Jose Russo *Editor*

Trends in Breast Cancer Prevention

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Preface

This volume is designed to inform all who wish to gain an understanding of how to prevent breast cancer—which affects the body, families, and the social environment.

In Chap. 1, Janssens discusses how the disease originates early in life, the impact of epigenomic imprinting, and the recognition that breast cancer is a family of related but distinct diseases. All of these are connected and serve as the basis for developing new paradigms in the prevention of breast cancer. The importance of the preclinical interval between generation of susceptibility and appearance of the diseases offers opportunities for primary prevention and presumably has a period when genetic control is modifiable. Early detection now becomes the priority and can be achieved through recognition of risk markers that reliably predict disease. High priority must be given to lifestyle research on affordable reversion of epigenetic alterations.

In Chap. 2, Lamartiniere and his colleagues provide scholarly evidence on the use of genistein—a phytochemical component of soy—for the prevention of breast cancer. Prepubertal exposure of rats to genistein via the mothers' milk alters mammary protein expressions whose actions are consistent with regulation of cell turnover and tissue remodeling. In mature rats exposed prepubertally to genistein, as well as in the absence of genistein, protein expressions are altered to reflect actions consistent with more differentiated terminal ductal structures, increased apoptosis, and reduced potential for carcinogenesis in the mammary gland. The basic concept is that genistein induces permanent and irreversible modifications that determine how the mammary gland responds later in life, even in the absence of the initial effector. From the blood of adolescent girls with high urine concentrations of genistein, they identify protein biomarkers of exposure and susceptibility. Toxicology studies with genistein in animals and epidemiology reports with soy demonstrate little or no toxicity. The authors of this chapter recommend clinical studies in adolescent girls to determine if soy and genistein can suppress mammary cancer.

In Chap. 3, Manni, Karam El-Bayoumy, Thompson, and Russo, and the distinguished members of their teams, address the role of omega-3 fatty acids in breast cancer prevention. Preclinical and epidemiological data suggest that omega-3 fatty

acids (*n*-3FA) protect against breast cancer. Preclinical data from their laboratories indicate that *n*-3FA potentiates the chemopreventive effect of the antiestrogen tamoxifen based on the complementarity of their mechanisms of antitumor action suggested by the signaling, genomic, and proteomic studies. Because of their antiestrogenic and antiinflammatory properties, *n*-3FA may be preferentially effective in preventing obesity-related breast cancer. In view of the hyperestrogenic and proinflammatory milieu present systematically and in the mammary glands of obese women, *n*-3FA may cooperate with weight loss induced by dietary energy restriction in reducing breast cancer risk in these subjects. Evidence-based combinatorial intervention trials targeting appropriately selected populations of women at risk are needed to establish the role of *n*-3FA in breast cancer prevention.

In Chap. 4, Maximov and Jordan discuss the role of raloxifene and tamoxifen in the prevention of breast cancer. The authors describe the history, the current role, and the deficiencies of tamoxifen and raloxifene in the prevention of breast cancer. The chapter clearly illustrates the potential of other SERMs and new approaches to hormone replacement to improve women's health and to reduce the risk of breast cancer.

In Chap. 5, Brodie and her collaborators Chumsri, Yu, Schech, and Sabnis describe the use of aromatase inhibitors for breast cancer prevention. Aromatase inhibitors offer a new treatment option for breast cancer prevention without increased risks of venous thromboembolism and endometrial cancer. Compared to placebo, both exemestane and anastrozole significantly reduced the risk of invasive breast cancer as well as noninvasive lesions.

In Chap. 6, Cavalieri and Rogan present a lucid description of the role of specific estrogen metabolites in cancer initiation and how the understanding of their mechanism of action has led their team to develop preventive strategies against breast and other types of cancer. Estrogens can initiate cancer by acting as chemical carcinogens and reacting with DNA. Specific metabolites of endogenous estrogens, the catechol estrogen-3,4-quinones, react with DNA to form depurinating estrogen–DNA adducts. Inhibiting formation of these estrogen–DNA adducts can, therefore, prevent cancer. The finding that high levels of estrogen–DNA adducts precede the presence of breast cancer indicates that formation of these adducts is a critical factor in breast cancer initiation. The discoveries of these two researchers led to the recognition that reducing the levels of estrogen–DNA adducts would prevent the initiation of breast and other types of human cancer.

Pereira, Su, and Russo, in Chap. 7, present the molecular basis of the preventive effect induced by pregnancy. It is well-accepted knowledge that pregnancy exerts a protective effect in women who delivered their first child before late twenties, when compared to women who never had a full-term pregnancy. In addition, multiple pregnancies significantly decrease the risk of developing breast cancer after age 50. The authors clearly explain the role of chromatin remodeling mechanisms in the long-lasting preventive effect of pregnancy against breast cancer and how to mimic this protective effect using pregnancy hormones or smaller targeting molecules. These concepts offer a new paradigm in the prevention of this disease.

In Chap. 8, Gronich and Rennert discuss the current evidence available for the association between breast cancer and commonly used drugs suggested in the literature as carrying potential preventive activity against breast cancer in vitro, in animal models, and in humans. These include vitamin D, bisphosphonates, statins, and metformin, all of which are used for a variety of noncancer-related indications. While all of these compounds have shown a high level of anti-breast cancer activity, in one or more of the different experimental platforms, none have been shown to be preventive in randomized controlled trials (RCTs). A common use of these compounds by the population, if they actually have a true preventive effect, would lead to reduction in incidence of breast cancer in the population at large by way of a "natural experiment." The current reduction in breast cancer incidence and mortality seen in many western countries can actually be attributed, at least in part, to an inadvertent effect of these drugs.

In Chap. 9, Shapira provides a comprehensive update on the evidence base that supports the potential for nutritional prevention of breast cancer. The chapter reviews difficulties in studying direct nutrition–BC correlations, critical periods in the life cycle, and their dietary implications for carcinogenic and patho-metabolic trajectories. Evidence-based risk factors include anthropometric measures—high birth weight, adult tallness, fatness (body mass index), weight gain, and reproductive events—early menarche, and late childbearing without breastfeeding. Gender-based nutrition explains women's specific risks, i.e., with high fatness, estrogen metabolism, and *n*-6 polyunsaturated fatty acid conversion to proinflammatory/carcinogenic mediators. Recent large-scale studies have confirmed effectiveness of evidence-based recommendations for reducing breast cancer risk, emphasizing low dietary energy density, nutritious plant-based diets, physical activity, and body/abdominal fatness management.

In Chap. 10, Czerniecki, Nocera, Lowenfeld, Showalter, and Koski introduce a fascinating new concept in breast cancer prevention—a vaccination against cancer cells. Vaccines have long been hailed as the most effective medical intervention to prevent a disease. While cancer vaccines have mostly been used therapeutically with little success in established breast cancer, their role in early breast cancer appears more promising, and primary prevention of breast cancer by vaccination is now being contemplated. Although there is no single cause of breast cancer, there are multiple subsets of breast cancers including Luminal A, Luminal B, HER-2, and subsets of basal-like cancer. Each of these types can be antigenically distinct and present immune targets that may be phenotype-specific or, to some degree, overlapping between subsets. Three general categories of such targets are being developed as breast cancer vaccines: oncodrivers, breast tissue-specific antigens, and cancer specific antigens. It is likely that combinations of these vaccine approaches may be best for treatment and prevention. Carriers of high-risk breast cancer mutations represent a potential target patient population for prevention. However, approximately 85 % of breast cancers occur in patients with no identified risk. Recent evidence suggests that a loss of natural immune responses against oncodrivers may identify

patients at risk for early breast cancer. Devising tests to identify subjects at risk for breast cancer is needed as such tests will enable the focus on prevention efforts, including vaccination, on those individuals where such resources are most needed. Preventive breast cancer vaccines may be achievable with an improved understanding of breast cancer biology and the immune response in breast cancer.

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Jose Russo M.D. is a senior member and director of the Irma H. Russo, M.D., Breast Cancer Research Laboratory; director of the Breast Cancer and the Environment Research Center at the Fox Chase Cancer Center; adjunct professor of pathology and cell biology at Jefferson Medical School; and professor of biochemistry at Temple Medical School in Philadelphia, Pennsylvania. He has authored more than 400 publications and serves on the editorial boards of several scientific journals. Dr. Russo has received numerous research awards for his original research in breast cancer from the National Cancer Institute of the National Institutes of Health (NIH), the American Cancer Society, and the Department of Defense. For the last 35 years he has been an active member of the NIH peer review system and has served as a special reviewer for the American Cancer Society, the National Science Foundation, the Department of Defense, and the US Department of Veterans' Affairs. He has trained 55 Ph.D. and M.D. investigators in cancer research. Dr. Russo, whose interests are broad-based, focuses on understanding the mechanisms that control the susceptibility of the breast epithelium to undergo neoplastic transformation, identifying markers of susceptibility, and developing strategies for breast cancer prevention.

Chapter 1
The Paradigms in Breast Cancer Prevention

Jaak Janssens

Abstract Three important recent models have shaped our current knowledge about breast cancer prevention: the accumulating evidence that the disease originates early in life, the impact of (epi-)genomic imprinting, and the recognition that breast cancer is a family of related but distinct diseases. The breast, the target organ, starting a unique intense growth after the first decade of life and involuting already during the third decade, is tremendously vulnerable to several endogenous and exogenous hormone disrupting molecules and other chemical and physical genotoxic factors. Lifestyle and toxins seem to generate long lasting (epi-)genomic marks especially in rapidly growing tissues such as the breast that can be reset for example by an early first full term pregnancy.

The preclinical interval between generation of susceptibility and appearance of the diseases offers opportunities for primary prevention and presumably has a period when genetic control is modifiable. Reversibility declines progressively when different premalignant or early malignant phenotypes appear. Early detection becomes now the priority and can be achieved through recognition of risk markers that reliably predict disease. Newer sophisticated imaging techniques detect the disease in phases where cure is expected. But these tools don't address the rapid mortal threat of breast cancer in the third world. This is now the prime concern for the next generations worldwide that probably are best served with affordable primary prevention. High priority must be given to lifestyle research on affordable reversion of (epi-) genomic alterations.

Keywords Hormone prevention • Early prevention • Secondary prevention • Diet • Vaccination • Chorionic gonadotropin • Anti-estrogens • Aromatases • SERM • Anti-inflammatory agents • Personalized prevention • Fitness

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Abbreviations

ADH	Atypical ductal hyperplasia
ASM	Allele-specific methylation
BMI	Body mass index
BRCA 1 and 2	Breast cancer gene 1 and 2
CE	Catecholestrogen
CT Scan	Computerized tomography scan
DCIS	Ductular carcinoma 'in situ'
DES	Diethylstilbestrol
DNA	Deoxyribonucleic acid
DDT	Dichlorodiphenyltrichloroethane
EBV	Epstein-Barr Virus
ER	Estrogen receptor
FFTP	First full term pregnancy
hCG	Human chorionic gonadotropin
HER2	Human epidermal growth factor receptor type 2
HR	Hormone receptors (both estrogen and progesterone receptor)
IDA	Invasive ductular adenocarcinoma
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
lncRNAs	Long noncoding ribonucleic acids
LOH	Loss of heterozygosity
miRNA	Microribonucleic acid
MRI	Magnetic resonance imaging
PET	Positron-emission tomography
PR	Progesterone receptor
rhCG	Recombinant human chorionic gonadotropin
SHBG	Sex hormone binding globulin
SR	Steroid receptor

1.1 Introduction

The human breast harbors one the most lethal cancers in the world, affecting up to one out of eight women. An estimated 1 million will be identified yearly and about 500,000 new and existing patients will die. Previously a malady of all times and mainly of Western countries, breast cancers are now everywhere. By 2020, 70 % of all cases will be in developing countries [1]. The global differences in survival reflect closely the diagnostic and therapeutic competences of local health care [2]. In countries where access to diagnosis is to the state of the art, survival is beyond 80 and even close to 90 %. If health care is poor, the number of patients detected with curable cancers is inferior to 50 % [3].

There is no treatment available that offers more cure than early detection. Breast clinics have been established for that purpose, concentrating on early detection and treatment. There is no conclusive evidence that they really improve outcome but standardization of care certainly is underway [4]. Unfortunately, breast clinics score low when it comes to primary prevention.

Although incidence rates are substantially higher for women age 50 and older, approximately 23–50 % of breast cancers are diagnosed in younger women. Before the age of 24, breast cancer is extremely rare. Age-specific incidence rates then rapidly increase until age 50 for all ethnic groups, and then continue to increase more slowly for Western countries. Incidence rates reflect a bimodal (early- and late-onset) curve, whereas Japan for example has primarily an early-onset age distribution. But in this curve the molecular phenotypes differ in relation to age; being much more heterogeneous for premenopausal women. Breast cancer incidence in young women is also increasing in Western countries [5].

Due to the belief that cancer is predominantly a local disease in its first stages, surgery became the cornerstone of treatment. Radiotherapy, a valued adjunct to surgery and successfully preventing major mutilations from surgery, became truly applicable in the 60s and developed with unmatched safety through computerization in the 90s. Both treatments now are highly effective for the treatment of local diseases. Adjuvant hormone and chemotherapy increase disease-free survival for another 10 %. The overall effect of chemotherapy on mortality is less well understood but several studies indicate a benefit between 10 and 15 % which is almost similar to the beneficial effect of screening [6].

As recent as since the 80s, an enormous evolution has been seen in diagnosis and staging of breast cancer. Ultrasound, computerized scans (CT), magnetic resonance imaging (MRI), Positron-Emission Tomography (PET) with merging in CT or MRI are now fully applied in breast clinics. As a result, cancer is better staged and, despite a disturbing increase in false positive rates, usually up-staged. Together, better adjuvant therapies, screening and improved diagnostics account for the improved cure rates.

However, once the disease become disseminated, cure is not possible; although long survivals are well known. This distressing observation has been the main incentive to search better treatments. Key developments in basic biomolecular research, such as microarray technology, have entered the breast clinic and will be useful for therapy guidance and selection of patients towards better products.

Molecular profiling separates breast cancer in different subtypes: Luminal A and B, HER2, and triple-negative. The classification has expanded our knowledge on how to treat the different types of breast cancer more logically. It is expected that this trend will expand further and that more subtypes will be discovered so that treatments for advanced diseases can be further fine-tuned.

Since 2000, an explosion of targeting new molecules became available to bloc or modify signal transduction and other cellular mechanisms that are crucial for cancer cell's survival and growth. Diagnosis and treatments can be further personalized based on the increasingly sophisticated molecular profiles. Consequently, overall

survival will increase for women and societies that can afford these new diagnostics and treatments; excluding the growing majority of those who can't [7].

With the continuous global escalation in incidence and prevalence, and the economical limitations of progress, there is real risk that overall mortality might go up in the future; in particular, since developing countries experience an explosion of cancer cases. Limitations of health care, need for economic priorities but also the unjustified trust in cure for newer systemic treatment modalities has given the consideration of better prevention a second thought. Besides, the power of prevention has been amply demonstrated. The urgent need to slow incidence and prevalence rates with affordable means makes that research on primary prevention becomes a priority once again.

Prevention in the past mainly relayed on innovations in epidemiology, statistics, internationalization of study work and relationship between lifestyle factors and the disease. Now there are new impulses that make this discipline even more powerful. The use of molecular biology, so warmly welcomed by clinical oncology, has created a new momentum in prevention studies. There is ample evidence that the different subtypes of breast cancer originate and grow differently. This model explains partly why prevention was less efficient in the past but provides openings both for study and implementation policies. In addition, health care has changed as well. New preventive drugs such as statins, anti-diabetic and anti-inflammatory drugs have provided new opportunities against those cancers that are related to the metabolic syndrome. The upcoming of molecular biology and safe new drugs has created new thrust in prevention. But also new insights in genomic imprinting, relating events early in life with chronic diseases, and targeted therapies towards altered gene products make prevention more exciting and probably the only affordable approach for developing countries.

