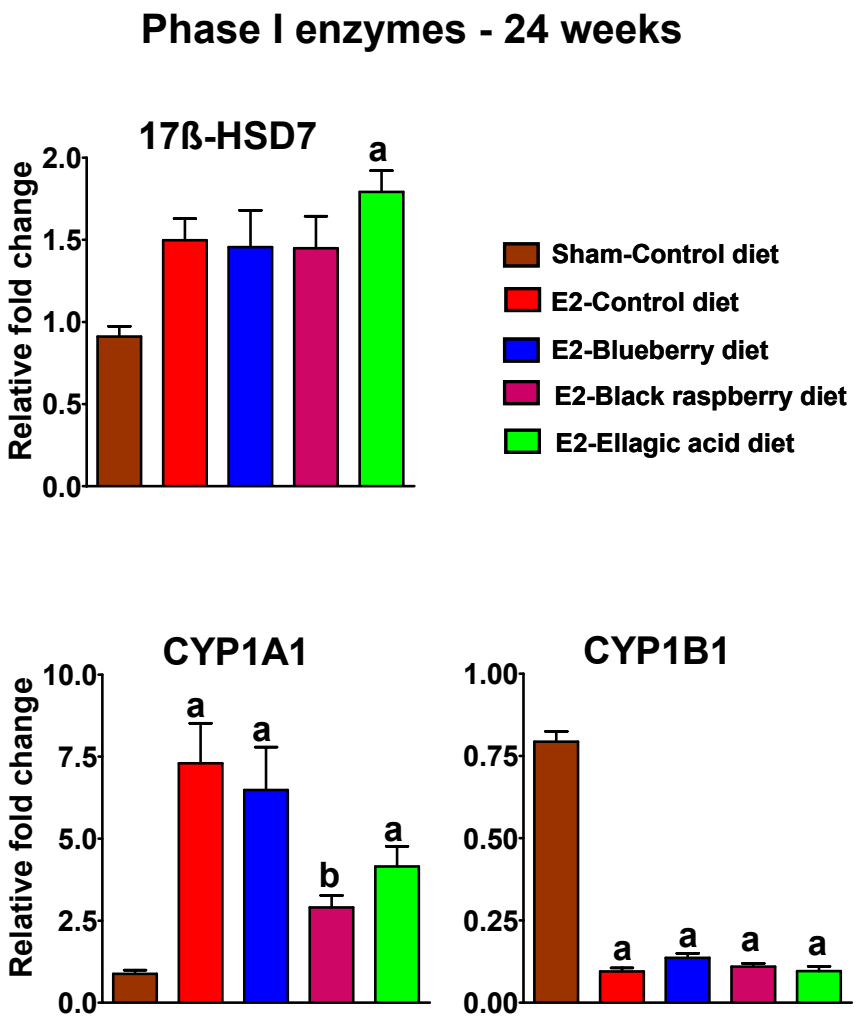


Figure 4.4. Effect of diets supplemented with indicated agents on the expression of genes involved in phase I estrogen metabolism, 6 weeks after estrogen treatment.

The relative fold change is expressed as mean ± SEM of n=6 per group. The results were compared using one way-ANOVA, followed by Tukey's test and the significant differences ($p<0.05$) are denoted as follows: a - significantly different from sham treated on control diet; b - significantly different from E_2 treated on control diet. 17ßHSD- 17ß hydroxy streroid dehydrogenase, type 7.

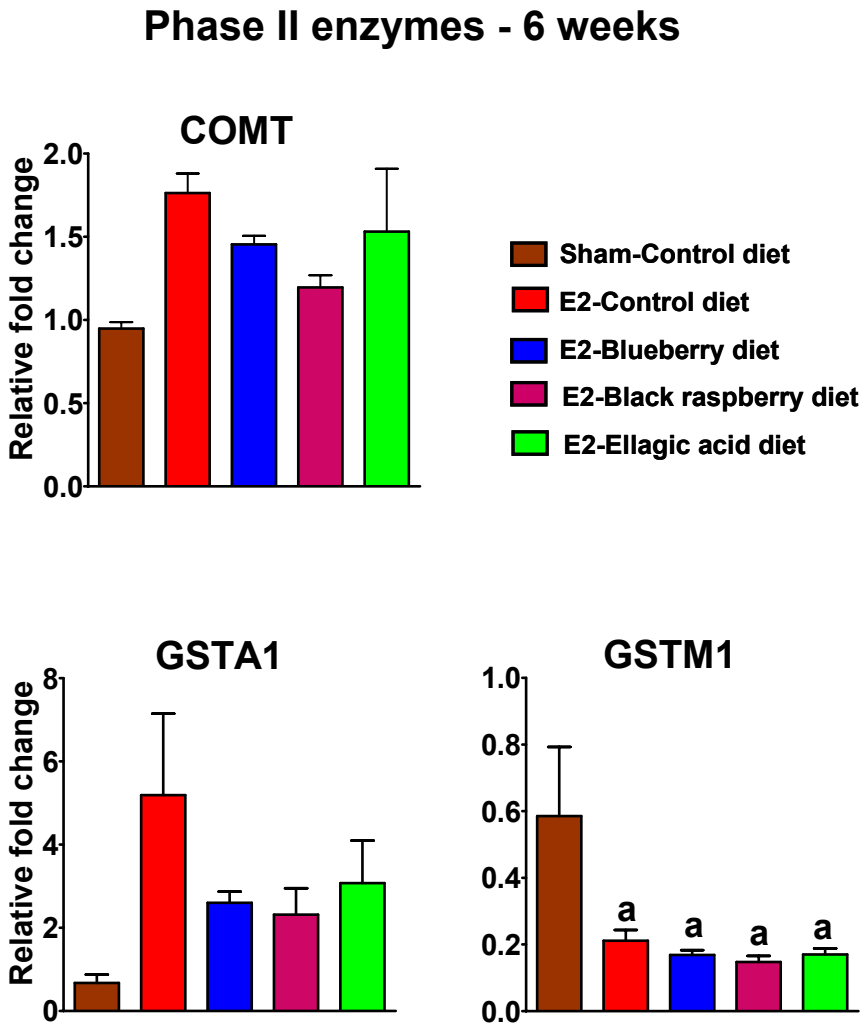


Figure 4.5. Effect of diets supplemented with indicated agents on the expression of genes involved in phase II estrogen metabolism, 6 weeks after estrogen treatment.

The relative fold change is expressed as mean ± SEM of n=6 per group. The results were compared using one way-ANOVA, followed by Tukey's test and the significant differences ($p<0.05$) are denoted as follows: a - significantly different from sham treated on control diet; b - significantly different from E_2 treated on control diet. COMT- Catechol-O-methyl transferase; GST-Glutathione-S-transferase.