1.2 Risk Factors

Because breast cancer appears from the third decade onwards, this can give the impression that it's a disease of young to middle aged women. In a sense that is true indeed for the clinical part of the disease. It means also that the origin must be explored mainly before the age of 24; i.e. at puberty and adolescence. The target organ—the breast—makes a remarkable entrance throughout woman's life. Unlike any other organ, the breasts are not present before puberty. But at puberty, they start an huge growth (Fig. 1.1) in a few years to become about the largest organ of the human body. At menarche, growth turns into differentiation and later in maturation. The development stops around the age of 25 or at first full term pregnancy (FFTP) and turns into an apoptotic process that involutes the parenchyma as can be observed in sequential mammographies. The involution continues until almost all parenchyma disappears after menopause. Amazingly, the first clinical breast cancers appear when breasts start the involutionary process. Reflecting on prevention, one

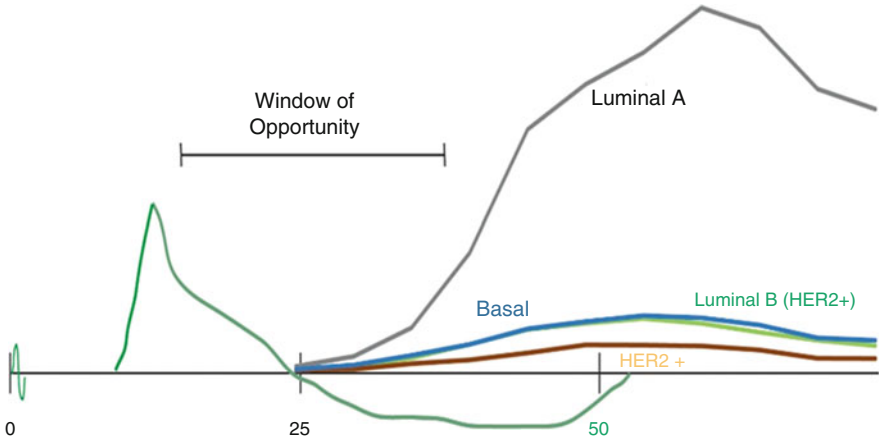

Fig. 1.1 Breast organogenesis and cancer. This schematic representation relates breast development with cancers throughout life. In the neonatal period a rudimentary breast is seen that disappears in the next months. At puberty the breast develops with a growth spurt during the first few years. Then growth slows down and maturation comes in. (The drawing above the line reflects growth and under the line regression.) Roughly at the age of 25 the breast starts to involute, a process that is normally finished at menopause or shortly thereafter and can be visualized by regression in breast density on mammography. The first cancers appear around the age of 24, with a substantial part basal (triple negative) and HER2 positive cancers. Luminal (ER + PR + HER2neg) cancers appear predominantly later in life

might consider influencing growth, differentiation and, in particular, maturation phases as configuring prerequisites for a cancer free apoptotic process.

Familial predisposition is truly the most important risk factor for breast cancer, however, not even 10 % of the breast cancer patient population has an inherited germ line gene mutation. Germ line mutations of the breast cancer susceptibility genes, BRCA1 and BRCA2, are well known and responsible for a large part of the familial breast and ovarian cancer syndromes. A child born with a germ line mutation in the BRCA1 has 70–80 % life time risk. For BRCA2, the penetration is somewhat less but still amounting to almost 60 % [8]. Most BRCA1 cancers have the basal cell phenotype. This phenotype is rarely found in BRCA2 carcinomas that tend to be estrogen receptor- and progesterone receptor-positive. Somatic mutations in the BRCA genes are rarely found in hereditary tumors. By contrast, BRCA1 and BRCA2 loss of heterozygosity (LOH) is found in almost all BRCA1 and BRCA2 carcinomas. Furthermore, all types of hereditary breast carcinomas have low frequency of HER2 expression [9]. Genetic modifiers of penetrance are under intense research [10]. Other germ-line mutations, such as Chek2, are less frequent and generally convey a lesser risk [11].

There is ample evidence that exposure to estrogens, whether endogenous or exogenous, is the main determinant of breast cancer risk. Estrogens have a dual stroke: direct hormonal action and genotoxicity (Fig. 1.2). Metabolic products, mainly the catechol estrogens (CE), are toxic to DNA [12].

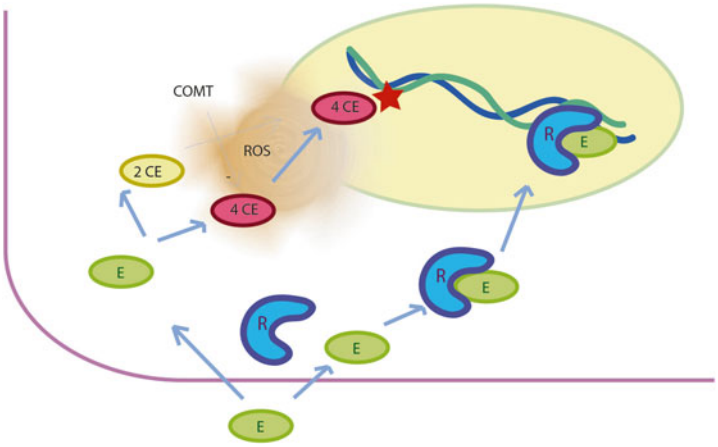

Fig. 1.2 Dual mechanism of genomic activity by estrogens. Estrogen activity at the genomic level comes mainly from the steroid receptor and catechol estrogen (CE) pathway. In the receptor pathway, estrogens (E) bind to the steroid receptor (R), which is activated to bind to nuclear constituents. In turn, cell proliferation is influenced (mostly stimulated). Estrogens (mainly estradiol and estrone) are converted to metabolites, particularly the CEs. Mostly the 4-CEs and related quinones can react with DNA to form depurinating adducts in a scene of reactive oxygen species (ROS). CE formation is blocked by COMT (Catechol-O-methyltransferase)

The prevailing model proposes that estrogens increase the rate of cell proliferation by stimulating estrogen receptor (ER)-mediated transcription and thereby the number of errors occurring during DNA replication. Estrogens behave different depending on the relative binding properties to ER alpha and beta. Diethylstilbestrol (DES) for example is a strong ligand for the ER alpha and causes high estrogen activity. Ethinylestradiol, the main compound in the contraception pills, behaves similar to the natural estradiol. Estriol is much less active for the breast where it can even be considered as an anti-estrogen [13].

An alternative hypothesis proposes that estrogens can be metabolized to quinone derivatives which react with DNA and then remove bases from DNA through a process called depurination (Loss of a purine base (A or G) to form an apurinic site). Error prone DNA repair then results in point mutations. DES metabolization for example causes high production of quinone derivatives or catechol-estrogens (CEs). Ethinylestradiol give much less genotoxic products in comparison with natural 17-beta-estradiol [14]. Mammary cancer development is primarily initiated by metabolism of estrogens to 4-CE and, then, to CE-3,4-quinones, which may react with DNA to induce oncogenic mutations [15]. Phytoestrogens in general seem to form more 2-CE that results in far less quinone production. Soy products are even able to change estrogen metabolism to less toxic CE. But the effects presumably are much more complex and still not quite clear [16].

These two relatively independent processes, increased cell proliferation and genotoxic metabolite formation, act in a complex additive or synergistic way to induce cancer. If correct and as a correlate, aromatase inhibitors could block both

processes whereas anti-estrogens would only inhibit receptor-mediated effects. Consequently, aromatase inhibitors would be more effective in preventing breast cancer than use of anti-estrogens. In particular for hormone-dependent post-menopausal breast cancers. Besides, aromatase inhibitors have lesser side effects. This supposition indeed has been confirmed in recent chemoprevention trials [17].

In previous decennia there has been a considerable search towards lifestyle factors that can explain the geographical and temporal variability of breast cancer. Human migration studies and laboratory animal work disclosed that food (e.g. fat, alcohol), hormonal medication and reproduction are related to risk. Recent cohort- and case control studies however are less convincing, probably because only adults were included. Indeed, limited attention was given to children [18].

The hypothesis that breast cancer originates mainly from lifestyle factors and the consideration that causation must precede clinical disease, gave attention to the period in life before the disease becomes evident. Immediately, puberty and the dynamically growing target organ come into focus (Fig. 1.1). Apoptotic failure of breast parenchyma combined with estrogen exposure seem the prime carcinogenic mechanisms. Lesser apoptosis is related to early menarche and late FFTP, and assumes that maturation of the breast remained incomplete [19].

Numerous scientific data support the hypothesis that the susceptibility originates during the organogenesis of the breast. For example, children undergoing thoracic radiation are tremendously at risk for developing cancer with odds ratios amounting to 40 when irradiation takes place immediately after the start of breast growth [20, 21]. Early removal of the ovaries on the other hand decreases effectively cancer risk [22]. Clearly, the growing breast is highly susceptible to genetic disruption. This is in a sense comparable to teratogenicity.

Environmental conditions early in life are known to influence biology and long-term health. The period of imprinting extends from preconception to (early) childhood and involves epigenetic and genetic reactions to environmental changes. They guide cell- and tissue-specific gene expression and even can be transmitted to the next generation. The (epi-) genomic changes might remain clinically silent for years [23, 24]. Identifying epigenetic deviations offers preventive opportunities: development of epigenetic biomarkers for early diagnosis, ability to identify susceptible individuals at risk, and development of novel preventive and curative measures that are based on diet and/or novel epigenetic chemopreventive drugs.

The relevant oncogenic lifestyle factors that initiate susceptibility are primarily related to estrogen exposure. Well known are early menarche, late first full term pregnancy (FFTP), and increased height and weight.

Little is known about the sequence of endocrine development and maturation early in life. Timing of puberty, breast development and menarche might give the impression of a natural occurring process determined by a biological clock that, once initiated, turns on a rather independent process of senological and gynecological development and maturation [25, 26]. This concept is endorsed by the recent finding that age at menarche is associated with parent-of-origin-specific allelic associations. This explains also the heritable trait association with obesity, type-2 diabetes, cardiovascular disease, breast cancer and all-cause mortality [15]. However,

age at menarche is also affected by nutrition because ovulation and menstruation need a critical weight. Conversely, children with an intense physical life, like ballerinas and young athletes, have a retarded menarche. Thus the biological clock, although partly inherited, is influenced by sociocultural, environmental and nutritional factors that alter the timing of the involved neuroendocrine mechanisms. If lifestyle factors might change pubertal milestones then prevention should start as early as possible in the hope for a stronger lifetime protection.

Both earlier menarche and adult tallness are markers of increased risk [27]. Earlier menarche in the Western hemisphere is usually associated with earlier onset of hyperinsulinemia, and numerous case-control studies report that fasting hyperinsulinemia too is a marker of increased breast cancer risk [28]. A 1-year decrease in age at menarche is estimated to increase breast cancer risk by at least 10 % [29].

The explanation why menarche has such a powerful influence on breast cancer risk is the exposure to estrogens. Children with a menarche before the age of 12 have twice the endogenous estrogen exposure compared to children with a menarche after the age of 13 [30]. The difference remains during the entire puberty and might be boosted or reduced by other lifestyle factors. One of the best known is the sex hormone binding globulin (SHBG) that binds free estrogens and hereby protects the body from direct estrogen activity. SHBG is decreased in obese infants. Hence, for the same total plasma concentration, the exposure to free estrogens is higher in obese children.

Overweighed and tall girls have an earlier menarche and earlier onset of puberty [31, 32]. The sooner the critical weight is acquired, the earlier a regular bleeding pattern starts [33]. Malnutrition and low body fat, or an altered ratio of lean mass to body fat seemed to delay the adolescent spurt and to retard the onset of menarche. Both earlier menarche and adult tallness are markers of increased cancer risk for both ER-negative and ER-positive malignancies, although the associations with HR-negative breast cancers were only borderline significant [34]. However, above average weight at age 12 can be inversely associated with risk of postmenopausal breast cancers [35].

A recent cohort study indicated that size at birth may be the etiological relevant factor in premenopausal breast cancer [36]. Higher weight of the mother predicts for higher birth weight [37, 38]. This mother-daughter relation can be associated with genomic imprinting in utero which influences pubertal weight and confers later risk for breast cancer [39]. Lifestyle variables that reduce age at menarche may contribute to the rising risk of breast cancer diagnosed after age 40, whereas earlier-onset cancers may be characterized by a distinct pathogenesis [40]. Advanced paternal and maternal age predicts for increased risk for early premenopausal breast cancer and identifies a novel population group at increased risk [41, 42]. Breast feeding reduces overweight in children and is inversely associated with cancer risk. Early genistein containing nutrition promotes cell differentiation that results in a less active epidermal growth factor signaling pathway in adulthood that, in turn, might be responsible for suppression of cancer risk [43].

Risk is related to exposure to chemicals during juvenile life [44, 45]. Dichlorodiphenyltrichloroethane (DDT) exposure in young women has related to

increased breast cancer risk [46]. Positive association exists between circulating levels of insulin-like growth factor-I (IGF-I) and IGF-binding protein-3 (IGFBP-3) as markers of insulin resistance and cancer risk [47].

Sporting girls have a later menarche although there was no relationship with onset of puberty. This can be explained by sporting activities being started at around 6–8, an age that is too late to influence puberty but in time for menarche. It was already known that hard physical work or intensive physical activity at young ages delays menarche [48–50]. Physical activity has been found to be associated with decreased risk [51] for postmenopausal women in the majority of epidemiologic studies, but the association is inconsistent in premenopausal women. A Scandinavian group studied the effect of physical activity at various ages on the incidence of breast cancer. Compared to inactive women, women with higher levels of physical activity had a similar risk [52] suggesting that physical activity is more important at premenarcheal ages and that the effect in adults can be overruled by other risk factors. Mononucleosis between 3 and 6 years induces earlier breast development probably because this infection is related with a prolonged period of low physical activity. Particles of the Epstein-Barr virus (EBV) are often found in breast cancer. EBV is an ubiquitous human herpes virus associated with lymphoid and epithelial tumors, including breast cancer [53]. Research on this topic is highly warranted; in particular to address the exploding cancer figures in developing countries.

Cancer seems to be related to an incomplete maturation of the breast tissue. Maturation is induced by an early first full term pregnancy (FFTP). The longer the period between menarche and FFTP, the more immature elements remain from which cancer can originate. An early FFTP confers a decreased risk most probably through restitution of (epi-) genomic imprinting at the chromatin level [54].

1.3 Genomic Imprinting

Sporadic breast cancers differ from those of germ-line mutations in a way that cancer associated genes are only present in affected cells. Mutated genes, that are present in most of the cells of the body, only confer a risk and are considered hereditary and germ-line. But cancer is as much an epigenetic disease as a genetic one and even the term hereditary implies both epigenetic and genetic anomalies. BRCA1 mutation related carcinogenesis is for a substantial part epigenetic [55]. Imprinted gene clusters are regulated by long noncoding RNAs (lncRNAs), CCCTC binding factor (CTCF)- mediated boundaries, and DNA methylation [56]. There is reason to believe that lncRNAs are implicated in breast carcinogenesis [57]. A large proportion of DNA-methylation variability can be explained by allele-specific methylation (ASM) that is either germ-line [58] or somatically acquired [59]. The concept that genes or epigenomic alterations, either additions or loss of imprinting [60], can adapt to situations early in life or even from one of the parents (monoalellic expression), and that these changes persist and play a pathogenic role later in life is relatively new [61, 62]. This phenomenon, called genomic imprinting, is a chromosomal

modification leading to parental-origin-specific gene expression in somatic cells [63], and explains various clinical observations such as racial differences [64] in tolerance and resistance to chemotherapy [65]. Early exposure to common environmental compounds has the potential to disrupt fetal and postnatal health through epigenetic changes in the embryo and abnormal development of the placenta [66]. There is reason to believe that many chronic diseases have their origin early in life although confirmation of this hypothesis is only beginning to appear.

Aberrant DNA-methylation of imprinted genes is associated with hormone receptor status [67]. Conversely, epigenetic repression of the imprinted gene cyclin-dependent kinase inhibitor 1C is mediated by estrogens [68]. Frequently dysregulated gene-products in breast cancer are the Insulin-like growth factor type 1 receptor (IGF1R) [69] and IGF2 [70]. Imprinted regions contain tumor-suppressing miRNAs that, together with the methylation status drives carcinogenesis and metastasis. As such, they can be useful as preventive, diagnostic, and therapeutic tools [71]. Specific sets of miRNAs were associated with HER2 overexpression, p16INK4a or E-cadherin mutation or E-cadherin methylation status, which implies that these miRNAs may contribute to the driver role of these genetic aberrations [72]. While these data are only beginning to develop, they could have a meaningful influence on contemporary thinking about breast carcinogenesis and primary prevention models.

1.4 Molecular Type Dependent Carcinogenesis

There is ample evidence that breast cancer reflects similar but distinct diseases with regard to causation, biological behavior, and response to treatment. The finding that breast cancer is a mixture of different diseases originating from the same organ has resulted in a marked improvement of diagnosis and therapy. Probably, many more subtypes will be detected and characterized with proper adapted diagnostic tests [73].

Breast cancer subtypes most probably originate different as well and, when true, should be prevented accordingly. But while data relating prevention to tumor type only starts to appear, one could think of two carcinogenetic pathways. Either transformed stem cells differ or the subtypes split from a common stem cell after one or more stages during carcinogenesis [74]. In the latter case, premalignant precursors might not develop consistently on the cell level (some cells in a mixture develop more than others) or modify on the chromatin level (e.g. dedifferentiation). One reason to believe that subtypes arise from similar stem cells is that premalignant lesions show much less differentiation. For example, most DCIS (ductular carcinoma ‘in situ’) show expression for HER2 while in the IDA’s (invasive ductular carcinoma’s), this is only 10–15 %. On the other hand, most basal types or triple negative cancers might be so different that they may originate from different stem cells.

The change in molecular type during carcinogenesis offers possibilities for prevention. Although the precise nature of premalignant evolution is controversial, especially the earliest stages, most models agree that cancers could develop in a nonobligatory mode through an increasingly abnormal series of hyperplasias, atypical hyperplasias, and noninvasive or 'in situ' carcinomas. Certain lesions have significant premalignant potential, including atypical ductal hyperplasia (ADH) and atypical lobular hyperplasia, and their more advanced counterparts ductal and lobular carcinoma 'in situ' [74]. Atypical ductal hyperplasia shows increase in ER level in contrast to the normal breast [75]. The condition is linked to postmenopausal hormonal substitution and xenoestrogens and difficult to distinguish from DCIS.

Some DCIS lesions are believed to rapidly transit to invasive ductal/lobular carcinomas, while others remain unchanged. Existing classification systems for DCIS fail to identify those lesions that transit to invasiveness. DCIS may be classified in a similar manner as invasive cancer, and defining the relative frequency of different subtypes in DCIS and invasive disease may shed light on factors determining disease progression. There is also a role for Bcl-2 in classifying DCIS [76]. A set of genes independent of grade, ER-status and HER2-status are identified. The genes that differentiate between DCIS that progress and not suggest several processes related to the re-organization of the microenvironment [77].

Metabolic syndrome represents a modifiable risk factor for postmenopausal cancer and is more related to luminal breast cancers. Body Mass Index (BMI) alone is associated to luminal A subtype risk. Waist circumference >88 cm has been shown to be associated to the more aggressive HER2+ breast cancer subtypes in postmenopausal women. Insulin resistance showed association to HER2+ and luminal B tumors.

Ductal carcinoma 'in situ', particularly high-grade ductal carcinoma in situ, has more HER2 overexpression/amplification (50–60 %) than in invasive cancers (25–30 %); while normal tissues and premalignant lesions show almost no expression. In the majority of mammary cancers with HER2 overexpression, E2f3a is upregulated, raising the possibility that E2f3a is a critical effector of the HER2 oncogenic signaling pathway. Loss of E2f1 or E2f3 led to a significant delay in tumor onset, whereas loss of E2f2 accelerated oncogenesis driven by Myc-overexpression [78]. The HER2 oncogene is a bi-functional locus encoding the membrane receptor and a functional miRNA gene [79]. Low PRKAR1A/high SRC expression defines basal-like and HER2 breast tumors [80]. The pivotal role of Dmp1 in quenching growth signals from HER2 to the Arf-p53 pathway seems to act as a safety mechanism to prevent carcinogenesis [81].

Basal-like BC represents approximately 15–20 % of invasive breast cancers and has been strongly associated with younger age at diagnosis (Fig. 1.1), BRCA1 mutation, and African-American race of patients. It is a heterogeneous disease characterized by the expression of basal cell markers, no estrogen or progesterone receptor expression and a lack of HER2 overexpression and amplification. Studies have linked activation of the Wnt/β-catenin pathway, and its downstream target, Myc, to basal-like breast cancer. β-Catenin-induced stem cell amplification and tumorigenesis rely ultimately on the Myc pathway activation and reinforce once

more the hypothesis that basal stem/progenitor cells may be at the origin of a subset of basal-like breast tumors [82]. Oral contraceptives seem to be associated with increased risk for triple-negative breast cancer in premenopausal women [83]. APRIL (a proliferation-inducing ligand), providing growth to tumor cells through paracrine and autocrine signaling, is distinctly associated with basal types [84]. Increased hypermethylation of GSTP1, ID4, TWIST, DAPK, PAX5 and HIN-1 genes is seen [85]. Cancers arising in previously irradiated breast tissue were more likely to be basal type in contrast to age-matched sporadic invasive cancers and are less likely to be HR positive [86]. The tumor suppressor p53, being the most frequently mutated gene in human cancers, is mutated in 25–30 % of breast cancers. However, mutation rates differ according to breast cancer subtype. Aggressive estrogen receptor-negative tumors, the basal-like and HER2-amplified subtypes, have higher percentages of p53 mutation. miR-146a is highly expressed in p53 mutant basal-like breast cancers [87].

1.5 Window of Opportunity for Breast Cancer Prevention

The efficacy of early cancer prevention and screening seem to be mainly influenced by the awareness of risk knowledge among healthy women, indisputably a result of increased media attention. Recommendations for the improvement of prevention programs include targeting understanding of lifetime risk of breast cancer, age as a risk factor, survival from cancer and hormonal causal factors. There is a need to separately address the perceptions of women depending on age, social status, geographical area, and educational levels. The finding that risk factors are already present at early life remains however largely unknown.

Given that the dual peak of clinical breast cancers appearance is between 50 and 60 years of age and that breast cancer susceptibility induction is at early puberty, a fairly large window of opportunity of nearly 50 becomes available (Fig. 1.1). During that window, genomic and epigenetic alterations might be detectable and can serve as intermediary risk factors that show reversible and later irreversible modifications; and can be used to monitor implementation of primary preventive programs and identify women at increased risk [88].

The largest impact might come from the identification of reliable risk biomarkers and polymorphisms that could be monitored and identify high risk groups. If childhood factors cause DNA derangements or epigenetic alterations that confer cancer risks, they will remain detectable in the next years before breast cancer becomes clinically overt. These biomarkers can be direct gene expression assessments but might be indirect as well, like breast density. The identification at young ages makes it possible to steer prevention implementation policies and activities. Crucial in the latter type is the detection of damaged genes, epigenetic alterations or gene products expression as early as before the age of 20. These intermediate risk factors might be useful in determining risk groups for secondary prevention as well. In addition, risk markers have an important role in clinical disease management. They might show

the way of how cancer appeared and the pathways that were necessary to derange the cellular metabolism. Consequently, therapeutic strategies might be designed upon data from this gene profiling.

Epidemiologic models used for cancer risk prediction, such as the Gail model [89], are validated for populations undergoing regular screening but often have suboptimal individual predictive accuracy. Risk biomarkers from tissue samples [90] and body fluids may be used to improve predictive accuracy and, to the extent that they are reversible, and to assess response in prevention trials.

Common intermediate risk biomarkers include high mammographic breast density [91], premalignant lesions [92], intra-epithelial neoplasia [93], and cytomorphology with associated molecular markers such as Ki-67 [94] and hormonal metabolic products [95]. Biomarkers suitable for the prevention of breast cancers must be extremely sensitive, easily detectable and reliably correlated with the disease. They should also be expressed in the reversible phase of carcinogenesis. Among the large number of candidate tumor-associated proteins, those related to the estrogen/chorionic gonadotropin/insulin pathway seem to be of most interest because these can be causally implicated and are relevant to the most frequent (luminal) subtypes. They presumably are the first to express differently and are open to hormonal treatments. The biomarkers that give information on membrane receptor-modulated signal transduction, such as HER2 and epidermal growth factor receptor mechanisms, should be considered as well.

Up to now, only tamoxifene has shown some preventive activity, suggesting that the estrogen pathway is useful indeed. Fenretinide and recombinant human chorionic gonadotropin (hCG) are also promising [96, 97]. But the financial requirements and the very long assessment periods largely prevent current research. This is precisely why research need to give priority to molecular biology that identifies intermediate markers and expectedly will reduce significantly follow-up time and associated costs in clinical trials. There is widespread belief that advanced proteomics together with increased informatics can provide specific combinations of disease-related expression profiles that could identify high-risk groups with much more reliability and that allows monitoring preventive strategies on short notice.

Most of the intermediate risk biomarkers in breast cancer are related to tissue components i.e. premalignant lesions [98]. The assessment of these markers is less evident compared to non-invasive techniques. However, new tissue acquisition techniques become increasingly available leading to improved sample quality (specific to the lesion of interest), better patient tolerance and affordable applications [99]. In particular, the improved patient comfort has given the tissue based molecular markers a new impetus in prevention.

1.6 Primary Prevention

Primary prevention, measures to minimize risk of attaining the disease, relies on the rapidly increasing knowledge of carcinogenesis. Although there is a large window between initiation of susceptibility and the clinical disease (Fig. 1.1), primary

prevention should be ideal in a phase were susceptibility is still reversible. Presumably this is at young age and before the age of 25 for women that have no early FFTP. The opportunities lie in lifestyle changes and intervention.

Lifestyle changes are shown to be important in the prevention of breast cancer. Diet, physical activity, smoking, alcohol use, vitamins, and minerals are key factors. Because these factors are linked to each other, it is difficult to assess their individual roles [100].

Some direct measures can be undertaken to protect children from susceptibility induction like exposure to toxins during breast development. The radiation-induced breast cancer risk increases with longer follow-up, higher radiation dose and younger age of exposure. The risk for cancer following irradiation for lymphomas or benign diseases is well known. Particularly, women with a BRCA1/2 mutation might be more sensitive for the deleterious effects of ionizing radiation due to an impaired capacity of repairing double strand DNA breaks [101]. In an era of increased medical consumption one could protect children from high radiation exposures imposed by CT scanning whenever possible. The choice for less toxic methods such as ultrasound and magnetic resonance should be encouraged in early puberty.

High energetic food and drinks, in particular soft drinks, ought to be avoided whenever possible. They also affect age at puberty and menarche. Soft drinks in children are related to obesity, under-nutrition (compensation of calories from nutrition with sugars), hyperinsulinemia syndrome, early menarche and thus cancers that are related to the metabolic syndrome [102]. Many countries already implemented guidelines to minimize soft drink sales in schools but emphasis on sugar containing drinks has to be continued especially since their use in developing countries is staggering. The huge variety of food products available should make this measure feasible.

Increase in physical activity is another evident way for prevention. Convincing epidemiologic evidence indicates that physical activity is inversely associated with cancer risk. In addition, both nutrition and physical activity are not only important for cancer prevention but have a larger impact on many diseases and conditions as well. Decreasing the exposure to estrogens might also be feasible during puberty by nutrition and physical activity.

Studies in adults however, did not show significant effects on risk [103]. Greater adult BMI was not associated with increased breast cancer risk, but some measures of early-life body size and abdominal obesity were associated with risk [104]. Physical activity reduces risk of invasive breast cancers that lack HER2 overexpression [105]. Having a high score on an index of combined healthy behaviors (diet, physical activity, smoking, alcohol consumption and anthropometry) reduces the risk of developing breast cancer among postmenopausal women [106]. Alcohol reduction, although not immediately relevant to teenagers, might be considered for the younger adult. Presumably, only part of women belonging to distinct pleiomorphisms should be warned. The general feeling is that women can only have maximum of one alcoholic beverage per day [107]. More on nutrition and cancer is found in this book.

Despite the low impact of protection measures for adults, new opportunities seem to come up for adults such as medication for the metabolic syndrome and non-steroidal anti-inflammatory products. They are described in more detail in this book. Similarly, chemoprevention and vaccination, based on recently developed risk models will be amply contemplated on in next chapters.

1.7 Secondary Prevention

Early detection of breast cancer relies on medical imaging. The technologies developed very recently. Mammography has become available at the end of the 70s and is now increasingly replaced by digital platforms. Ultrasound is an invention of the 80s and MRI of the late 90s. All these innovations rely on digital systems. It is expected that fusion technologies will become available soon. Altogether, secondary prevention is based on rather recent technology and is still in a learning mode. The exponential budding technologies however have a price that developing countries can't afford. Considering the steep increase in breast cancer in these countries with parallel mortality, one should prioritize more on affordability instead of sophistication. Breast cancer screening is debatable and improvements of therapy results makes the discussion much more intense [108]. Also for Western Countries with easy access to health care, the effect of screening seems rather small and becomes questionable if false positive results with newer techniques would further increase. Finally, less aggressive tumors are preferentially detected creating a disturbing bias for both mortality and incidence.

The gap between susceptibility induction and clinical appearance of the disease, being extremely large (Fig. 1.1), enables to detect (epi-) genomic imprints, risk factors and pre-malignancies. These intermediate risk factors are usually tissue based. Hence the need for tissue acquisition technologies that offer high quality samples to the different biomolecular and histology platforms, and also guarantee comfort to the patient. Such technologies have been developed [99]. It is expected that the combination of better imaging and targeted tissue acquisition will identify selectively patients at high risk, hereby personalizing and tailoring screening.

1.8 Optimal Strategy

It might be clear that breast cancer prevention in the twenty-first century must be adjusted to a complex strategy with focus on childhood, window of opportunity, and challenges of secondary prevention in developing countries. In addition, focus on etiology of different molecular types and identification of high risk women would improve our diagnostic and preventive capabilities.

Since susceptibility originates during childhood increased attention must be given to risk factors that induce genomic imprints and cause cancer later in life.

Such conditions are obesity, the related early sexual development (puberty, menarche), lifestyle, environmental toxins, radiation, physical activity etc. In this book the role of genistein [109], omega 3 [110] and nutrition are explored. Altogether, what we know as "healthy lifestyle" should be implemented as early as from the postnatal period and up to at least to the third decade of life.

Apart from some protecting measures during childhood, interventions during childhood and early adulthood could be useful. An early FFTP could be mimicked by treating young women with hCG or rhCG. Clinical trials are underway in high risk (BRCA1 and 2) women [111]. A vaccine against HER2 is logic since HER2 expression is common in the early breast cancers and, in particular, in premalignant lesions. Anti-hormonal therapies such as raloxifene, tamoxifene, and aromatase inhibitors that inhibit the estrogen metabolism pathways have [112] already been extensively studied in high risk patients. This approach is also getting acceptance to a broad population [113].

The thought that adults could only poorly reduce risk might not be completely true. Certainly postmenopausal breast cancer could be reduced by proper treatment of the metabolic syndrome through common medications such as statins and oral antidiabetics [114]. Alcohol consumption should be moderate to low, at least for some pleiomorphisms. Chronic anti-inflammatory medication could provide some protection as well.

Screening, with attention to lower social classes and developing countries, along with awareness campaigns should give an answer to the steep rise in breast cancer. Digital mammography seems still to be the first choice and affordable to larger populations.

Further research is mandatory but difficult to steer. Overlooking the priorities one should aim at prevention during childhood and adolescence, the use of molecular intelligence, but most importantly to look at screening with affordable means. Sophistication of medical technology is no longer the ultimate and should be replaced by concentrating on easily implementable and affordable technology that can be used to address the breast cancer plague in developing countries.

1.9 Conclusions

Breast cancer is largely a preventable disease. Susceptibility to develop the disease originates very early in life or even before life and is biologically translated into (epi-) genomic imprinting. The large window between susceptibility development and the disease offers opportunities for primary and secondary prevention. The large array of possible measures has to be tailored to address the different types of cancers and the epidemic in developing countries.

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Chapter 2
Genistein: Programming Against Breast Cancer

Coral A. Lamartiniere, Sarah B. Jenkins, and Jun Wang

Abstract Soy and its primary isoflavonic component, genistein, have been demonstrated to act *via* a novel mechanism for breast cancer chemoprevention, i.e. programming. Programming is defined as developmental modifications at the molecular level that result in permanent and irreversible modifications that determine how cells and tissues respond later in life, even in the absence of the initial effector. Depending on the chemical effector and the changes in the biochemical blue-print the adult host may be rendered more or less susceptible for biochemical insult. Exposure of prepubertal rats to physiological concentrations of genistein *via* the diet protects against chemically-induced mammary cancer. Genistein during the prepubertal period increases mammary protein expression of p-AKT, annexin A2, EGF-receptor, gelsolin and GTP-cyclohydrolase-1, while decreasing expression of cleaved-caspase-3 and protein disulfide-isomerase A3, actions consistent with increased cell proliferation and differentiation, cell turnover, and tissue remodeling. In mature rat mammary glands, cleaved-caspases-3 and 9, cleaved-poly(ADP-ribose) polymerase, fetuin B, β-casein and Ki-67 were increased, while tyrosine hydrolase, annexin A2, EGF-receptor, phosphoglycerate kinase-1, steroid receptor co-activators 1–3, and vascular endothelial growth factor-receptor-2 were down-regulated, actions consistent with increased apoptosis and reduced potential for carcinogenesis. Recent epidemiology reports confirm the laboratory findings on carcinogenesis, demonstrating that adolescent girls ingesting soy containing genistein are at reduced risk for breast cancer. Toxicology studies in animals and epidemiology with genistein and soy demonstrate little or no toxicity. We recommend clinical studies in adolescent girls to determine if soy and genistein can suppress mammary cancer development by programming for cell/tissue differentiation.

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2.1 Introduction

Soybeans (Glycine max) have been cultivated in China for over 13,000 years. The soybean is the basis for soy milk, tofu, tempeh, and soy protein. While it has been used in other regions of Asia as a food source, it was not until the early twentieth century that the soybean was used for more than animal feed in the West. Soy contains numerous phytochemicals, including the diphenolic isoflavones, genistein and daidzein, of which genistein (4,7,4′-trihydroxyisoflavone) is the predominant and bioactive isoflavone of soy diet. In soy foods, the isoflavones are in the form of glycosides. After ingestion, the isoflavones undergo enzymatic hydrolysis to release the bioactive aglycones, genistein and daidzein (Fig. 2.1).

Soy has been touted as a health supplement with little or no toxicity. It is a good source of protein, low in fat and calories, cholesterol-free, and provides bone-healthy minerals like calcium, potassium, and magnesium. Regularly eating soy appears to reduce the risk of diabetes, especially in people who are overweight. The carbohydrates in soy are complex, hence they break down slowly in the body, limiting their impact on blood sugar [1]. People with diabetes are at increased risk of heart and kidney diseases, and soy is beneficial against these diseases. It can lower levels of low density lipoproteins (LDL), a benefit for heart health. Anderson et al. [2] reported that the consumption of soy protein rather than animal protein significantly decreased serum concentrations of total cholesterol, LDL cholesterol, and triglycerides. Soy lowers the amount of proteinuria, which is a common complication of diabetes. For postmenopausal women concerned about osteoporosis, soy can be a valuable dietary addition.