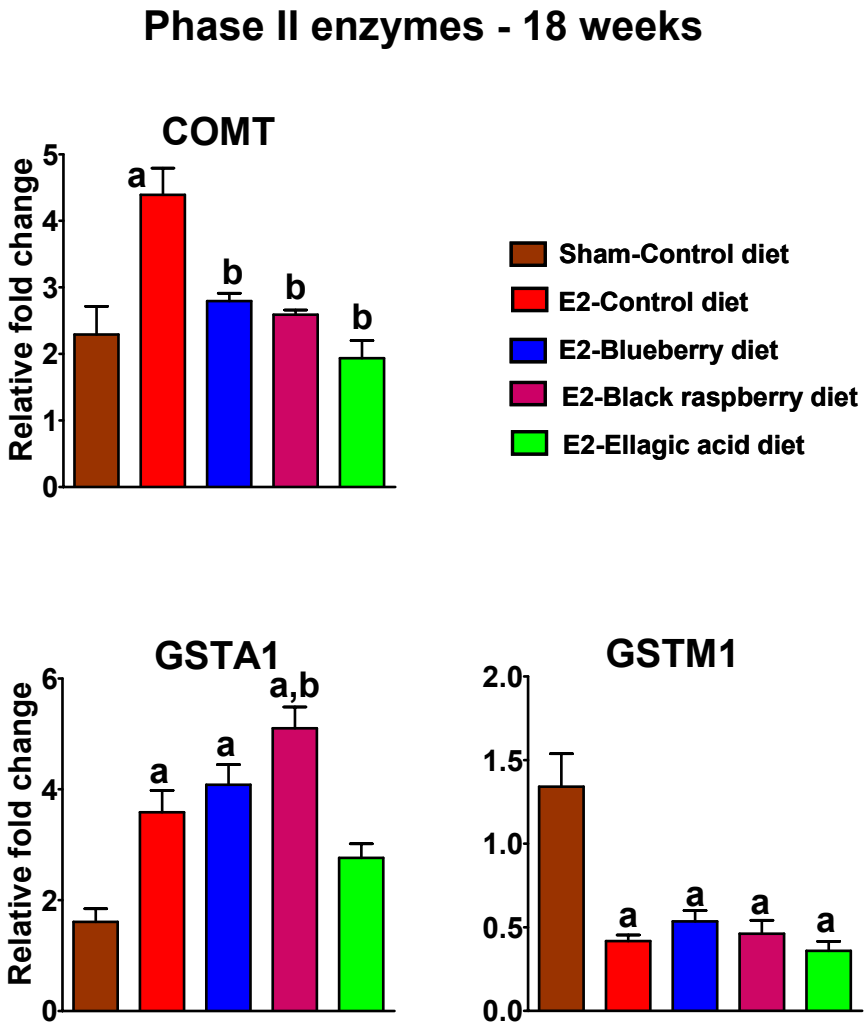


Figure 4.6. Effect of diets supplemented with indicated agents on the expression of genes involved in phase II estrogen metabolism, 18 weeks after estrogen treatment.

The relative fold change is expressed as mean ± SEM of n=6 per group. The results were compared using one way-ANOVA, followed by Tukey's test and the significant differences ($p<0.05$) are denoted as follows: a - significantly different from sham treated on control diet; b - significantly different from E_2 treated on control diet. COMT- Catechol-O-methyl transferase; GST-Glutathione-S-transferase.

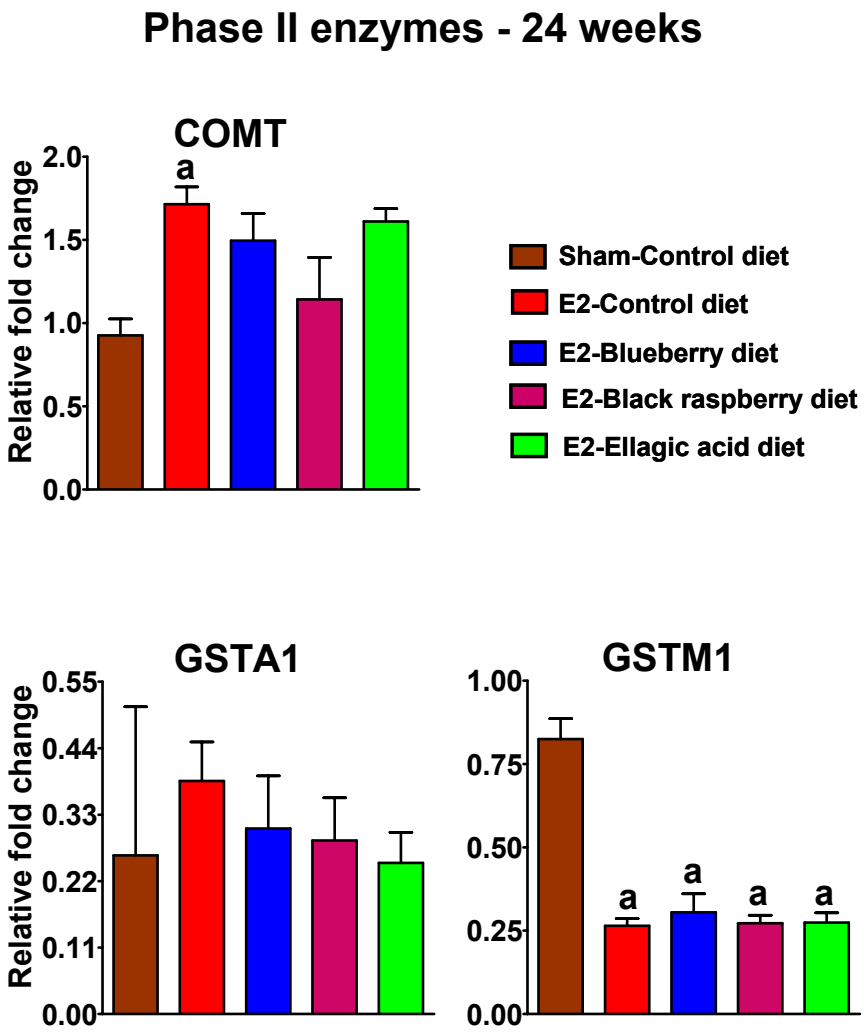


Figure 4.7. Effect of diets supplemented with indicated agents on the expression of genes involved in phase II estrogen metabolism, 24 weeks after estrogen treatment.

The relative fold change is expressed as mean ± SEM of n=6 per group. The results were compared using one way-ANOVA, followed by Tukey's test and the significant differences ($p<0.05$) are denoted as follows: a - significantly different from sham treated on control diet; b - significantly different from E_2 treated on control diet. COMT- Catechol-O-methyl transferase; GST-Glutathione-S-transferase.

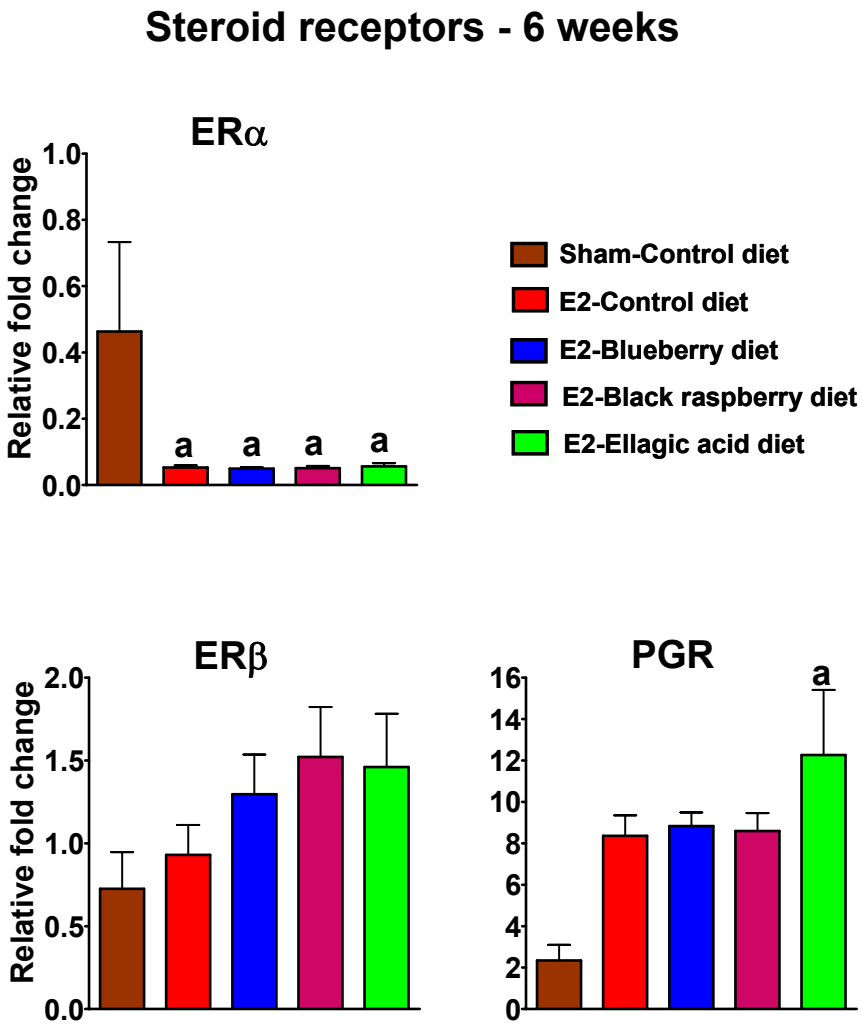


Figure 4.8. Effect of diets supplemented with indicated agents on the expression of steroid receptors, 6 weeks after estrogen treatment.