In women, phytoestrogens have been found to have weak estrogen-like activity. Isoflavones appear to work by binding and stimulating estrogen-receptor sites on cells and blocking out the natural estrogens. They can be helpful in improving symptoms of estrogen depletion such as postmenopausal syndrome. Due to the phytoestrogen content of soy, many women decide to include it in their diet as they enter menopause. During the menopause, the body's natural production of estrogen declines and symptoms may ensue. As phytoestrogens act as a weak estrogen, they

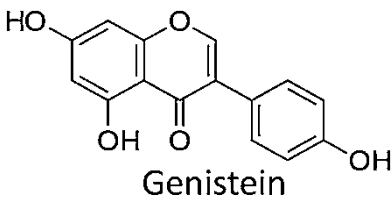

Fig. 2.1 Structure of Genistein

help relieve symptoms by providing a source of weak estrogen stimulation. Soy isoflavones, especially genistein, have attracted a great deal of research and studies suggest that women consuming a soy-rich diet have a lower risk of breast cancer.

2.2 Genistein *In Vitro* Studies

In cell culture, genistein has been reported to have growth promoting properties at nanomolar concentrations and inhibitory effects at micromolar concentrations [3, 4]. It has been demonstrated that genistein binds to the estrogen receptors-α and -β, with a higher affinity for the latter [5]. Genistein has been reported to be an antioxidant and to inhibit protein tyrosine kinases [6, 7], topoisomerase [8], cell proliferation and angiogenesis [9]. In ovariectomized female SCID mice (immunocompromised) implanted with human MC-7 breast cancer cells, genistein was reported to stimulate cell growth [10]. However, in mice with intact ovaries, genistein had no effect on the growth of human tumor cell growth [11]. Many of the studies resulting in toxicity are either carried out in *in vitro* systems using supra-physiological concentrations and/or animals administered genistein in a *non-physiological manner (injections),* the latter not taking into account bioavailability.

2.3 Genistein *In Vivo* Studies

Epidemiological studies show that Asian women consuming a diet high in soy products have a lower lifetime incidence of breast cancer [12, 13]. Yet, Asians who immigrate to the United States and adopt a Western diet lose this protection. Using rodents [14], researchers have reported soy-containing diets protecting against chemically-induced mammary cancer in animal models [15–17]. Subsequently, researchers investigated the potential of early exposure to injections of genistein in female rats to protect against chemically-induced mammary cancer using the chemical carcinogen, 7, 12-dimethylbenz(a)anthracene (DMBA). The reason for trying genistein was threefold: (1) it was previously reported that estrogen administered during the neonatal period protected against mammary tumor development [18, 19], (2) the structural similarity of genistein to estrogen, and (3) the epidemiology reports that Asian women consuming a traditional diet high in soy have a lower incidence of breast cancer but when they migrate to the U.S. the second, but not the first, generation lose this protection [13]. Fortuitously, genistein injected neonatally (days 2, 4 and 6 postpartum) to Sprague-Dawley rats did suppress DMBA-induced mammary cancer [20, 21]. This was followed by demonstrating that injections of genistein during the prepubertal period (postnatal days 16, 18 and 20) also suppressed DMBA-induced mammary cancer in rats [22, 23]. Subsequently, Hilakivi-Clarke et al. confirmed that prepubertal injections of genistein suppressed DMBA-induced mammary cancer in rats [24].

2.4 Dietary Genistein

Switching to a more physiological means of genistein administration, three groups of female rats (n = 30/treatment group) were fed 0, 25, and 250 mg genistein/kg AIN-76A diet starting two weeks before breeding and continuing through conception and parturition, until being discontinued at the time of weaning (21 days postpartum [25]. From day 21 postpartum, all female offspring from the three treatment groups were fed AIN-76A diet only, which is free of phytoestrogens. At day 50 postpartum, DMBA (80 mg/kg BW) was administered by gavage to all female offspring to induce mammary tumors. These specific dietary concentrations of genistein were chosen because, in a rodent model, they yielded serum concentrations of total genistein (aglycone and glycoside) similar to serum concentrations of total genistein found in men eating a diet high in soy [26, 27].

Control animals (no genistein in the diet) developed 8.8 tumors per rat, whereas dietary genistein suppressed DMBA-induced mammary tumor development in a dose-dependent manner (Fig. 2.2). Rats exposed to 25 and 250 mg genistein/kg AIN-76A diets had 7.1 and 4.4 mammary tumors per rat, respectively. We concluded that the chemoprevention of perinatal dietary genistein [25] was similar to our previous reports of neonatal and prepubertal genistein injections rendering a protective effect against DMBA-induced mammary cancer [20–23]. Demonstrating that life-

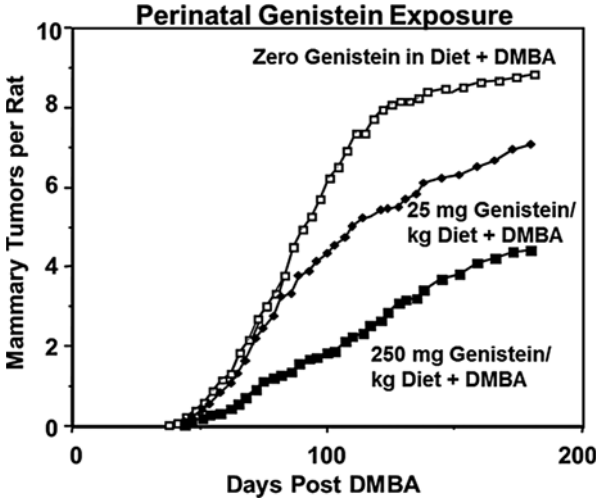

Fig. 2.2 Ontogeny of palpable mammary tumors in female Sprague-Dawley CD rats exposed perinatally to genistein in the diet from conception until 21 days postpartum. After weaning, the offspring were fed AIN-76A diet only. On day 50 postpartum, all animals were treated with 80 mg DMBA/kg body weight [25]. Reprinted with permission from Fritz WA, Coward L, Wang J, Lamartiniere, CA (1998) Dietary genistein: perinatal mammary cancer prevention, bioavailability and toxicity testing in the rat. Carcinogenesis 19:2151–2158 (Oxford University Press)

time protection against mammary cancer could be achieved by perinatal exposure to genistein in the diet suggests a differentiation/programming effect on the mammary target tissue [22, 25].

2.5 Prenatal Genistein Treatment

To determine whether the prenatal period was the sensitive period for genistein to program against chemically-induced mammary cancer, we provided one group of pregnant females with 250 mg genistein/kg diet and the other group (controls) with no genistein in the diet during pregnancy. At parturition, both groups of dams and their offspring were fed the base diet, AIN-76A, containing no genistein. At day 50 postpartum, both groups of female offspring were gavaged with DMBA. Both groups developed the same number of tumors/rat, demonstrating that *the prenatal period did not influence DMBA-induced mammary cancer chemoprevention* [28]*, and strongly suggesting that the early postnatal period (neonatal/prepubertal) is responsible for the chemoprevention that we observed in our perinatal dietary genistein study* [25].

2.6 Bioavailability

Our prenatal genistein findings proved to be contrary to that of Hilakivi-Clarke et al. [29] who reported that injecting pregnant rats with genistein resulted in the adult female offspring having increased susceptibility for DMBA-induced mammary cancer. We speculated that this contradiction was due to different routes of administration and bioavailability between the two studies. Measuring blood genistein concentrations from 21 day fetal-, 7 day neonatal-, and 21 day prepubertal rats exposed perinatally to 250 mg genistein/kg AIN-76A diet, we determined the circulating genistein concentrations to be 43, 726, and 1810 nmol/L, respectively [25, 30]. The 21 day fetal blood concentration [29] was 2.4 % that of the 21 day old prepubertal blood concentration [25]. This demonstrated that dietary genistein had good bioavailability during postnatal life, but poor bioavailability prenatally. It is noteworthy to point out that prepubertal rats start eating solid feed out of the jars at 14–16 days postpartum accounting, in part, for the high genistein concentration at day 21 postpartum. Furthermore, we determined that approximately 46 % of circulating total genistein was free genistein 24 h after injection of rats with genistein [31]. This is in contrast to less than 2 % being aglycone (free) genistein from dietary administration [25]. *Thus, the bioavailability of injected genistein is substantially greater than that of oral genistein (23-fold), and this supraphysiological concentration can account for increased DMBA-induced mammary tumors in the Hilakivi-Clarke et al. report* [29]. *An awareness of timing of exposure, route of administration, metabolism, bioavailability, and biological action explains why prenatal injections of genistein to rats resulted in increased mammary tumorigenesis, while prenatal dietary genistein does not change susceptibility to DMBA-induced mammary cancer in the offspring* [25, 28, 30, 31].

2.7 "Reading the Blueprint"

Because most breast cancers have been demonstrated to be estrogen dependent, we were concerned that genistein, an isoflavone phytoestrogen, may contribute to mammary cancer development. More specifically, women who have been diagnosed with breast cancer inquire whether soy products, including genistein, will protect from, or cause a recurrence of their cancer. To address this concern in the laboratory, rats were fed AIN-76A diet ± 250 mg genistein/kg diet at three time periods, and all females were treated with DMBA at day 50 to induce mammary cancer. As seen in Fig. 2.3, rats exposed to the control diet, AIN-76A only, from birth until the end of the experiment (Zero/DMBA/Zero) had the highest average number of tumors (8.9 tumors/rat) [28]. Rats exposed to genistein from days 1 to 21 postpartum only (Gen/DMBA/Zero) developed 4.3 tumors, which confirmed

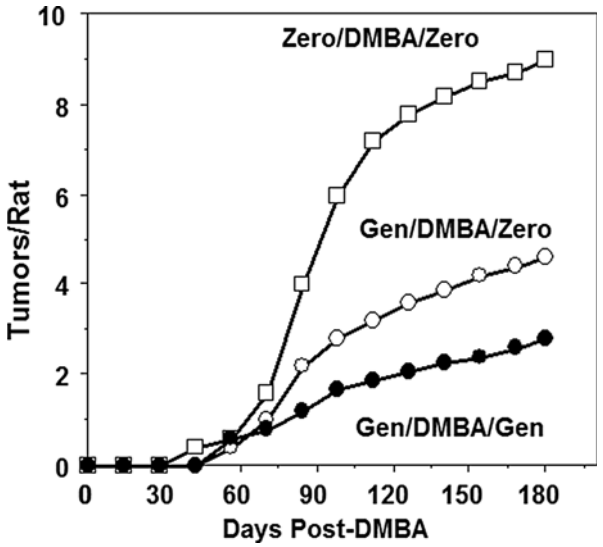

Fig. 2.3 Ontogeny of palpable mammary tumors in female Sprague-Dawley CD rats exposed prepubertally and/or as adults to genistein in the diet. Group 1 was fed control AIN-76A diet starting after parturition and continued throughout the study (Zero/DMBA/Zero). Group 2 was fed AIN-76A diet containing 250 mg genistein/kg diet, starting after parturition through day 21 only and then AIN-76A onward (Gen/DMBA/Zero). Group 3 was fed genistein-containing diet after parturition through day 21, AIN-76A only through day 100 postpartum, and genistein-containing diet (Gen/DMBA/Gen) from day 100 onward. All animals received 80 mg DMBA/kg body weight at day 50. Each group consisted of 25 rats. Reprinted with permission from Lamartiniere CA, Cotroneo MS, Fritz WA, Wang J, Mentor-Marcel, R-M, Elgavish A (2002) Genistein chemoprevention: timing and mechanisms of action in murine mammary and prostate. J Nutr 132:552S-558S (American Society for Nutritional Sciences)

Table 2.1 Dietary genistein, timing of exposure and mammary cancer chemoprevention

Exposure period	Tumor multiplicity[1]
No genistein	8.9
Prenatal genistein only[2]	8.8
Adult genistein only (after tumors)[3]	8.2
Prepubertal genistein only[4]	4.3
Prepubertal and adult genistein[3,4]	2.8

Diets contained ± 250 mg genistein/kg AIN-76A feed.
[1]All rats were treated with 80 mg DMBA/kg body weight at day 50 post-partum.
[2]Prenatal treatment is throughout gestation.
[3]Adult treatment was initiated at 100 day postpartum.
[4]Prepubertal treatment was from day 1 to 21 postpartum.
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the earlier work of Fritz et al. [25]. Furthermore, rats exposed to genistein from days 1–21 and 100–180 (Gen/DMBA/Gen) developed the fewest number of tumors (2.8 tumors/rat). The later genistein feeding was initiated 50 days after the DMBA treatment, the time of onset of palpable mammary tumors. The results showed that genistein fed to adult rats previously exposed prepubertally to genistein provided these animals with additional protection against mammary cancer (Table 2.1). Prepubertal genistein exposure appears to permanently affect the mammary gland in a way that determines how that individual later responds to the same or similar chemical stimuli, i.e. the "blue-print" for gene and protein expression is set. *In this case, genistein acquired via the diet during the prepubertal period programmed future (adult) genistein response against mammary cancer susceptibility* [25, 28].

2.8 Mammary Gland Development

Via the elegant studies of Jose and Irma Russo, we know that developmental alterations to the mammary gland can determine cancer susceptibility. The development of the mammary gland in rats starts *in utero*. From birth through the first week postpartum in the rat, the mammary gland is composed of a single primary or main lactiferous duct that branches into 3–5 secondary ducts [32, 33]. During the second week, further sprouting of ducts occurs up to the sixth generation. This sprouting of ducts causes an increase in density of terminal end buds in the growing periphery of the mammary gland (Fig. 2.4). Some of the terminal end buds differentiate in response to each estrous cycle, giving rise to alveolar buds which can be found in

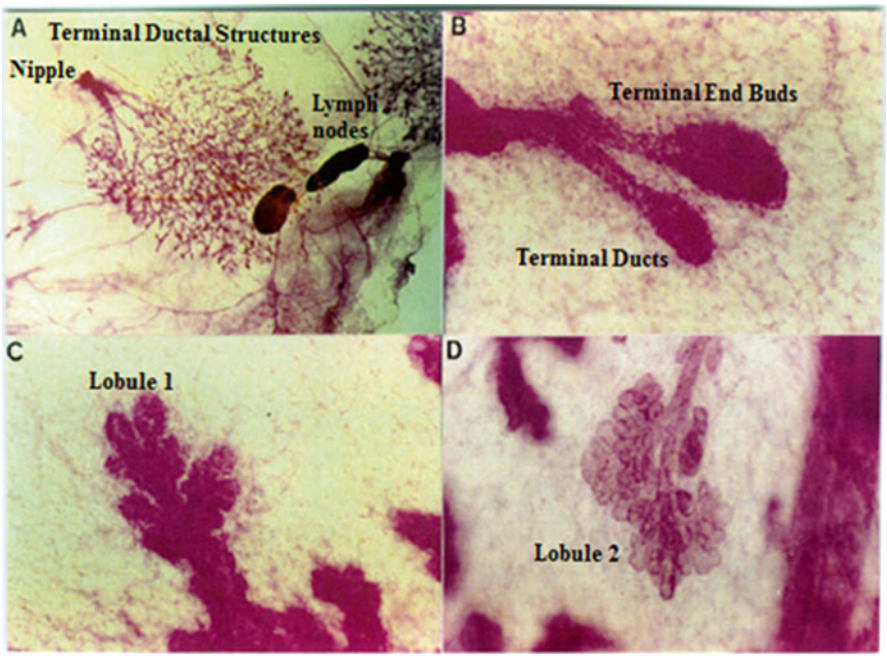

Fig. 2.4 Terminal ductal structures in rat mammary glands. (**A**) Whole mounts of fourth abdominal mammary glands from female Sprague Dawley CD rats. Note nipple at *upper corner* and lymph nodes at *bottom right*. (**B**) The *upper* structure is a terminal end bud, the *lower* structure is a terminal duct, (**C**) Lobule I, (**D**) Lobule II. Reprinted with permission from Brown NM, Manzolillo PA, Zhang J-X, Wang J, Lamartiniere CA (1998) Prenatal TCDD and predisposition to mammary cancer in the rat. Carcinogenesis 19(9):1623-1629 (Oxford University Press)

type I lobules. Type I lobules can mature to type II lobules. These lobules respond to hormones of pregnancy by differentiating further into type III lobules, which form the functional units of the lactating gland [34, 35]. The differentiation of terminal end buds to lobules appears to be a basic and protective mechanism against chemical carcinogenesis. Terminal end buds and terminal ducts are less differentiated structures that are more susceptible to chemical carcinogenesis than the more differentiated alveolar buds and lobules [32]. This is due to the increased mitotic activity of terminal end bud and terminal duct cells at day 21 as opposed to cells in alveolar buds and lobules in mature animals [34]. In the human, the development of the mammary gland is similarly initiated *in utero* [36]. Further development and differentiation requiring active cell proliferation takes place almost simultaneously with the formation of lobules type 1–4.