The relative fold change is expressed as mean ± SEM of n=6 per group. The results were compared using one way-ANOVA, followed by Tukey's test and the significant differences ($p<0.05$) are denoted as follows: a - significantly different from sham treated on control diet; b - significantly different from E_2 treated on control diet. ER- Estrogen receptors; PGR- Progesterone receptor.

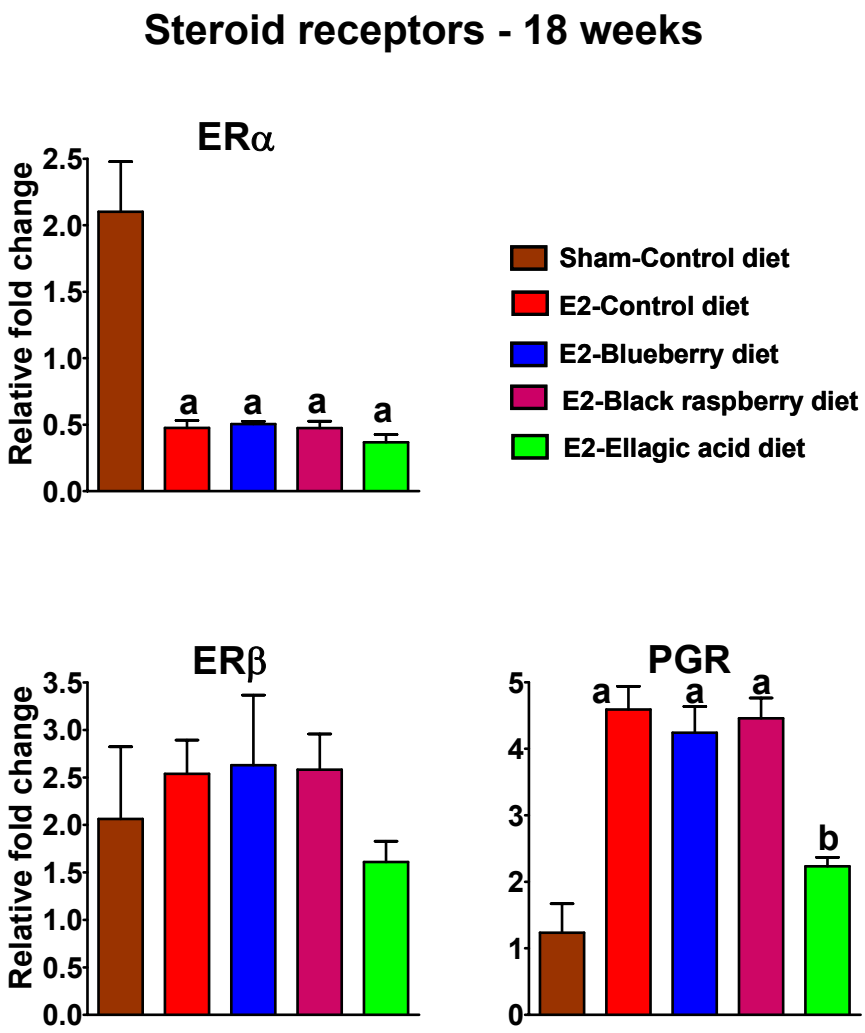


Figure 4.9. Effect of diets supplemented with indicated agents on the expression of steroid receptors, 18 weeks after estrogen treatment.

The relative fold change is expressed as mean ± SEM of n=6 per group. The results were compared using one way-ANOVA, followed by Tukey's test and the significant differences ($p<0.05$) are denoted as follows: a - significantly different from sham treated on control diet; b - significantly different from E_2 treated on control diet. ER- Estrogen receptors; PGR- Progesterone receptor.

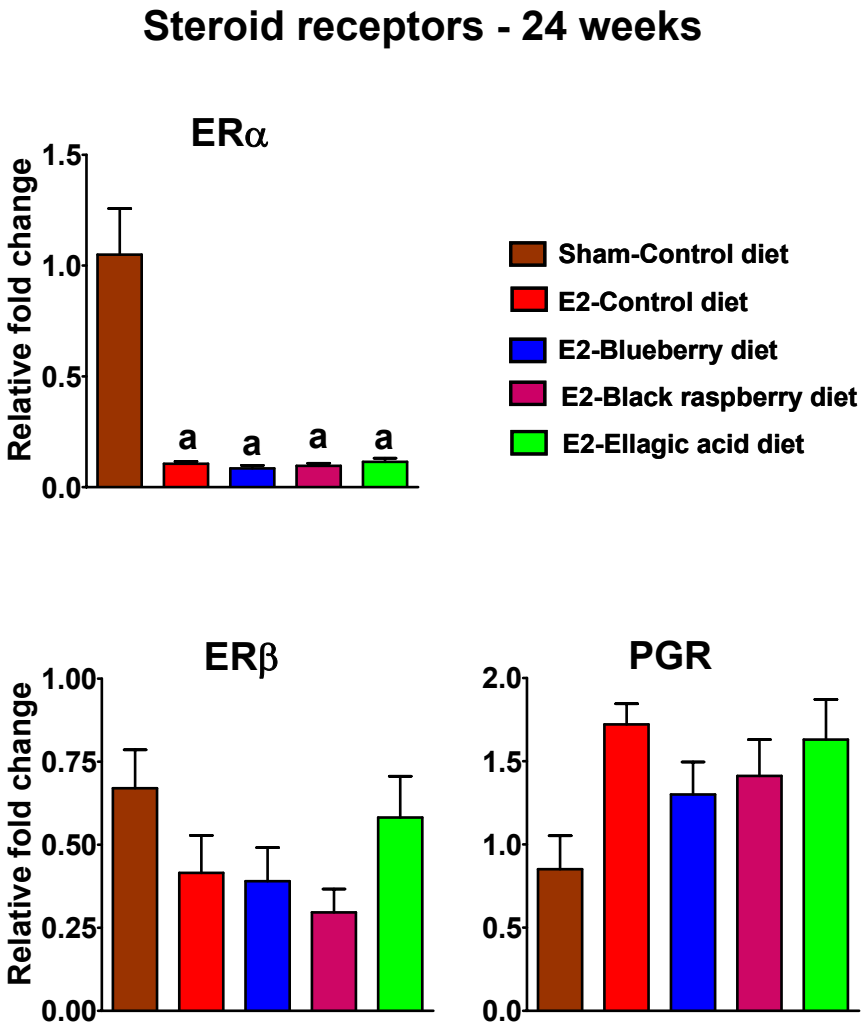


Figure 4.10. Effect of diets supplemented with indicated agents on the expression of steroid receptors, 24 weeks after estrogen treatment.

The relative fold change is expressed as mean ± SEM of n=6 per group. The results were compared using one way-ANOVA, followed by Tukey's test and the significant differences ($p<0.05$) are denoted as follows: a - significantly different from sham treated on control diet; b - significantly different from E_2 treated on control diet. ER- Estrogen receptors; PGR- Progesterone receptor.

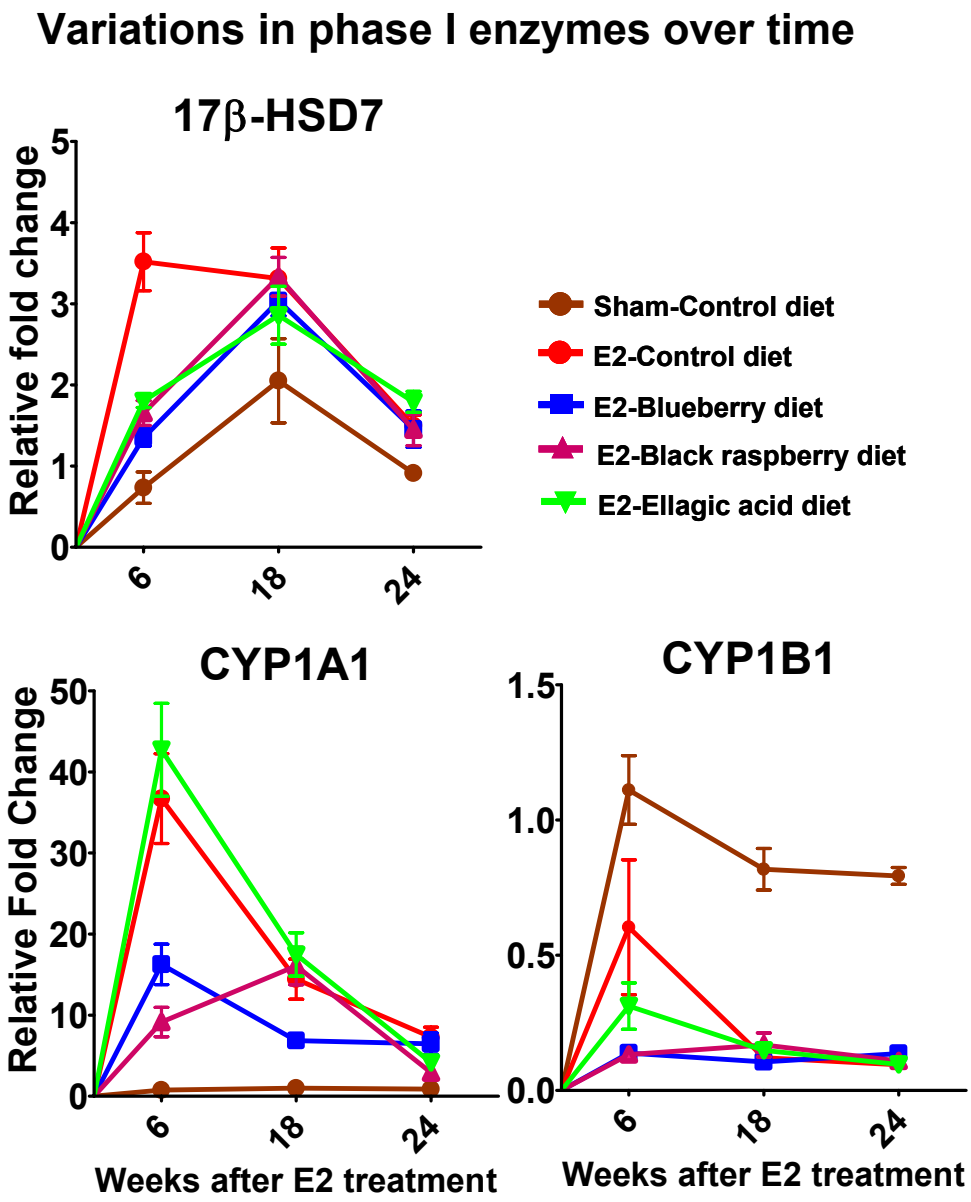


Figure 4.11. Variations in phase I enzymes of estrogen metabolism at different time points after estrogen treatment.

Six animals were randomized in to each group and were euthanized 6, 18 and 24 weeks after 17ß-estradiol implantation. RNA from the whole mammary tissue was analyzed for gene expression using quantitative real-time PCR. 17ßHSD-17ß hydroxy streroid dehydrogenase.

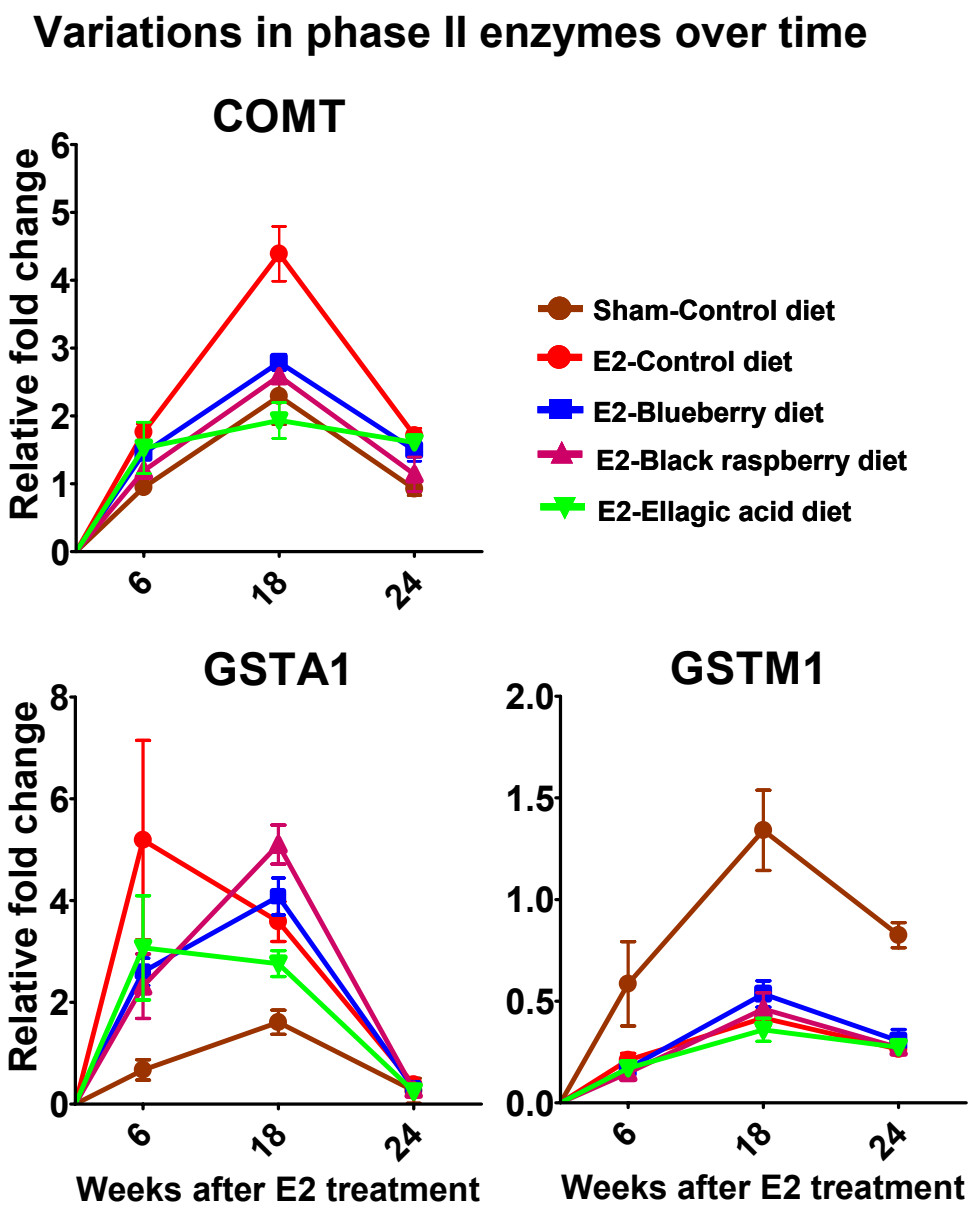


Figure 4.12. Variations in phase II enzymes of estrogen metabolism at different time points after estrogen treatment.