Evidence of age-related breast cancer susceptibility is evident from reports in girls exposed to cancer causing agents early in life. In female patients who were exposed to radiation *via* fluoroscopy an average of 102 times over a period of

several years, the greatest risk of breast cancer occurred among women who were first treated between the ages of 15 and 19 years, with no excess risk being associated with women who were over 30 years old at the time of first exposure [37]. This increased breast cancer risk did not appear until 15 years after the initial exposure and was present at the end of 40 years of observation. Also, after World War II bombing of Hiroshima and Nagasaki [38], young girls who were 10–19 years old when exposed to the ionizing radiation of the atomic bomb were followed and found to have a higher lifetime risk of breast cancer after the age of 35. These reports suggest that the early period of a women's life is critical for predisposition to, or for protection against, breast cancer.

2.9 Cellular Mechanism of Action

Analysis of mammary gland morphology in mature rats exposed to genistein early in life revealed that its cellular mechanism of action is enhancement of mammary gland differentiation [21, 22, 25, 28, 30, 39, 40]. We studied this in 50 day old rats since this is the time that the carcinogen, DMBA, is administered in the rat model. We found reduced number of terminal end buds and increased number of lobules in adult animals exposed neonatally or prepubertally to genistein (before administering the DMBA). Mammary terminal end buds are terminal ductal structures found primarily in young animals (and humans) and contain many undifferentiated epithelial cells [32, 34]. They are characterized by having a high mitotic index, hence they are most susceptible to chemical carcinogens [33]. While terminal end buds are the structures most susceptible to chemical carcinogenesis in the mammary glands, lobules are the most differentiated and least susceptible to chemical carcinogens. Importantly, a similar cellular mechanism of action involving increased mammary terminal ductal differentiation also occurs in the rat mammary and human breast *via* hormones of pregnancy [34–36].

Further evidence that genistein enhances differentiation was obtained by measuring β-casein in mammary glands. β-Casein is a milk protein and biomarker of mature mammary glands and differentiated cells [34]. Using western blot analysis, we found that prepubertal genistein treatment increased beta-casein expression in mammary glands of prepubertal and adult rats [28]. In the adult rats beta-casein was measured 30 days after genistein treatment, so even in the absence of circulating genistein, the rat mammary gland still produced β-casein, indicating permanent, non-reversible differentiation.

The potential of the soy components, daidzein and equol, have also been investigated for potential to alter mammary gland development and for mammary cancer prevention. Daidzein is the second most plentiful of the isoflavones, and equol is an intestinal bacterial metabolite of daidzein. Perinatal exposure of female rats *via* 250 and 1000 mg daidzein/kg AIN-76A diet did not alter mammary gland development

at day 50 or the ontogeny of chemically-induced mammary tumors in rats treated with DMBA on day 50 [41]. Brown et al. investigated the potential of 250 mg equol/kg diet during the neonatal (0–21 days) or prepubertal (21–35 days) period to alter mammary gland development and predisposition for mammary cancer. By day 50, early equol exposure resulted in a decrease in immature terminal end structures and an increase in mature lobules [42]. Despite these morphological changes to the mammary gland, neonatal and prepubertal exposures to equol had no long-term chemoprevention against mammary tumors induced by DMBA. *The fact that equol enhanced mammary gland differentiation, but did not render chemoprevention, suggest that there is more than gland differentiation as the mechanism for genistein chemoprevention.*

2.10 Genistein: Cell Proliferation and Apoptosis

Cell turnover is a key process involved in mammary gland development and the pathogenesis of tumor formation. Hence, we investigated if early prepubertal exposure to genistein treatment impacted normal cell proliferation and apoptosis in the mammary gland. At postnatal day 21, rats prepubertally exposed to dietary genistein exhibited a 53 % increase in cell proliferation and a 45 %, but not statistically significant decrease in apoptosis in mammary terminal ductal structures (Fig. 2.5A & B) [43]. Using the ratio of cell proliferation to apoptosis to estimate cell turnover in mammary terminal ductal structures, genistein exposure increased the ratio compared with controls by 2.6-fold (Fig. 2.5C). The latter is suggestive of active remodeling in the mammary gland from the presence of genistein.

Because Sprague Dawley rats are most susceptible to chemically-induced mammary cancer at day 50, we also investigated cell proliferation and apoptosis at that age. This time point is 30 days after the last dietary genistein treatment and a time point that, due to metabolism and disposition, animals are free of circulating genistein. While cell proliferation was not changed in mammary glands of 50 day old rats exposed prepubertally to genistein (Fig. 2.5D), cell apoptosis was increased over two-fold (Fig. 2.5E) [43]. Cell turnover was calculated to be decreased by 55 % (Fig. 2.5F). Summarily, genistein administered to rats during the prepubertal period stimulates mammary cell proliferation at day 21, but not at day 50, while apoptosis is increased at the latter age. The increase in cell proliferation during early postnatal mammary gland development correlates with differentiation of the mammary terminal ductal structures (from day 21 to day 50), and is to be associated with less chemically-induced cancer in the adult animals [21, 22, 25, 28, 30]. Of added importance is that increased rate of apoptosis at time of carcinogen exposure probably contributes to chemoprevention by killing DNA-damaged cells.

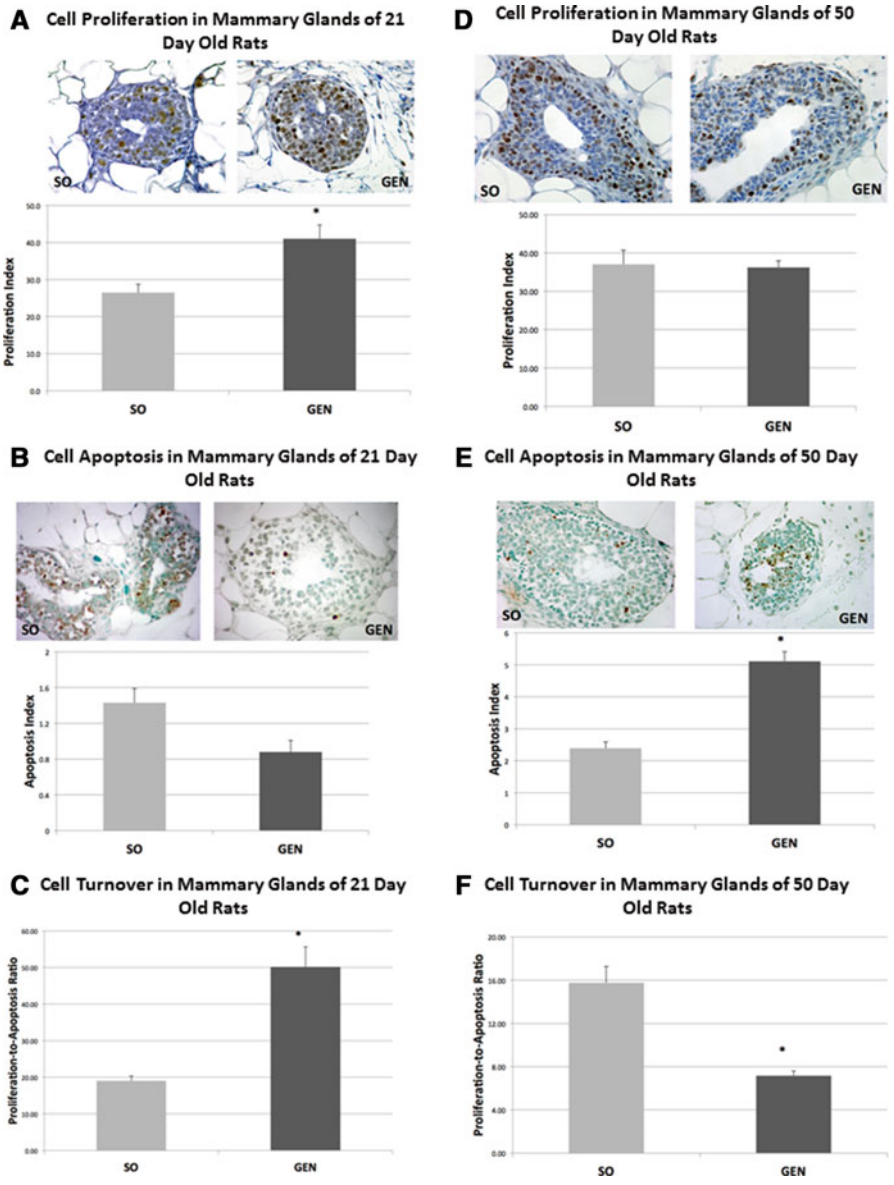

Fig. 2.5 Cell Proliferation, apoptosis and cell turn-over in mammary gland terminal end bud epithelial cells of 21 and 50 day old rats exposed prepubertally *via* lactating dams fed genistein in the diet from days 2–21 postpartum. The pictures in sections (**A & D**) illustrate cell proliferation *via* immunohistochemical staining for antigen Ki-67, and sections (**B & E**) are for apoptosis *via* ApopTag *in situ* labelling kit. Sections (**C & F**) reflect cell turn over, i.e. ratio of cell proliferation to apoptosis. Reprinted with permission from Wang J, Jenkins S and Lamartiniere CA (2014) Cell proliferation and apoptosis in rat mammary glands following combinational exposure to bisphenol A and genistein. BMC Cancer 14:379 doi:10.1186/1471-2407-14-379 (BioMed Central)

2.11 Molecular Mechanism of Genistein Action

Day 21 mammary glands. One reason cancer researchers have investigated genistein as a chemoprevention agent are the reports that it inhibits protein tyrosine kinases activity *in vitro* [6, 7]. One such tyrosine kinase protein is the epidermal growth factor (EGF)-receptor. The EGF-receptor and its ligands play essential roles in normal and pathological mammary gland development. Early in development, the EGF-signaling pathway plays an essential role in cell differentiation, development, and ductal morphogenesis [44, 45]. Accordingly, we investigated the potential of genistein to regulate the EGF-receptor *in vivo*. In 21 day old rats treated prepubertally with genistein, we found increased EGF-receptor expression in mammary terminal end buds (Fig. 2.6A & B) [40]. Not only was this finding contrary to the *in vitro*

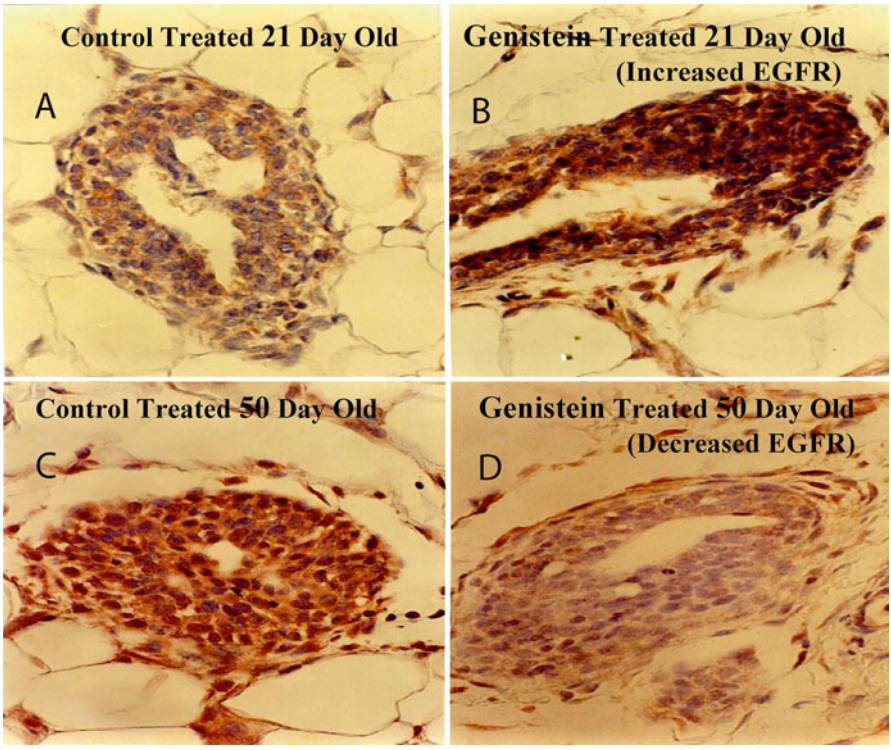

Fig. 2.6 Immunohistochemical staining for the EGF-receptor in mammary terminal end buds. Photographs (**A**, **B**) show staining for the EGF-receptor in terminal end buds of 21 day old rats treated prepubertally with (**A**) vehicle, or (**B**) genistein. Photographs (**C**, **D**) show staining for the EGF-receptor in terminal end buds of 50 day old rats treated prepubertally with (**A**) vehicle, or (**B**) genistein. Note the dark immunohistochemical staining for the EGF-receptor in panels B and C (40). Reprinted with permission from Brown NM, Wang J, Cotroneo MS, Zhao, Y-X, Lamartiniere CA (1998) Prepubertal genistein treatment modulates TGF-α, EGF and EGF-receptor mRNAs and proteins in the rat mammary gland. Mol Cell Endocrinol 144:149–165 (Elsevier)

reports [6, 7], but this was surprising to us because we expected a chemoprevention agent to down-regulate the expression of this cancer-related growth factor signaling pathway. However, as discovered later, in mammary glands of 50 old rats exposed prepubertally to genistein the expression of the EGF-receptor was down-regulated (Fig. 2.6C & D) [40, 46]. We surmise that exposure to genistein early in postnatal life alters the "molecular program" from which the mammary gland of 50 day old rats responds to later. This will be discussed in more detail in the next section.

To increase the number of mammary biomarkers, we used two-dimensional gel electrophoresis coupled with mass spectrometry to separate, quantitate, and identify mammary gland proteins that are changed in response to prepubertal genistein treatment [46, 47]. For those proteins that we were able to obtain commercially available antibodies, we pursued validation *via* western blot analysis, and extended our use of immuno-blots to identify and quantitate proteins that play a role in cell proliferation, apoptosis, or other signaling pathways related to carcinogenesis. In mammary glands of 21 day old rats exposed prepubertally to genistein, expression of phospho-Akt and annexin A2 were increased compared to controls (Table 2.2) [46]. Like the EGF-receptor, these two proteins have been associated with mitotic activity, and their actions are believed to play a significant role in the cell proliferation observed in the presence of genistein in mammary glands of prepubertal rats. In mammals, cell proliferation is required for embryogenesis, growth, and differentiation of cells and tissues, including the mammary gland. Metabolic effects of the PI3K/Akt/mTOR pathway include enhanced uptake of glucose and essential amino acids and protein

Table 2.2 Differentially regulated proteins in mammary glands of 21 day old rats exposed prepubertally to genistein

Protein	Summary of mechanism of action or molecular function	Regulation	Reference
p-AKT	Phospho-protein kinase B: regulates cell proliferation, motility, glucose homeostasis, and cell survival	UP	43, 46
Annexin A2	Associated with DNA synthesis, cell proliferation and differentiation, and angiogenesis	Up	46
c-caspase-3	Involved in cell differentiation and apoptosis, and inflammation	Down	43
EGF-receptor	Enhances cell proliferation and differentiation, development, and ductal morphogenesis	Up	40, 46
Gelsolin	Regulates stromal–epithelial communication for cell differentiation, and mammary ductal growth	Up	46
GTPCH-1	GTP-cyclohydrolase-1: rate-limiting enzyme in the production of BH4, and regulates catecholamine levels	Up	47
PDIA3	Protein disulfide-isomerase A3 (glucose regulated protein): plays a role in protein folding and differentiation	Down	46

translation that can contribute to cell motility, and cell survival [48]. While not prominent in the field of mammary cancer, annexin A2 has been shown to play a role in DNA synthesis, cell differentiation and neoangiogenesis [49].

GTP-cyclohydrolase-1 (GTPCH-1) and gelsolin are two other proteins that were found to be up-regulated in mammary glands of 21 day old rats treated prepubertally with geinistein [46, 47]. GTPCH-1 is the rate limiting enzyme in the production of tetrahydrobiopterin (BH4) and to the production of catecholamines, which regulate differentiation and development of cells [50]. Gelsolin is an actin filament-capping protein that has been shown to play a key role in cell migration. It is required in the mammary stroma for proper ductal morphogenesis and promotes mammary ductal growth through stromal-epithelial communication [51]. Also, gelsolin has been reported to be an inhibitor of apoptosis, and overexpression of gelsolin results in the lack of activation of caspase-3 [52].

Gelsolin being up-regulated is consistent with our finding that cleaved caspace-3 protein expression was reduced in mammary glands of 21 day old rats exposed prepubertally to genistein [43]. Previously, Qian et al. [53] showed that genistein treatment reversed ischemia-induced mitochondrial dysfunction by decreasing mitochondrial reactive oxygen species, preventing cytochrome C release, and inhibiting caspase 3 activation. Protein disulfide-isomerase A3 (PDIA3), also known as glucose-regulated protein or GRP58/ERp57, was reduced in mammary glands of 21 day old rats exposed prepubertally to genistein *via* the diet [54]. PDIA3 is reported to play a role in protein folding and differentiation [52]. We found decreased PDIA3 expression in mammary glands of 21 day old rats, but unchanged at day 50. We speculate that up-regulated PDIA3 in mammary glands of prepubertal rats supports cell differentiation and gland maturation.