Six animals were randomized in to each group and were euthanized 6, 18 and 24 weeks after 17ß-estradiol implantation. RNA from the whole mammary tissue was analyzed for gene expression using quantitative real-time PCR. COMT-Catechol-O-methyl transferase; GST-Glutathione-S-transferase.

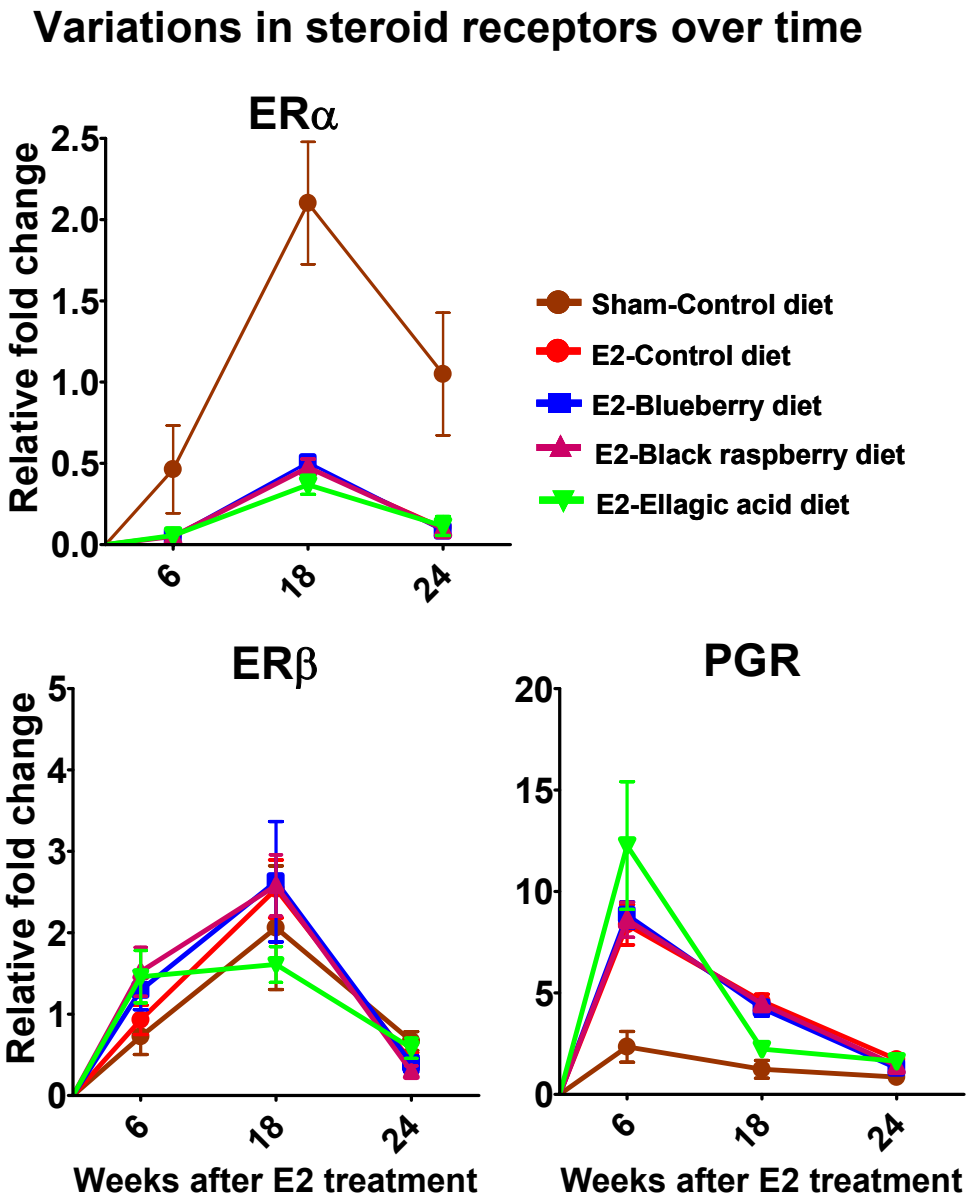


Figure 4.13. Variations in steroid receptor expression at different time points after estrogen treatment.

Six animals were randomized in to each group and were euthanized 6, 18 and 24 weeks after 17ß-estradiol implantation. RNA from the whole mammary tissue was analyzed for gene expression using quantitative real-time PCR. ER- Estrogen receptors; PGR- Progesterone receptor.

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Table 4.1. Experimental protocol – effect of berries and ellagic acid on estrogen metabolism during estrogen-induced mammary tumorigenesis in ACI rats.

	Group description	Treatment	Intervention
1	SH-CD	Sham	Control diet
2	E2-CD	17ß-estradiol	Control diet
3	E2-BB		2.5% (w/w) blueberries
4	E2-BRB		2.5% (w/w) black raspberry
5	E2-EA		400 ppm ellagic acid

Eighteen animals were randomized in to each group and fed respective diets from 2 weeks prior to estrogen treatment until the end of the study. Six animals from each group were euthanized at 6, 18 and 24 weeks after estrogen treatment.

Table 4.2. Primer sequences for quantitative real-time PCR.

Gene	Forward	Reverse
17ß HSD	5' - CTTTATCCTGATTCGGGAACTG - 3'	5' - GTCCTCAAGACTGAAGTTAGA C - 3'
CYP1A1	5' - TGGAGACCTTCCGACATTCAT - 3'	5' - GGGATATAGAAG CCATTCAGACTT G - 3'
CYP1B1	5' - AACCCAGAGGACTTTGATCCG - 3'	5' - CGTCGTTTGCCCACTGAAAA - 3'
COMT	5' - GGATGCAGTGATTCGGGAGTA - 3'	5' - GCAGCGTAGTCAGGGTTCATCT - 3'
GSTA1	5' - CCAGCCTTCTGACCTCTTTCC - 3'	5' - TCTTCGATTTGTTTTGCATCCA - 3'
GSTM1	5' - TCTTGACCAGTACCACATTTTTGA G - 3'	5' - TCGAAAATATAGGTGTTGAGAGGTAGTG - 3'
ERα	5' - GGCACATGAGTAACAAAGGCA - 3'	5' - GGCATGAAGACGATGAGCAT - 3'
ERß	5' - CTCCTTTAGCGACCCATTGC - 3'	5' - CTCCCACTAAGCTTCCTCTTCAGT - 3'
PGR	5' - TCACAACGCTTCTATCAACTTACAAA - 3'	5' - GGCAGCAATAACTTCAGACATCA - 3'
ß-Actin	5' – GCCAACCGTGAAAAGATGAC - 3'	5' - ACCCTCATAGATGGGCACAG - 3'

Primers were designed using Primer Express® software across exon boundary for the following genes: 17ßHSD7- 17ß hydroxyl steroid dehydrogenase 7;CYP1A1-Cytochrome P450 1A1; CYP1B1-Cytochrome P450 1B1; COMT-Catechol-O-methyl transferase; GSTA1- Glutathione-S-transferase A1; GSTM1-Glutathione-S-transferase M1; ERα-Estrogen receptor α; ERß-Estogen receptor ß; PGR- Progesterone receptor.

Table 4.3. Effect of diets supplemented with indicated agents with on expression of genes involved in estrogen metabolism and signaling in ACI rat mammary 6 weeks after estrogen treatment

Gene	Fold Change (mean ± SEM)	SH-CD *(n=3)*	E2-CD *(n=6)*	E2-BB *(n=6)*	E2-BRB *(n=6)*	E2-EA *(n=5)*
17ßHSD	Relative	0.73±0.2	3.5±0.4	1.3±0.1	1.6±0.2	1.5±0.4
	Absolute	1	4.8[a]	1.8[b]	2.2[b]	2.5[a,b]
CYP1A1	Relative	0.75±0.09	36.7±5.5	16.3±2.5	9.2±1.8	42.7±5.7
	Absolute	1	48[a]	21[b]	12[b]	56[a]
CYP1B1	Relative	1.1±0.1	0.6±0.2	0.1±0.01	0.1±0.01	0.3±0.1
	Absolute	1	1.8↓	11[a]↓	11[a,b]↓	3.6[a]↓
COMT	Relative	0.9±0.04	1.8±0.1	1.4±0.05	1.2±0.1	1.5±0.4
	Absolute	1	2	0.7	1.3	1.6
GSTA1	Relative	0.7±0.2	5.2±2.0	2.6±0.3	2.3±0.6	3.1±1.0
	Absolute	1	7.4	3.7	3.3	4.4
GSTM1	Relative	0.6±0.2	0.2±0.03	0.2±0.01	0.1±0.01	0.2±0.02
	Absolute	1	3[a]↓	3[a]↓	6[a]↓	3[a]↓
ERα	Relative	0.5±0.3	0.05±0.01	0.05±0.004	0.05±0.001	0.06±0.01
	Absolute	1	10[a]↓	10[a]↓	10[a]↓	8.3[a]↓
ERß	Relative	0.7±0.2	0.9±0.2	1.3±0.2	1.5±0.3	1.5±0.3
	Absolute	1	1.3	1.8	2.1	2.1
PGR	Relative	2.3±0.8	8.3±0.9	8.8±0.6	8.6±0.8	12.3±3.1
	Absolute	1	3.6	3.8	3.7	5.3[a]

Female ACI rats were treated with subcutaneous sham implants (SH-CD) or those containing 27 mg 17ß-estradiol (E2-CD) and fed control diet or diets supplemented with 2.5% w/w blueberries (E2-BB); black raspberries (E2-BRB) or 400 ppm ellagic acid (E2-EA) and euthanized 6 weeks after treatment. Relative and absolute gene expression changes were calculated as described in materials and methods. The results were compared using one way-ANOVA, followed by Tukey's test and the significant differences ($p<0.05$) are denoted as follows: a - significantly different from sham treated on control diet (SH-CD); b - significantly different from E2 treated on control diet (E2-CD). Down–regulation of genes is denoted by a downward arrow (↓)

Table 4.4. Effect of diets supplemented with indicated agents with on expression of genes involved in estrogen metabolism and signaling in ACI rat mammary 18 weeks after estrogen treatment

Gene	Fold Change (mean ± SEM)	SH-CD *(n=5)*	E2-CD *(n=6)*	E2-BB *(n=6)*	E2-BRB *(n=6)*	E2-EA (*n=6)*
17ßHSD	Relative	2.1±10.5	3.3±10.4	3.0±0.2	3.3±0.2	2.9±0.4
	Absolute	1	1.6	1.4	1.6	1.4
CYP1A1	Relative	0.98±0.2	14.4±2.5	6.8±0.7	16.1±2.3	17.5±2.7
	Absolute	1	14.7[a]	6.9	16.4[a]	17.8[a]
CYP1B1	Relative	0.8±0.1	0.1±0.01	0.1±0.01	0.2±0.04	±0.02
	Absolute	1	8[a]↓	8[a]↓	4[a]↓	8[a]↓
COMT	Relative	2.3±0.4	4.4±0.4	2.8±0.1	2.6±0.1	1.9±0.3
	Absolute	1	1.9[a]	1.2[b]↓	1.1[b]↓	1.1[b]↓
GSTA1	Relative	1.6±0.2	3.6±0.4	4.1±0.4	5.1±0.4	2.8±0.3
	Absolute	1	2.25[a]	2.6[a]	3.2[a,b]	1.75
GSTM1	Relative	1.3±0.2	0.4±0.04	0.5±0.1	0.5±0.1	0.4±0.1
	Absolute	1	3.25[a]↓	2.6[a]↓	2.6[a]↓	3.25[a]↓
ERα	Relative	2.1±0.4	0.5±0.05	0.5±0.02	0.5±0.05	0.4±0.05
	Absolute	1	4.2[a]↓	4.2[a]↓	4.2[a]↓	5.2[a]↓
ERß	Relative	2.1±0.8	2.5±0.4	2.6±0.7	2.6±0.4	1.6±0.2
	Absolute	1	1.2	1.2	1.2	1.3
PGR	Relative	1.2±0.4	4.6±0.3	4.2±0.4	4.5±0.3	2.2±0.1
	Absolute	1	3.8[a]	3.5[a]	3.75[a]	1.8[b]

Female ACI rats were treated with subcutaneous sham implants (SH-CD) or those containing 27 mg 17ß-estradiol (E2-CD) and fed control diet or diets supplemented with 2.5% w/w blueberries (E2-BB); black raspberries (E2-BRB) or 400 ppm ellagic acid (E2-EA) and euthanized 18 weeks after treatment. Relative and absolute gene expression changes were calculated as described in materials and methods. The results were compared using one way-ANOVA, followed by Tukey's test and the significant differences ($p<0.05$) are denoted as follows: a - significantly different from sham treated on control diet (SH-CD); b - significantly different from E2 treated on control diet (E2-CD). Down–regulation of genes is denoted by a downward arrow (↓)

Table 4.5. Effect of diets supplemented with indicated agents with on expression of genes involved in estrogen metabolism and signaling in ACI rat mammary 24 weeks after estrogen treatment

Gene	Fold Change (mean ± SEM)	SH-CD *(n=5)*	E2-CD *(n=6)*	E2-BB *(n=5)*	E2-BRB *(n=6)*	E2-EA *(n=6)*
17ßHSD	Relative	0.9±0.1	1.5±0.1	1.5±0.2	1.4±0.2	1.8±0.1
	Absolute	1	1.6	1.6	1.5	1.9[a]
CYP1A1	Relative	0.9±0.1	7.3±1.2	6.5±1.3	2.9±0.4	4.2±0.6
	Absolute	1	8.1[a]	7.2[a]	3.2[b]	4.6[a]
CYP1B1	Relative	0.8±0.03	0.09±0.01	0.14±0.01	0.1±0.01	0.1±0.01
	Absolute	1	4[a]↓	5.7[a]↓	8[a]↓	8[a]↓
COMT	Relative	0.9±0.1	1.7±0.1	1.5±0.2	1.1±0.3	1.6±0.1
	Absolute	1	1.8[a]	1.6	1.2	1.7
GSTA1	Relative	0.3±0.2	0.4±0.1	0.3±0.1	0.3±0.1	0.3±0.05
	Absolute	1	1	1	1	1
GSTM1	Relative	0.8±0.1	0.3±0.02	0.3±0.05	0.3±0.02	0.3±0.03
	Absolute	1	2.6[a]-↓	2.6[a]-↓	2.6[a]-↓	2.6[a]-↓
ERα	Relative	1.05±0.2	0.1±0.01	0.08±0.01	0.09±0.01	0.1±0.01
	Absolute	1	10.5[a]↓	13.1[a]↓	11.6[a]↓	10.5[a]↓
ERß	Relative	0.7±0.1	0.4±0.1	0.4±0.1	0.3±0.1	0.6±0.1
	Absolute	1	1.75↓	1.75↓	2.3↓	1.2↓
PGR	Relative	0.8±0.2	1.7±0.1	1.3±0.2	1.4±0.2	1.6±0.2
	Absolute	1	2.1	1.6	1.75	2

Female ACI rats were treated with subcutaneous sham implants (SH-CD) or those containing 27 mg 17ß-estradiol (E2-CD) and fed control diet or diets supplemented with 2.5% w/w blueberries (E2-BB); black raspberries (E2-BRB) or 400 ppm ellagic acid (E2-EA) and euthanized 24 weeks after treatment. Relative and absolute gene expression changes were calculated as described in materials and methods. The results were compared using one way-ANOVA, followed by Tukey's test and the significant differences ($p<0.05$) are denoted as follows: a - significantly different from sham treated on control diet (SH-CD); b - significantly different from E2 treated on control diet (E2-CD). Down–regulation of genes is denoted by a downward arrow (↓).