Day 50 mammary glands. Turning our attention to proteins in 50 day old rats exposed to genistein during the prepubertal period, we found higher β-casein protein expression in mammary glands of 50 day old rats exposed prepubertally to genistein (Table 2.3) [28]. This finding is consistent with our reports of increased number of lobules (more differentiated terminal ductal structures) in 50 day old animals exposed prepubertally to genistein [22, 23, 25, 28, 30, 40]. β-Casein is a milk protein and considered a marker of differentiation in mammary glands [34]. This confirms that prepubertal genistein exposure enhances mammary gland cell differentiation.

Also, we were able to confirm by western blot analysis that EGF-receptor expression was decreased in mammary glands of 50 day old rats exposed prepubertally to genistein compared to those with no genistein in the diet [46]. This was previously demonstrated by immunohistochemical staining [42]. Importantly, in comparing increased mammary EGF-receptor expression from the 21 day old rats being exposed to genistein to that of reduced mammary EGF-receptor expression in 50 day old rats where genistein treatment had been discontinued since day 22 postpartum, we see the dramatic effect of direct genistein action in the prepubertal period to that of an apparent delayed, but permanent effect on specific protein expression. We interpret this to mean that early in postnatal life (days 1–21) genistein up-regulated the EGF-receptor to stimulate mammary gland development and cell differentiation that resulted in enhanced mammary gland maturation later in life. Reduced

Table 2.3 Differentially regulated proteins in mammary glands of 50 day old rats exposed prepubertally to genistein

Protein	Summary of mechanism of action or molecular function	Regulation	Reference
Annexin A2	Associated with DNA synthesis, cell proliferation differentiation, angiogenesis and tumorigenesis	Down	46
β-Casein	Milk protein, marker of differentiated mammary cells	Up	28
c-caspase-3	Involved in cell differentiation, apoptosis, and inflammation	Up	43
c-caspase-9	Cleaved cysteine-dependent aspartate-specific protease-9: initiates apoptosis	Up	43
EGF-receptor	Regulates development, cell proliferation and differentiation	Down	42, 46
Fetuin B	Anti-angiogenic properties and tumor suppressor	Up	46
Ki-67	Marker of cell proliferation	Down	46
c-PARP	Cleaved-poly(ADP-ribose) polymerase: a nuclear enzyme involved in DNA repair	Up	43
PGK1	Phosphoglycerate kinase 1: involved in glycolytic pathway and breast cancer	Down	46
SRCs 1-3	Steroid receptor co-activators 1–3: involved in cell proliferation and steroid dependent carcinogenesis	Down	43
Tyrosine Hydroxylase	Rate-limiting step in the catecholamine pathway Dopamine product is inversely related to tumorigenesis	Up	47
VEGFR2	Vascular endothelial growth factor receptor-2: involved in angiogenesis and tumorigenesis	Down	46, 47

EGF signaling and decreased cell proliferation at day 50, at which time the DMBA was given, coincides with the more mitotically inert lobules and thus reduced susceptibility to chemical carcinogenesis [21, 22, 25, 30, 40]. Developmental modifications by a hormonally-active chemical that results in altered biochemical or behavioral responses later in life has been defined as organizational, imprinting or programming effects [55–57]. We speculate that down-regulated EGF-receptor signaling in mammary terminal end buds at the time of carcinogen exposure plays a role in reduced mammary cancer development.

Not only is the EGF signaling pathway important for cell proliferation and differentiation, and mammary gland development and carcinogenesis, but so are estrogen receptors. Regulation of steroid receptor action is complex due to a number of transcriptional regulatory molecules, including steroid receptor co-activators (SRCs), which can determine signaling specificity and intensity, resulting in pleiotropic biological effects, including cancer causation [58–60]. We assessed the expression of ER-α, ER-β and SRC proteins known to play a role in estrogen signaling and breast cancer in mammary glands of 50 day old rats exposed prepubertally

to genistein in the diet. No significant differences were observed in ER-α and ER-β expression (data not shown), but we found all three members of the p160 family of steroid receptor coactivator proteins, SRC-1, SRC-2 (GRIP-1: glucocorticoid receptor-interacting protein) and SRC-3 (AIB1: amplified in breast cancer) to be significantly down-regulated in mammary glands of 50 day old rats following prepubertal genistein exposure when compared controls (Table 2.3) [43]. Just as a large variety of steroid hormone dependent cancers overexpress SRCs, down regulation of steroid receptor-coactivators is viewed as consistent with suppressing cancer development.

In mammary glands of 50 day old rats with prepubertal exposure to genistein, we found annexin A2 expression to be decreased [46]. As opposed to the decreased expression seen here, increases in annexin A2 have been reported in cancer invasion and progression processes, and observed in cancers of the breast and prostate [61, 62]. This suggests that annexin A2 possess cancer promoting properties. On the other hand, the opposite result, reduced expression of annexin A2 as noted in mammary glands of 50 day old rats could be viewed as contributing to reduced cell proliferation and thus reduced potential for cancer. This pattern is similar to what was evidenced with the EGF-receptor. Both protein expressions were increased in mammary glands of 21 day old rats and decreased at day 50. Likewise, in the presence of genistein during the prepubertal period, both may contribute to cell proliferation and cell differentiation, and in the more differentiated and mature mammary glands (in the absence of genistein) there is less annexin A2 and EGF-receptor signaling, properties that are less conducive for carcinogenesis.

The caspases are a family of evolutionarily conserved cysteinyl proteases that mediate both apoptosis and inflammation through aspartate-specific cleavage of a wide number of cellular substrates. Caspase biology has been extended to cellular responses such as cell differentiation and proliferation. We selected two caspases as potential biomarkers, caspace-3 and caspace-9. Caspases involved in apoptosis have been classified by their mechanism of action and are either initiator caspases (caspase-9) or executioner caspases (caspase-3) [63–65]. We also measured c-PARP, a nuclear enzyme involved in DNA repair which is a well-established substrate for caspase-3 [66]. Cleaved PARP is considered to be a hallmark of apoptosis [66–68]. Activated-caspace-3, activated-caspace-9 and activated PARP were all up-regulated at day 50 [43]. These results are consistent with increased potential for apoptosis, which was determined by an *in situ* apoptosis assay in mammary terminal end buds of 50 day old rats exposed prepubertally to genistein [43]. This would suggest that DNA damaged cells would undergo apoptosis and serve as another level of chemoprevention.

Fetuin B has been reported to possess anti-angiogenic properties, and its overexpression in skin squamous carcinoma cells leads to suppression of tumor growth in nude mice [69]. We found that prepubertal genistein exposure resulted in increased fetuin expression by 67 % compared to controls in mammary glands of 50 day old rats [46]. Therefore, fetuin B could be acting as a tumor suppressor in the rat mammary gland. Also, Cabanes et al. [70] reported that injections of prepubertal genistein resulted in increases in the tumor suppressor BRAC1 mRNA in

prepubertal and 8 week old rats. It would be interesting to determine if the mRNA of this tumor suppressor gene is translated to the protein in a dietary model.

Genistein exposure decreased the levels of PGK1 by 54 % compared to control [46]. PGK1 is involved in the glycolytic pathway. PGK1, like annexin A2 is a component of the primer recognition complex that stimulates the activity of DNA polymerase [71, 72]. Therefore, decreased expression of PGK1 could explain, in part, the reduced rate of cell proliferation observed in the mammary gland.

As a follow-up to our finding that GTPCH-1 protein expression was up-regulated in mammary glands of 21 day old rats treated with genistein, we investigated GTPCH-1 and tyrosine hydrolase expression in mammary glands of 50 day old rats. At 50 days, there was no change in GTPCH-1 protein, but tyrosine hydroxylase expression was increased [47], a factor that could lead to increased dopamine. Interestingly, Teunis et al. [73] reported that rats with high dopaminergic activity had a reduction in tumor size compared with rats with low dopaminergic activity. Associating elevated dopamine levels with suppressed mammary tumorigenesis, they noted that the angiogenic response to the vascular endothelial growth factor receptor (VEGFR) could be inhibited by administration of dopaminergic agonists. Basu et al. [74] showed that dopamine acts through the dopamine 2 receptor to induce the endocytosis of VEGFR and thereby inhibit or prevent VEGF binding, receptor phosphorylation, and subsequent signaling steps. They reported that immunohistochemical studies did not find tyrosine hydroxylase–positive nerves in tumors, and the dopamine concentration in malignant tumors was significantly reduced compared with concentrations in controls. Furthermore, Ferguson et al. [75] reported that lifetime exposure to genistein could potentiate dopamine levels in striata of amphetamine-exposed animals. In addition, researchers found that genistein decreased both transcription and protein levels of VEGF and that this decrease is involved in the loss of angiogenesis. Heffelfinger et al. [76] demonstrated that inhibition of VEGFR2 will prevent DMBA-induced mammary tumors in rats. As a follow-up, we found VEGFR2 expression to be decreased in mammary glands of 50 day old rats exposed prepubertally to genistein [46, 47]. These results complemented the finding that tyrosine hydroxylase levels were elevated in the mammary glands of 50 day old rats from prepubertal genistein treatment. We speculate that increased tyrosine hydroxylase expression results in dynamic up-regulation of catecholamines, which, in turn, decrease the VEGFR2 levels, resulting in decreased ability to promote angiogenesis. Ortega et al. [77] implicated VEGFR2 in mediating cell proliferation. Therefore, a decrease in VEGFR2 may decrease the overall proliferative potential of the mammary gland. The absence of a demonstrable change in dopamine concentrations may mean that the concentration is dynamic or that changes in concentration within the microenvironment may not manifest in detectible or significant change in the larger sample (whole mammary gland). Observed decrease in VEGFR2 at the time of carcinogenesis could reduce cell proliferation, angiogenesis, and cell invasion, and favor breast cancer prevention.

Not surprisingly, we found the Ki-67 protein to be down-regulated in mammary glands of 50 day old rats [46], thus adding validity to differentially regulated proteins involved in cell proliferation. The fact that the Ki-67 protein is present during

all active phases of the cell cycle, but is absent from resting, makes it an excellent marker for determining the so-called growth fraction of a given cell population [78]. Ki-67 protein expression is an absolute requirement for progression through the cell-division cycle and is an excellent indicator of cell proliferation.

2.12 Programming Against Breast Cancer

Programming or differentiation in the mammary gland is to be contrasted to *activational effects* that are often described in conventional mechanisms of action following exposure to a chemical modulator. For example, when a chemical such as genistein is provided to an animal and the result is an up or down expression of a specific protein, followed by reversal to previous expression level when the chemical is withdrawn, this is an activational effect, i.e. an effect that is reversible. On the other hand, when hormones of pregnancy or genistein is given to a young female rat, not only can activational effects take place, but so can *organization effects that can be termed programming or imprinting* [55–57]. An example is cell and mammary gland differentiation whereby terminal end buds are transformed to the more mature lobules that can eventually produce milk. These lobules can now be characterized as having different gene and protein expressions from those of terminal end buds. Some of these changes are permanent and irreversible changes at the molecular level. Hence, we refer to these permanent changes in gene and protein expressions as the "*blue-print*" from which these mammary cells respond. When the term imprinting is used, this most often refers to an epigenetic modification, i.e. as a consequence of changes in DNA methylation or histone acetylation. But, proteins can undergo post-translational modifications that can alter function. Because we do not yet know the exact nature of genistein's action in promoting mammary cell and gland differentiation, and subsequent biological actions that render the mammary to be resistant to cancer, we use the term programming. In our proteomic studies we observed several examples of activational effects. In mammary glands of 21 day old rats exposed to genistein, we found that p-AKT, gelsolin, GTPCH-1 and PDIA3 were differentially regulated in the presence of genistein. However, in adults, in the absence of genistein, the expression of these specific proteins were similar to those of controls, i.e. the effect was not sustained at 50 days.

On the other hand, in mammary glands of 50 day old rats exposed prepubertally to genistein we observed 14 proteins whose expressions were different from controls (Table 2.3). The importance of this observation is that neither group of adult animals was in the presence of genistein at the time of measurement. *Hence*, *exposure to genistein during the prepubertal period*, *a critical time for mammary gland development*, *set the biological* "*blue print*" *or the stage for permanent manifestations that determine how the mammary gland develops and responds later to chemical exposures such as hormones*, (*pro*)*carcinogens and effectors of cell proliferation and apoptosis. Critically*, *programming can in the mammary predetermine susceptibility for disease*, *including breast cancer*.

Does the programming mechanism apply to all tissues? No. For example, we investigated the potential of dietary genistein exposure in transgenic mice designed to spontaneously develop prostate tumors (TRAMPs), and chemically-induced prostate cancer in Lobund-Wistar rats. Our prostate cancer studies demonstrated that genistein exposure during the neonatal and prepubertal periods only did not suppress prostate cancer development in adult TRAMPs [79]. On the other hand, genistein in the diet to adult TRAMP mice resulted in a 29% decrease in poorly-differentiated adenocarcinomas. More effective was life-time (weeks 1–28 postpartum) genistein treatment. It resulted in a 50% decrease in poorly differentiated prostate tumors. With both of these genistein treatment groups, the chemoprevention was associated with suppressing the rate of cancer development as evidenced by increased percentage of prostate cancer manifested as moderately differentiated tumors, (44–60%). Similar results were obtained with N-methylnitroso urea (NMU)-induced prostate cancer in Lobund-Wistar rats [80], i.e. adult exposure was more effective than neonatal/prepubertal genistein exposure only, and life-time use of genistein, starting in the first week was more effective in suppressing prostate cancer. In both prostate cancer models, the direct presence of genistein was necessary to suppress prostate cancer development.

2.13 Toxicology Studies

In laboratory studies, genistein has been reported to stimulate tumor growth in athymic ovariectomized mice subcutaneously implanted with MCF-7 breast cancer cells, albeit six fold lower than positive controls provided by estrogen [81]. But, the latter is contradictory to the report where genistein administered to intact athymic mice orthotopically implanted with MCF-7 cells suppressed the growth of resulting tumors [82]. The significance of ovariectomy as a model of menopause and the use of mice lacking proper cellular surveillance immunity remains to be discerned.

Since the perinatal period is the most sensitive time for toxicity to the endocrine and reproductive systems, we have carried out toxicology studies in rats exposed perinatally to genistein. We chose to administer genistein *via* the diet, which is the primary means of soy and genistein exposure. The dietary genistein doses were selected on the basis of a previous report that rats fed 25 mg genistein/kg AIN-76A diet (phytoestrogen-free) resulted in total genistein concentrations of 252 pmol/ml in the serum [26]. This was comparable with the total genistein concentration (276 pmol/ml) in the blood of Asian men eating a traditional diet high in soy [27]. A dose one order of magnitude higher was also selected for the purpose of investigating potential toxicity of dietary genistein and for dose-response and bioavailability studies. The numbers of male and female offspring, anogenital distances, time of testes descending and vaginal opening were not significantly different from controls [25]. The body weight, uterine weights and mammary gland size were not significantly different compared to control exposed animals at postnatal days 21 and 50. Perinatal genistein in the diet did not alter percent of time in each phase of the estrous cycle of the female offspring. The numbers of primordial normal follicles

and corpora lutea were not significantly different in females exposed perinatally to genistein. Also, *histomorphological analysis of vaginal, uterine and ovarian tissues in 50 and 100 day old female rats exposed perinatally to genistein did not reveal significant alterations compared to controls* [25].

Flynn et al. [83] have carried out toxicology investigations using dose response studies and evaluated morphometric measurements and sexually dimorphic behaviors in rats. They reported that *dietary genistein* at 25 and 250 mg/kg AIN-76A diet fed to pregnant rats, beginning on gestational day 7 and the offspring continued until postnatal day 77, *did not significantly alter gestational duration*, *total offspring/litter*, *total sex ratio*, *live pups/litter*, *live sex ratio*, *and average weight/live pup*.

Studies with humans show that isoflavonic phytoestrogens are normal constituents of human urine from subjects consuming large amounts of soy products (tofu, soy flour, soy milk, tempeh, soy nuts, soy bars, etc). Yet, *little or no toxicity is associated with soy/genistein consumption* [84]. Infants are able to absorb isoflavones, and infants fed soy formula were demonstrated to have plasma isoflavone blood levels exceeding those of Japanese adults several-fold [85]. Soy-based infant formula can result in plasma concentrations of isoflavones in infants that are 13,000–22,000 times higher than endogenous estrogen concentrations in infants [86]. Infants consuming soy-based formula are exposed to 6–11 mg isoflavones /kg per day (4–7 mg total genistein/kg) that result in circulating total genistein levels of approximately 1–5 μM. In contrast, adults consuming a moderate to large amount of soy in the diet are exposed to ~1 mg total genistein/kg per day resulting in circulating total genistein levels of approximately 0.5 μM [87]. *Even though infants ingesting soy milk are exposed to high concentrations of genistein*, *little toxicity has been reported*. The most noted consequence is hypothyroidism in infants with already compromised thyroid function, a situation that is remedied by fortifying soy milk with thyroid hormone supplement [88]. On the other hand, a plethoria of publications have investigated the potential of soy and it components for health benefits.

To address the potential of soy formula to result in toxicity to children, the *National Toxicology Program* convened an expert panel to determine the level of concern for soy infant formula on infants and child development. The Expert Panel of the 2010 NTP Brief on Soy Infant Formula focused on soy infant formula and the potential developmental toxicity of its major isoflavone components, e.g. genistein, daidzein (and estrogenic metabolite, equol), and glycitein. They expressed minimal concern for adverse developmental effects in infants fed soy infant formula. The NTP *concurred with the expert panel that there is minimal concern for adverse effects on development in infants who consume soy infant formula* [89].

2.14 Epidemiology

Early chemoprevention work with soy and genistein has been driven by epidemiology reports of high soy diets being protective against breast cancer in women [12, 13]. Since then, a multitude of epidemiology publications have supported these

publications, and some have not. On the other hand, none report that soy or genistein promote new estrogen-dependent breast or reproductive cancers. One of the most comprehensive meta-analyses of soy and risk for breast cancer was carried out by Trock et al. [90]. They performed a meta-analysis of 18 epidemiology studies (12 case–control and six cohort or nested case–control) published from 1978 through 2004 that examined soy exposure and breast cancer risk. Pooled relative risk estimates were based on either the original soy exposure measure defined in each study or on an estimate of daily soy protein intake. They found that risk estimates, levels and measures of soy exposure, and control for confounding factors varied considerably across studies. In a pooled analysis, among all women, high soy intake was modestly associated with reduced breast cancer risk (odds ratio (OR) = 0.86, 95 % confidence interval [CI] = 0.75–0.99); the association was not statistically significant among women in Asian countries (OR = 0.89, 95 % CI = 0.71–1.12). Among the ten studies that stratified by menopausal status the inverse association between soy exposure and breast cancer risk was somewhat stronger in premenopausal women (OR = 0.70, 95 % CI = 0.58–0.85) than in postmenopausal women (OR = 0.77, 95 % CI = 0.60–0.98). However, eight studies did not provide menopause-specific results, six of which did not support an association. When exposure was analyzed by soy protein intake in grams per day, a statistically significant association with breast cancer risk was seen only among premenopausal women.

More intriguing, but convincing, are the four epidemiology reports showing an association between soy intake of adolescents and reduction in breast cancer that are consistent with the laboratory demonstrations that genistein exposure during the prepubertal period suppresses chemically-induced mammary cancer in rats [22–25, 28, 30]. In 2001, Shu et al. [91] analyzed data from a population-based case-control of 1459 breast cancer cases and 1556 age-matched controls and showed that *high soy food intake during adolescence (age 13–15) resulted in an inverse association with breast cancer risk in both pre- and postmenopausal Chinese women*. Shortly thereafter, Wu et al. [92] reported a population-based, case-control study of breast cancer risk among Chinese, Japanese and Filipino women in Los Angeles County to investigate the role of soy, focusing on soy intake during adolescence and adult life among Asian-American women. *Women who reported soy intake at least once per week during adolescence showed a significantly reduced risk of breast cancer, and there was a significant trend of decreasing risk with increasing soy intake during adult life. Furthermore, high soy intake during both adolescence and adult life showed the lowest risk for breast cancer.*

Also, Korde et al. [93] reported *Asian-American women with high soy intake as children (between the ages of 5 and 11 years) with the greatest reduction in breast cancer risk (58 %), followed by exposures at adolescence (age 12–19),* and as young adults age 20 to approximately 27, *furthermore illustrating how important early postnatal development for reduction in breast cancer risk.* The *epidemiologic reports by Wu et al.* [92] *and Korde et al.* [93] *support our laboratory report that female rats exposed to genistein via the diet from parturition through day 21 and then from day 100 until the end of the study at day 180 had fewer mammary tumors than those provided genistein only during the prepubertal period or as adults only* [28]. More

recently, a population-based case–control study evaluated the association between adolescent dietary phytoestrogen intake and adult breast cancer risk among women in Ontario, Canada. *Higher phytoestrogen intake during adolescence was associated with a reduced breast cancer risk, and a monotonic trend was observed from the lowest to the highest quartile* [94]. Frankly, it is remarkable how consistent the prepubertal genistein laboratory data and the adolescent soy epidemiology data are. Furthermore, the reports of adolescents exposed to soy having reduced breast cancer risk [91–94] explain why many earlier epidemiology reports had less than stellar results, i.e. adult only soy exposure matters only if adolescent plus adult exposure takes place.

Realizing that the most likely way towards cancer prevention is *via* early exposure to soy or genistein, Maskarinec et al. [95] investigated the compliance of young girls to soy intervention. They used an eight week dietary intervention, and urine samples were collected from eight to 14-year-old girls. The girls were asked to consume one daily serving of soymilk, soy nuts, or tofu. 17/20 of the girls completed the study. The serving sizes provided at least 30 mg isoflavones/day. Daily soy intake logs indicated a mean intake of 6.28 servings out of a maximum of seven servings per week. The food records revealed a six-fold increase in isoflavone intake during the study period ($P < 0.01$) which was confirmed by urinary isoflavone concentrations of 23.3 nmol/mg creatinine prior to intervention and 142.1 nmol/mg creatinine during intervention. *The adolescent girls demonstrated compliance, and no health complications related to soy consumption were reported.*

2.15 Blood Proteomics of Prepubertal Girls

Our focus on cancer biomarkers breaks from the accepted dogma of using genomic markers and moves to *a more practical aspect of biomarkers that actually reflects function, proteins.* Proteins, as enzymes, cofactors and regulators, actually carryout the enzymatic actions and support many metabolic processes. Although there are a plethora of papers that examine gene expression, the latter may not always translate into protein action. Recently, we developed methods to identify protein biomarkers of effect and susceptibility from blood using Isobaric Tandem Mass Tags and quantitative mass spectrometry (TMT-MS) combined with MudPIT technology. We used blood sera from prepubertal girls whose urine had been subjected to mass spectrometry analysis for soy isoflavones, phenols and phthalates. In prepubertal girls, urine concentrations of genistein, bisphenol A (BPA), mono-ethyl hexylphthalate (MEHP) and mono-benzyl phthalate (MBzP) were used to identify girls in the top quintile of exposure for each of these environmental chemicals, and age-matched prepubertal girls with urine analyte concentrations below the median [96]. Blood samples of these girls were depleted of the seven most abundant proteins using human-specific affinity spin columns. Using TMT-MS, 34, 51, 57 and 47 differentially expressed proteins were identified from the blood of prepubertal girls with high urine concentrations of genistein, BPA, MEHP and MBzP, respectively, compared to controls. Using bioinformatics and focusing on cancer as a disease, we also identified cancer biomarkers

of susceptibility for genistein and BPA exposures. *The differentially regulated cancer associated proteins in genistein and BPA girls are especially convincing in light of divergent functions and the literature demonstrating that genistein and BPA exposures are associated with mammary cancer prevention and causation, respectively.*

In blood of girls with high genistein concentrations in their urine, two proteins with cancer associations were down-regulated: endothelin-converting enzyme (ECE-1) and eukaryotic translation initiation factor 3 subunit J (EIF-3) [96]. ECE-1 has been implicated in the pathogenesis of a range of disease states including breast, gynecological and urological cancers, cardiovascular disease and Alzheimer's disease [97]. EIF-3 has been found elevated in human breast, cervical, esophageal, and lung cancers, suggesting a potential role in malignant transformation and cell growth control [98]. On the other hand, nucleolar 7 and PR domain zinc finger 5 (PRDM5) are proteins that are up-regulated in genistein girls. Nucleolar 7 and PRDM5 have been reported to regulate the cell cycle. The nucleolar 7 gene is reported to be a candidate tumor suppressor gene in cervical cancer that modulates the angiogenic phenotype [99]. PRDM5 has growth suppressive activities and is silenced in breast, ovarian, liver, lung, colon, and other cancers [100]. All four proteins could be considered as biomarkers of susceptibility for genistein/soy and cancer prevention. Interestingly, *from PANTHER analysis of biological functions, the genistein group had the highest response on apoptotic process* [96]*, a finding that corresponds very well with our report of apoptosis being increased in mammary glands of rats exposed prepubertally to genistein* [43]. In fact, the differentially regulated cancer associated proteins in girls with high concentrations of genistein and BPA (details not provided for BPA here) are consistent with reported roles in mammary cancer prevention and causation, respectively.

2.16 Summary and Conclusions

The concept of programming against breast cancer is both intriguing and challenging. Intriguing, because the mechanism of action is unique, but it is based on solid research that has been well documented, and the critical experiments have been confirmed by different laboratories. Proving it *via* the scientific method in humans will be the biggest challenge. It is not always easy to carryout clinical studies in humans, especially when it means children.

To summarize, (1) dietary genistein provided during the prepubertal period suppresses chemically-induced mammary cancer in adult rats, and this has been independently confirmed, (2) four epidemiological studies show that adolescent girls eating a diet high in soy are at reduced risk for breast cancer, (3) in rats, the cellular mechanism of action has been described as early cell and mammary terminal ductal structure differentiation, a mechanism similar to mammary gland differentiation that follows from early pregnancy in young women, (4) identification of genistein mechanisms of action at the molecular level (Seven proteins are identified as playing a role in enhancing cell and mammary gland differentiation, cell turnover and tissue

remodeling in presence of genistein. On the other hand, 13 proteins are associated with increased apoptosis, decreased cell turnover, and potential for carcinogenesis in mammary glands of mature animals.), and (5) *in vivo* toxicology studies with genistein in animal models and epidemiology reports in humans demonstrate little or no toxicity.

Accordingly, the time has come for soy/genistein to be tested in adolescent girls for prevention of mammary cancer. Let's consider the facts. One in eight women will be diagnosed with breast cancer in their lifetime. Breast cancer is the most commonly diagnosed cancer in women, and it is the second leading cause of death among women. Each year it is estimated that over 220,000 women in the United States will be diagnosed with breast cancer and more than 40,000 will die. The protocol for programming against breast cancer may sound unusual, but children consuming soy milk, tofu, soy nuts or soy bars is not unusual, and they can easily incorporate soy or genistein in one or two meals a day.

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Chapter 3
The Role of Omega-3 Fatty Acids in Breast Cancer Prevention

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Abstract Preclinical and epidemiological data suggest that omega-3 fatty acids (n-3FA) protect against breast cancer, although controversy still exists in the literature. In view of the heterogeneity of human breast cancer, we believe that n-3FA should be a component of a multi-targeted approach for effective chemoprevention. Preclinical data from our laboratories indicate that n-3FA potentiates the chemopreventive effect of the antiestrogen Tamoxifen based on the complementarity of their mechanisms of antitumor action suggested by our signaling, genomic, and proteomic studies. Because of their anti-estrogenic and anti-inflammatory properties, n-3FA may be preferentially effective in preventing obesity-related breast cancer. In view of the hyperestrogenic and pro-inflammatory milieu present systematically and in the mammary glands of obese women, n-3FA may cooperate with weight loss induced by dietary energy restriction in reducing breast cancer risk in these subjects. Evidence-based combinatorial intervention trials targeting appropriately selected populations of women at risk are needed to establish the role of n-3FA in breast cancer prevention.

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3.1 Success and Challenges in Breast Cancer Prevention

Despite a decline in the incidence of breast cancer in the United States in recent years, breast cancer remains the most common cancer and the second most common cause of cancer-related mortality in American women [1]. In the United States, the age standardized rate per 100,000 subjects is in excess of 242.9 with regard to incidence and about 15 with regard to mortality [2]. Clearly, multiple factors contribute to breast cancer risk which includes hormones, particularly estrogen and progesterone [3, 4], genetics, lifestyle habits, and most likely, a multitude of environmental factors whose direct role in mammary carcinogenesis in humans still remains to be clearly defined [5]. Interference with these contributing factors to breast cancer risk in an effective and safe manner has been and continues to be exploited in the attempt to optimize prevention which represents the optimal method to reduce breast cancer morbidity and mortality. It is now well-established that inhibiting estrogen action or estrogen biosynthesis is not only effective in treating hormone-dependent breast cancer, but also in reducing its incidence [6–8]. The two selected estrogen receptor modulators, Tamoxifen and Raloxifene, have been shown to be effective chemopreventive agents by reducing the incidence of estrogen receptor positive breast cancer by 50 % and 38 %, respectively [6, 7]. However, they are not widely accepted even by women at high risk because of fear of toxicity particularly venous thromboembolism [9]. In addition, both agents are not effective in reducing the incidence of estrogen receptor negative tumors which are more aggressive and associated with shorter survival [6, 7]. The steroidal aromatase inhibitor examestane has been shown to reduce the incidence of invasive breast cancer by 65 % after a median follow-up period of 3 years [8]. Whether this drug will be more acceptable to the general public remains to be determined.

Lifestyle modifications have also been shown to be effective in reducing breast cancer risk. Such interventions are particularly attractive since they are not associated with toxic effects but rather with health promoting effects which go beyond just breast cancer prevention. The French E3N Prospective Cohort Study involving 64,732 women has shown that compliance with five modifiable lifestyle behaviors including abstinence from smoking, maintaining a normal BMI, consuming less than one alcoholic beverage per day, consuming more than five servings of fruit and vegetables per day, and maintaining a recreational physical activity level above 20 met/hour per week, reduces postmenopausal breast cancer incidence by 6.3 % (0.5–12.1 %) [10]. The Women's Health Initiative Observational Study involving 65,838 women showed that optimal adherence to American Cancer Society (ACS) cancer prevention guidelines regarding body weight, physical

activity, diet, and alcohol consumption was associated with a reduction in breast cancer incidence of 22 % and actually a reduction in breast cancer mortality of 33 % [11]. In a more limited trial including 2905 women from the high risk Breast Cancer Family Registry in New York, adherence to three ACS recommendations including at least 150 min of moderate intensity physical activity per week, alcohol intake of less than one drink per day, and maintaining a body mass index of <25, was associated with a 44 % lower incidence of breast cancer mortality in women unaffected by breast cancer at baseline and a 53 % reduction in women with breast cancer at baseline [12]. These associations remained significant after stratification by age, race, and BRCA status. Overall, these results support the conclusion that women at high risk, similar to women at average risk, may have substantial benefits from adhering to the lifestyle ACS guidelines.

Among the lifestyle habits, diet is probably a major determinant of breast cancer risk, although its specific role still remains somewhat elusive. Dietary habits have been shown to modify the personal risk of breast cancer, even among subjects at high risk such as carriers of BRCA-1 and/or BRCA-2 mutations [13, 14]. Also, diet is one of the major differences between industrialized and underdeveloped countries which differ significantly in breast cancer risk. Among the different components of the diet, the contribution to mammary carcinogenesis of polyunsaturated fatty acids has received considerable attention in the literature. Among the fatty acids, omega-3 fatty acids (n-3FA) and omega-6 fatty acids (n-6FA) have been postulated for a long time to decrease and increase breast cancer risk, respectively [15].

In this chapter, we will initially review the epidemiological data reporting on the possible association between n-3FA and breast cancer risk. We will then primarily focus on our research testing the tumor protective effects of n-3FA in preclinical models of mammary carcinogenesis. We will also summarize our data supporting the potential benefit of the combination of n-3FA and antiestrogens for inhibition of breast cancer development based on the complementarity of their mechanisms of action. As we will discuss in this chapter, we believe that the addition of n-3FA to antiestrogens will increase the spectrum of molecular subtypes of breast cancer which can be prevented in addition to the estrogen receptor positive tumors. Finally, we will discuss the possible preferential effects of n-3FA in reducing obesity-associated breast cancer risk.