Chapter Five: General Discussion and Conclusions

Millions of women in the world are chronically exposed to 17ß-estradiol. This hormone is highly important for regular development but also increases the risk for breast cancer. Since women cannot be completely protected from exposure, prevention is an effective course of action to reduce the incidence of breast cancer. Many of the current treatment strategies evolve around this concept. Currently, tamoxifen therapy is the standard care for women who are at a high risk for breast cancer and involves 5 years of preventive therapy (Morrow & Jordan, 2000). However, this therapy does not come without costs, several side effects of tamoxifen treatment include increased incidence of blood clots, depression in some cases, endometrial cancer in post-menopausal women, etc,. (Reviewed by Anthony, Williams and Dunn, 2001). Raloxifene, another selective estrogen receptor mediator (SERM) is effective in reducing certain risks associated with tamoxifen therapy is poorly bioavailable and rapidly excreted from the body (Jordan, 2007). Thus, the search for an ideal estrogen receptor modulator that will have the nuero- and osteo-protective effects of estrogen and also prevent cancer in estrogen-target tissues has become the holy grail of modern breast cancer prevention research.

Most cells in the human body possess an estrogen receptor (ER) and the gene transcription function of estrogen was thought to be mediated via its interaction with this receptor. However, it is now documented that estradiol can act via non receptor-mediated pathways to cause changes in cell function (Reviewed in Coleman and Smith, 2001). Ideally, a SERM can interact with any or all of these pathways and its structural similarity to estrogen determine the interactions. Phytonutrients, such as polyphenols can act on several different pathways that overlap with estrogen/steroid signaling, such as MAPK, PKC, etc., suggesting that they may posses SERM effects beyond actions on the classic ER (Reviewed by Rushmore and Tony Kong, 2002).

The estradiol-induced mammary tumor in ACI rats provides an apt *in vivo* testing system to test several SERMs. Although most breast cancer drugs have traditionally been tested against 7,12, dimethyl benze[*a*]anthacene-induced mammary tumors, estrogen-induction causes several signal transduction cascades that can only occur in the presence of excess estradiol. In fact, the results from studies presented herein show that ERα in the mammary is down-regulated by estradiol treatment. Thus E_2-induced mammary carcinogenicity in the ACI rat may involve non receptor-mediated action of E_2. Tamoxifen (40 mg in subcutaneous cholesterol pellets) completely abrogates E_2-induced tumors in ACI rats (Li *et al.*, 2002b). However, this study showed significant increases in various ERα isoforms by immunohistochemical detection and increased levels of both progesterone receptor (PGR) isoforms (Li *et al.*, 2002b). The contrasting results with respect to ERα expression can be attributed to difference in the methods of detection (qRTPCR versus immunohistochemistry) and the presence and differential regulation of different isoforms of ER, which may be detected at the protein level but not at the mRNA level unless specific primers are designed for this purpose.

The importance of other organ systems, such as the liver must also be taken in to account when analyzing the mechanisms by which E_2 induces mammary tumors. For example, both phenobarbitol (PB) and clofibrate (CF) when administered together with E_2 have differential effects on the metabolism of E_2 in the liver (Mesia-Vela *et al.*, 2004; Mesia-Vela *et al.*, 2006). PB acts synergistically with E_2, while CF antagonizes its action. Further, PB effectively reduces the mammary tumor burden. This may be due to the direct effect of PB on E_2 metabolism in the mammary or via indirect mechanisms due to changes caused in other organs such as liver as reported (Mesia-Vela *et al.*, 2006). Studying the effects of berries and ellagic acid on hepatic metabolism may yield more clues about the mechanisms by which these agents prevent mammary tumors. Indeed, both red raspberry and ellagic acid when provided via the diet have significant effects on hepatic gene transcription in CD-1 mice (Chapter 2). It

remains to be seen whether similar and additional effects are present in the ACI rats also.

The mechanisms involved in E_2-induced mammary tumorigenesis in ACI rats are complex. Some mechanisms have been elucidated, while several others need to be clarified. Foremost, ACI rats are the only rat strain that is completely susceptible to E_2-induced mammary tumors (Dunning *et al.*, 1953; Shepel & Gould, 1999). In all of the prevention studies, the serum E_2 levels do not differ between the different groups, suggesting that preventive agents can act without altering the circulating E_2 levels (Li *et al.*, 2002b; Mesia-Vela *et al.*, 2006); Chapter 3). Further, prolactin is considered to play a major role in the development of these tumors (Holtzman *et al.*, 1981). Tamoxifen, which abrogates mammary tumors in female ACI rats, also reduces pituitary tumors and serum prolactin levels in male ACI rats treated continuously with E_2 (Lyle *et al.*, 1984). The effect of tamoxifen on the pituitary of female rats has not been reported, however, it significantly reduces the uterine wet-weight (Li *et al.*, 2002b), which could be an indicator of and effect on both direct estrogen action and effect via pituitary hormones. Further evidence for the role of pituitary in mammary tumor development can be garnered from estradiol-dose response studies done in our laboratory and also presented in this work. When the dose of E_2 is reduced 3-fold (from 27 mg to 9 mg), the tumor burden as well as tumor number increase (Chapter 3, Tables 3.5 and 3.6; Ravoori *et al.*, 2007). There is a striking correlation between the ratios of serum E_2 and plasma prolactin levels between the two doses. When the E_2 dose is reduced by 3-fold, the circulating E_2 and prolactin levels fall by 2.8- and 2.5- fold respectively at 12 weeks and by about 1.7 fold at termination of the study (Ravoori *et al.*, 2007).This correlation exemplifies the direct effect of E_2 on prolactin levels. It is unclear whether prolactin impedes or improves tumor development since lower levels of prolactin, caused by lowering the E_2 dose, increases tumor burden but the absence of a prolactin response results in non-induction of mammary tumors (Holtzman *et al.*, 1981). However, the effect of complete inhibition of prolactinomas with bromocriptine or tamoxifen in female ACI rats and subsequent effects on

mammary tumor incidence has not been reported. Further investigation is warranted to elucidate the exact role of prolactin in mammary cancer development in ACI rats.

There is collective evidence that black raspberry, blueberry and ellagic acid may prevent E_2–induced tumorigenesis by interrupting the effects of pituitary. First, the pituitary wet weights are slightly, but insignificantly reduced by dietary intervention at both doses of E_2 treatment (Chapter 3, Tables 3.5 and 3.6). Second, compared to sham treatment, the combined wet weight of ovary and uterus increased significantly in animals treated with 27 mg E_2 for 24 weeks (870 ± 50 mg versus 620 ± 80 mg) ($p<0.01$). This increase in tissue weight was significantly reduced after dietary intervention ($p<0.05$; blueberry diet – 650 ± 18; black raspberry diet- 600 ± 77; ellagic acid diet – 650 ± 42 mg). These results are similar in trend but not in magnitude to tamoxifen treatment (Li *et al.*, 2002; S. Li, personal communication). Moreover, all dietary interventions significantly offset the overexpression of 17ßHSD, a gene known to be controlled by prolactin, at 6 weeks (Duan *et al.*, 1997). However, the levels of prolactin must be measured in the serum of animals on the supplemented group to confirm this notion. Taken together, these facts suggest that both berries and ellagic acid may act as SERMs, but at a much lower capacity compared to a classic antiestrogen like tamoxifen. Further studies are required to confirm the SERM effects of these dietary agents in the various estrogen-responsive organs of the ACI rat including the pituitary, ovary, uterus, adrenal and the mammary.

The contribution of the classic ER pathway in the mammary tissue of these animals is not clear since E_2 treatment seems to down-regulate ERα expression by more than 10 fold beginning at 6 weeks until the end of the experiment. Furthermore, it is not known if estradiol signals through other pathways to cause molecular changes. Also, estradiol is known to cause chromosomal instability and *c-myc* amplification in these rats (Li *et al.*, 2002a). It is not clear yet if the interventions affect any of these pathways and further studies are required to elucidate this.

Estrogen is an endobiotic. The importance of estrogen metabolism in the causation of breast cancer has been understood based on several data. Polymorphisms in one or more genes that are involved in estradiol metabolism increase the carrier's risk of breast cancer (Thompson & Ambrosone, 2000; Gallicchio *et al.*, 2006). Also, there is an imbalance in the metabolic profile between cancer-free and cancer-prone women. In addition, tumor tissue contains higher levels of metabolites such as 4-hydroxy estradiol ($4E_2$) than in the surrounding normal tissue (Reviewed by Liehr, 2000). To support these observations, laboratory studies have also proven the pro-carcinogenic effects of $4E_2$ (Liehr *et al.*, 1986; Russo *et al.*, 2002), suggesting that $4E_2$ and CYP1B1 may play the major role in E_2-induced carcinogenesis. However, CYP1A1 is a mixed function oxygenase, which can form both $2E_2$ and $4E_2$ (Cribb *et al.*, 2006). It is clear from our findings that berries consistently offset E_2-induced up regulation of CYP1A1 and of CYP1B1 at 6 weeks only. Ellagic acid, however, is effective against CYP1B1 only. The transcriptional regulation of these enzymes is controlled by various nuclear receptors via interaction with antioxidant response elements (AREs) and electrophile response elements (EpREs) (Reviewed by Honkakoshi & Negishi, 2000 and by Hollenberg, 2002). Further research is required to elucidate the exact mechanisms of action of berry phytochemicals. Studies suggest that ellagic acid decreases the activity of hepatic CYP1A1 without increasing its expression and also increases the expression and activity of quinone reductase, an enzyme involved in phase II metabolism of estrogen metabolites (Barch & Rundhaugen, 1994; Barch *et al.*, 1994; Ahn *et al.*, 1996). The effect of both berry anthocyanins and ellagic acid on quinone reductase in the mammary must be investigated. Berries vary widely in their distribution of anthocyanins and ellagic acid. Of the berries tested, blueberry has a wider spectrum of anthocyanins but in lower quantities, while black raspberry contains predominantly one anthocyanin but in much higher amounts (Wu *et al.*, 2006). Whether these differences influence their preventive efficacy is not known. This can be tested by using bilberries, which are high in anthocyanin content and also have a wide variety of polymers (Wu *et al.*, 2006).

To summarize the results presented herein, 17ß-estradiol induces mammary tumors in ACI rats when administered alone. Berries and ellagic acid prevent the growth of these tumors by up to 70%. Also, these agents favorably alter estrogen-metabolizing enzymes in the mammary tissue. Although, there are some differences in the trends between berries versus ellagic acid 2 conclusions seem to emerge - 1) both berries act via similar mechanisms; 2) ellagic acid acts via a different mechanism to produce the same protective effect. Further, both berries and ellagic acid effectively reduce baseline DNA damage and induce DNA repair enzyme expression, in the liver of CD-1 mice. In addition, ellagic acid is the most effective polyphenol to reduce $4E_2$-induced DNA damage *in vitro*. Taken together these results suggest that both berries and ellagic acid may act via multiple mechanisms to prevent E_2–induced mammary tumorigenesis.

The most important conclusion of this thesis is that regardless of the mechanisms involved, both berries and ellagic acid beneficially influence mammary tumorigenesis in ACI rats. This provides evidence that a low dose of whole foods can be used in the prevention of breast cancer as effectively as relatively higher dose of a pure constituent. Although no reduction in mammary tumor incidence was seen in this investigation, the better quality of life in berry- and ellagic acid-fed animals, as evidenced by reduced mortality, weight loss etc., and significant reductions in tumor burden, provide strong support for the use of these agents as an adjuvant alongside traditional modes of cancer therapy. The safety and applicability of such an intervention is being tested for esophageal cancer (Kresty *et al.*, 2006). Preclinical studies looking at the interaction between classic prevention therapy such as tamoxifen and berries are required before proceeding to clinical trials. Also, studies assessing whether berries, berry anthocyanins and ellagic acid will antagonize the adverse actions of tamoxifen must be conducted. Nevertheless, the use of berries as an adjuvant in breast cancer prevention holds much promise.

APPENDIX

List of abbreviations

2-AAF	2-acetylaminofluorine
2-E_2	2-hydroxy estradiol
3-MC	3-methylcholantherene
4-E_2	4-hydroxy estradiol
8-oxodG	8-oxo-2' deoxy Guanosine
17ßHSD	17-ß-hydroxysteroid dehydrogenase
ACI	August-Copenhagen-Irish-hooded
B[*a*]P	Benzo[*a*]pyrene
BMI	Body Mass Index
CF	Clofibrate
COMT	Catechol-O-methyl transferase
COX-2	Cyclooxygenase -2
CYP	Cytochrome P450
DB[*a,l*]P	Dibenzo[*a,l*]pyrene
DCIS	Ductal carcinoma *in situ*
DMBA	7,12-dimethylbenz(*a)*antharcene
DMSO	Dimethyl sulphoxide
DNL3	DNA ligase 3
E_2	17ß-estradiol
EGCG	Epigallocatechin gallate
ERα	Estrogen receptor Alpha
ERß	Estrogen receptor Beta
ERCC5	Excision repair cross complementation group 5
ERK	Extracellular signal related kinase
ERKO/*wnt*-1	Estrogen receptor knock-out/*wnt*
GST	Glutathione-S-transferase
HAA	Heterocyclic aromatic amines
HNE	Hydroxy nonenal
HRT	Hormone replacement therapy
Lob 1	Lobules type 1
MAPK	Mitogen activated protein kinase
MAPKK	MAP kinase kinase
MDA	Malodialdehyde
NADP	Nicaonitamide dinucleotide phosphate
NFκB	Nuclear factor κ B
NMU	1-methyl-1-nitrosourea
PAH	Polycyclic aromatic hydrocarbons
PB	Phenobarbital
PCNA	Proliferating Cell Nuclear Antigen
PEI	Polyethyleneinimine
PGR	Progesterone Receptor
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
PKC	Protein Kinase C
PRL	Prolactin

ROS	Reactive Oxygen Species
SD	Sprague Dawley
SERM	Selective estrogen receptor modulator
SOD	Superoxide Dismutase
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TDLU	Terminal Ductal Lobular Unit
TLC	Thin layer chromatography
TST	Thiosulphate sulphur transferase
VEGF	Vascular endothelial growth factor
XPA	Xeroderma Pigmentosum group A complementing protein

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PROFESSIONAL PUBLICATIONS

Abstracts

1. **Aiyer HS**, Vadhanam MV and Gupta RC (2002). Efficacy of Cancer Chemopreventive Agents in Protecting Against Oxidative DNA Damage from Cu2+- Mediated Activation of 4-Hydroxy Estradiol. 93rd Annual meeting of AACR April 6 -10, 2002, San Francisco, CA. **Aiyer HS**, Caprio GD, Stoyanova R, Clapper ML and Gupta RC (2003). Modulation of mouse endogenous DNA adducts and gene expression by dietary intervention. 94th Annual meeting of AACR. July 7-14, 2003, Washington D.C, DC.
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Publications

1. **Aiyer HS**, Srinivasan C and Gupta RC. Dietary berries and ellagic acid diminish estrogen–induced mammary tumorigenesis in ACI rats. Manuscript submitted to Nutrition and Cancer, 2007.

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