3.2 Omega-3 Fatty Acids and Mammary Carcinogenesis

3.2.1 Bioavailability of n-3FA and n-6FA Through Dietary and Endogenous Sources

The 18-carbon, n-3FA alpha-linolenic acid (ALA) and the 18-carbon, n-6FA linoleic acid (LA) can only be derived by dietary sources since the human body totally lacks the enzymatic capacity to synthesize these two essential fatty acids. As can be seen

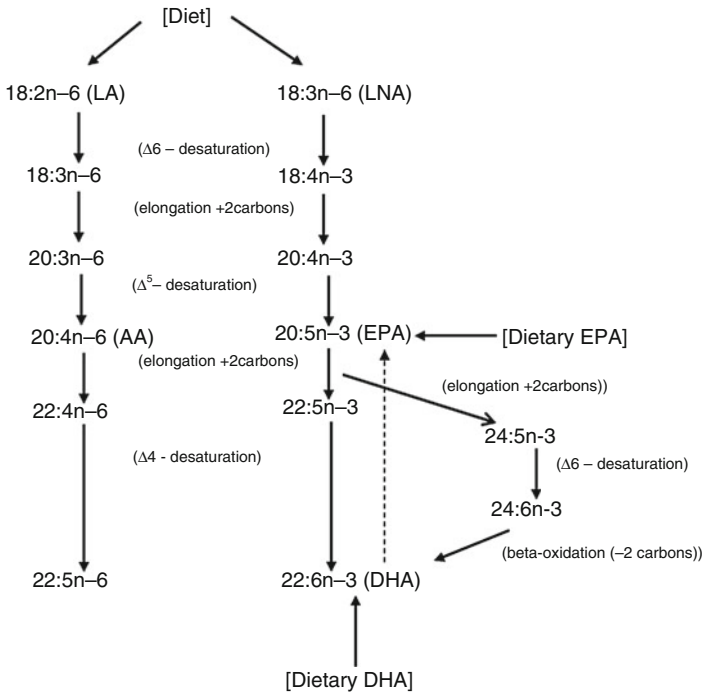

Fig. 3.1 Desaturation, elongation, and retroconversion of n-6FA and n-3FA

in Fig. 3.1, these two fatty acids compete for the same enzymes for desaturation and elongation to generate either n-6FA acids such as arachidonic acid (AA) or n-3FA such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Furthermore, AA and EPA and DHA are substrates for the same enzymes including cyclooxygenases, lipoxygenases, and CYP epoxygenases, to generate metabolic products which influence multiple cellular functions critically involved in the development of a variety of inflammatory and degenerative chronic diseases including cancer [16]. Importantly, while AA derived products such as PGE_2 are pro-inflammatory and tumor promoting, n-3FA derived metabolic products have opposite protective functions [16, 17]. Given the competition between n-3FA and n-6FA for the same biosynthetic and metabolic enzymes, a tumor protective effect might be expected by increasing the availability of n-3FA and/or reducing the amount of n-6FA. Evidence generated by two recent meta-analyses indeed suggests that both the n-3FA:n-6FA ratio as well as the absolute amount of n-3FA, may be important for tumor protection [18, 19]. Unfortunately, the bulk of polyunsaturated fatty acids consumed in the western diet are predominantly n-6FA. Of particular relevance to breast cancer is the observation that women have higher overall delta-6

desaturase activity than men resulting in higher levels of both AA and DHA in the plasma and adipose tissue [20]. Therefore, consumption of a large amount of LA may increase the risk of breast cancer in women by increasing the n-6FA:n-3FA ratio. Furthermore, their risk may be augmented by the high delta-6-desaturase activity in the mammary gland leading to formation of AA from LA [21]. In addition, AA has been shown to inhibit the formation of DHA from EPA which already proceeds with poor efficiency (<4 %) [22]. Therefore, due to the limitation in the production of DHA through the consumption of the precursors ALA or EPA, exogenous supplementation of DHA represents the best approach to provide an adequate tissue level of this n-3FA for a tumor protective effect.

3.2.2 Epidemiological Studies

Epidemiological studies have been inconclusive relative to the protective effect of n-3FA against breast cancer development. While some studies have shown an association between n-3FA intake and reduction in breast cancer risk, others have not shown this association and one has actually reported an increase in breast cancer risk with high n-3FA intake [23]. As recently discussed by Witte and Hartman in their review [24], multiple factors could contribute to the inconsistencies in the findings reported in the epidemiological studies. For instance, the association between omega-3 fatty acid intake and breast cancer risk can be altered by other dietary components as suggested in the French and E3N study population [25]. In addition, in dietary studies, fish and n-3FA consumption are often equated whereas fish vary considerably with regard to their content of n-3FA [26]. This point is illustrated by a recent meta-analysis of data from 21 independent prospective cohort studies which revealed that dietary intake of marine n-3FA was associated with a 14 % reduction in breast cancer risk [18]. Importantly, a dose-response effect was noted with a 5 % lower risk of breast cancer per 0.1 g/day increment of n-3FA intake [18]. In contrast, no association was detected between fish consumption and breast cancer risk [18]. In addition, based on a recent overview of consumption of dietary fats [27], it is possible that the lack of significant difference in breast cancer risk between “high” and “low” consumers may be due to the fact that the entire population effectively consumes inadequate n-3FA to modify breast cancer risk.

In summary, because of these multiple confounding variables, it is not surprising that epidemiological studies have failed to firmly establish whether there is a correlation between dietary n-3FA intake and breast cancer risk. In our opinion, the protective effect of n-3FA against breast cancer development in humans can only be tested by intervention trials in appropriately selected populations at high risk using a well-characterized n-3FA preparation based on solid preclinical data as well as using validated biomarkers of human breast cancer risk.

3.2.3 Preclinical Studies

In experiments conducted both in a pre-pubertal [28] and post-pubertal [29] model of MNU-induced rat mammary carcinogenesis, we observed that administration of fish oil providing ratios of n-3FA:n-6FA between 0.5 and 2.3 had a marginal antitumor action. In line with a lack of significant tumor protective effect, we observed that these ratios of n-3FA:n-6FA did not have major effects on systemic oxidative stress biomarkers based on oxidative damage to DNA measured as 8-hydroxy-2 deoxyguansine (8-OH-dG) and lipid peroxidation assessed by thiobarbituric acid reactive substances (TBARS) [30]. Tissue levels of 8-isoprostane, on the other hand, were markedly reduced in fish oil-fed rats, possibly as a result of fish oil-induced depletion of AA [30]. When similar clinically relevant ratios of n-3FA:n-6FA were tested in transgenic models of mammary carcinogenesis, we observed a protective effect in the HER2-neu model, a well-established model of estrogen receptor negative breast cancer (unpublished observations) in agreement with a previous report [31] but no protection in polyoma middle T transgenic mice [32]. These results suggest that gene-diet interactions play a critical role in the development of breast cancer.

These variable results prompted us to perform a critical review of the preclinical data on the role of n-3FA in mammary carcinogenesis. Our review of the literature covering over 30 years of investigation produced mixed results [23]. We found that the quality of the experiments varied so markedly that it was difficult to compare results across studies. For instance, we observed that a rigorous evaluation of the influence of the n-3FA:n-6FA ratio on mammary carcinogenesis had not been performed. Therefore, we decided to formulate a series of purified diets modeled after the AIN-93G formulation but with the major exception that the level of dietary fat was modified to reflect that currently recommended in the U.S. dietary guidelines. Thus, diets were formulated to provide 30 % of dietary calories from fat and an equal amount of these calories from saturated, monounsaturated, and polyunsaturated fatty acids. Within the polyunsaturated fatty acids, we sought to vary the ratio of n-3FA:n-6FA from 25:1 to 1:25 to provide a robust evaluation of the role of this ratio in affecting the post-initiation phase of chemically induced mammary carcinogenesis [33].

In these experiments, at 21 days of age female Sprague-Dawley rats were injected with 50 mg of N-methyl-N-nitrosurea/kg body weight intraperitoneally. Seven days following carcinogen injections, all rats were randomized to the different diets (n = 30 rats/group). In these experiments, we observed that a significant chemopreventive effect on MNU-induced mammary carcinogenesis was observed at calculated n-3FA:n-6FA dietary ratios of 10:1 and 25:1 which corresponded to experimentally verified ratios of 5:1 and 15:1, measured by gas chromatography equipped with flame ionization detection. This finding highlights the concern with much of the existing literature in which dietary fatty acid data are based on vendor provided information rather than analysis. The most striking antitumor effect of high n-3FA:n-6FA ratio was the reduction of cancer burden defined as average

cancer mass per rat expressed in grams. While the cancer burden in rats fed the referent 1:1 n-3FA:n-6FA ratio was 1.44 ± 0.39 g (mean ± SEM), the cancer burden in rats fed the 25:1 n-3FA:n-6FA ratio was only 0.29 ± 0.09 gm. In these experiments, we also tested whether the dietary n-3FA:n-6FA ratio would reduce mammary gland density in the rat and whether this change would be predictive of the anticarcinogenic effect. Breast density is a recognized independent risk factor for breast cancer in women and has been reported to be subject to modulation by lifestyle factors such as diet [34–36]. However, to our knowledge, there has not been any previous attempt to use mammary gland density as a screening tool in preclinical models for breast cancer. We, indeed, observed that increasing the levels of dietary n-3FA resulted in a progressive reduction of mammary gland density ($r = -0.477$, $p = 0.038$) which was predictive of the carcinogenic response (Fig. 3.2a, b). In addition, we observed a significant relationship between plasma IGF-1 concentration and mammary gland density ($r = 0.362$, $p < 0.005$) (Fig. 3.2c) which points to the importance of the IGF-I pathway in mediating the antitumor action of n-3FA as further explored in subsequent experiments (see below). Table 3.1 summarizes in detail the effects of the n-3FA:n-6FA ratio on mammary gland density and multiple plasma analytes in these experiments. In addition to the reduction in breast density and IGF-I levels, tumor protective ratios of n-3FA:n-6FA reduced plasma level of leptin and increased the level of adiponectin. These changes are consistent with an antitumor action. However, we found that in contrast to changes in plasma IGF-I level, neither cytokine was predictive of mammary gland density. As expected, increasing the n-3FA:n-6FA ratio in the diet resulted in an increase in the plasma n-3FA:n-6FA ratio caused by a rise in n-3FA along with a reduction in n-6FA (Table 3.1). However, the changes in plasma fatty acids plateaued at a calculated n-3FA:n-6FA ratio of 5:1 which was not tumor protective. Increasing the n-3FA:n-6FA dietary ratio to 10:1 and 25:1 inhibited tumor development but did not further modify plasma fatty acid profile. Interestingly, in a clinical dose-response study conducted in healthy women at high risk of breast cancer, Yee, et al. [37] observed that daily administration of increasing amounts of EPA and DHA for six months (ranging from 0.84 to 7.56 g/day) caused an increase in serum and breast adipose tissue omega-3 fatty acid content which plateaued at the dose of 2.52 g/day. We hypothesize that the tumor protective effect of the higher ratios may be due to increased production of protective n-3FA metabolites which is not reflected by the levels of the parent n-3FA.

Following the n-3FA:n-6FA ratio study described above, we performed an extensive analysis of the molecular signature underlying inhibition of mammary carcinoma by dietary n-3FA [38]. In these experiments, we analyzed tumors obtained from rats which were fed diets in which the ratio of n-3FA:n-6FA was either 0.7 (low n-3FA, control) or 14.6 (high, n-3FA). As shown in Table 3.2, we observed that cell proliferation assessed by Ki67 immunostaining was reduced by 60 % in carcinomas from the high n-3FA:n-6FA (14.6 ratio) treatment group and was associated with a reduction in the levels of cyclin D1 and phospho-Rb as well as an increase in the levels of two cyclin-dependent kinase inhibitors, p21 and p27 as determined by western blotting and densitometric analysis. These changes are consistent with a block at the G1/S transition induced by the high n-3FA diet. The apoptotic index

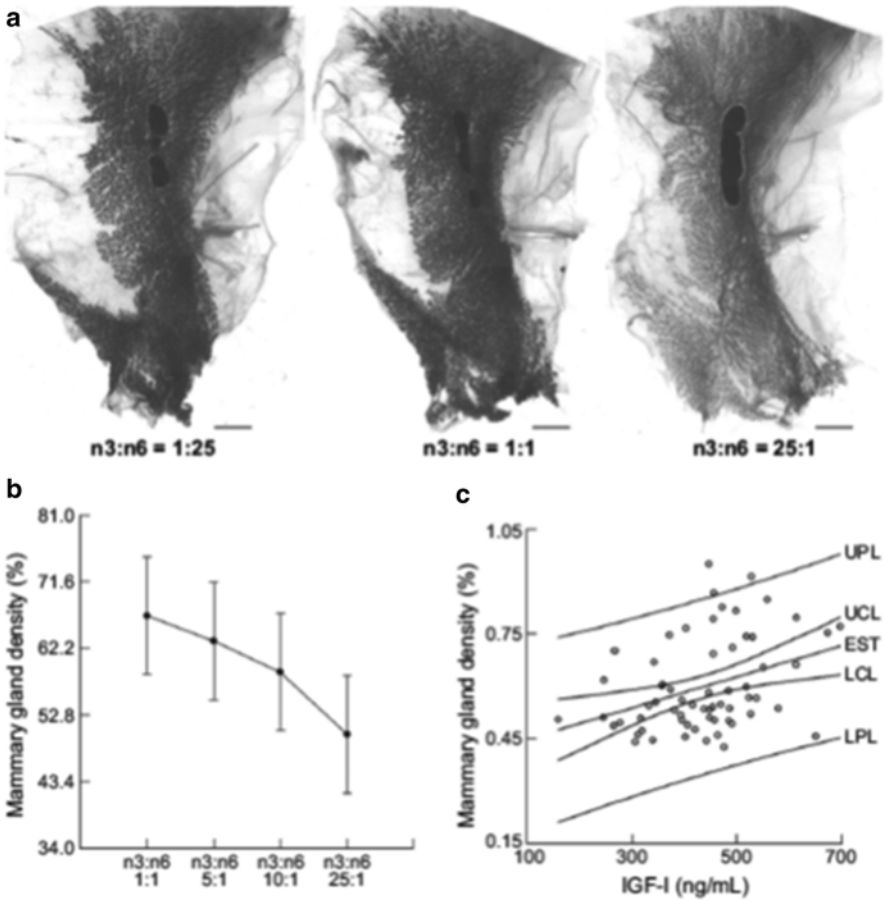

Fig. 3.2 (**a**) carmine-stained abdominal-inguinal mammary gland whole mounts depicting the effect of increasing dietary n-3FA:n-6FA on breast histology, bars = 0.5 cm. (**b**) Mammary gland density analysis shows a decreasing trend in density as n-3FA:n-6FA increases. The methodology for measurement of mammary gland density is described in detail in our publication [33]. Briefly, whole mounts of the abdominal-inguinal mammary gland chains were photographed and the images obtained were digitized. Digital images of the whole mounts were captured using a semiautomated image acquisition system. Images were evaluated for total area of the mammary fat pad occupied by mammary epithelium as well as total area of the fat pad encompassed by the mammary ductal tree. Area occupied by the mammary epithelium divided by the total area encompassed by the mammary ductal tree was calculated. (**c**) linear regression of mammary gland density and IGF-I upper prediction limit (UPL); upper confidence limit (UCL); estimate (EST); lower confidence limit (LCL); lower prediction limit (LPL). Reproduced with permission from [33]

(computed as the number of apoptotic cells divided by the total number of cells counted) was significantly increased by 29 % in carcinomas from the high n-3FA:n-6FA group (Table 3.2). Relative to apoptosis and consistent with the elevated apoptotic index observed in the high n-3FA diet group, the level of cleaved-PARP (PAR89/116 ratio) was elevated as were levels of Bax and Apaf-1, whereas the level

Table 3.1 Effect of n-3:n-6 dietary fatty acid ratio on mammary gland density and plasma analytes[a]

	n-3:n-6	n-3:n-6	n-3:n-6	n-3:n-6	n-3:n-6	n-3:n-6	n-3:n-6
Dietary treatment	25:1	10:1	5:1	1:1	1:5	1:10	1:25
Breast density (%)	50.1 ± 1.2^{b}	$58.9 \pm 3.5^{b,c}$	$63.7 \pm 5.3^{b,c}$	66.8 ± 5.0^{c}	60.4 ± 4.2^{c}	57.2 ± 4.2^{c}	62.6 ± 4.9^{c}
Leptin (pg/mL)	96.7 ± 27.9^{b}	118.5 ± 18.8^{b}	134.3 ± 16.8^{b}	$163.3 \pm 21.5^{b,c}$	$183.2 \pm 31.3^{b,c}$	264.6 ± 51.7^{c}	$152.3 \pm 20.5^{b,c}$
Adiponectin (μg/mL)	18.4 ± 0.6^{b}	17.5 ± 1.6^{b}	16.2 ± 1.1^{b}	$15.9 \pm 1.2^{b,c}$	$12.0 \pm 0.8^{c,d}$	$12.7 \pm 1.2^{c,d}$	10.9 ± 0.7^{d}
IGF-1 (ng/mL)	331.0 ± 34.7^{b}	$385.6 \pm 36.3^{b,c}$	481.0 ± 40.1^{c}	503.9 ± 31.9^{c}	481.9 ± 10.6^{c}	$432.9 \pm 33.5^{b,c}$	$443.5 \pm 30^{b,c}$
Linoleic acid (μmol/mL)	0.23 ± 0.07^{b}	0.30 ± 0.07^{b}	0.38 ± 0.08^{b}	0.69 ± 0.10^{c}	0.91 ± 0.17^{c}	0.93 ± 0.13^{c}	0.87 ± 0.21^{c}
Arachidonic acid (μmol/mL)	0.52 ± 0.10^{b}	0.50 ± 0.08^{b}	0.61 ± 0.16^{b}	0.66 ± 0.11^{b}	1.11 ± 0.38^{c}	1.34 ± 0.50^{c}	1.25 ± 0.45^{c}
Eicospentenoic acid (EPA; μmol/mL)	0.73 ± 0.17^{b}	$0.87 \pm 0.24^{b,c}$	0.99 ± 0.23^{c}	0.56 ± 0.13^{d}	$0.34 \pm 0.15^{b,e}$	0.18 ± 0.05^{e}	N.D.
Docosohexoenoic acid (DHA; μmol/mL)	$0.33 \pm 0.09^{b,d}$	0.44 ± 0.10^{c}	0.53 ± 0.13^{c}	$0.33 \pm 0.7^{b,d}$	$0.23 \pm 0.04^{d,e}$	$0.23 \pm 0.05^{d,e}$	0.19 ± 0.06^{e}
Total n-3 fatty acids (μmol/mL)	$1.14 \pm 0.25^{b,c}$	$1.39 \pm 0.33^{b,c}$	1.58 ± 0.35^{b}	0.95 ± 0.19^{c}	0.62 ± 0.16^{d}	0.47 ± 0.08^{d}	0.24 ± 0.07^{e}
Total n-6 fatty acids (μmol/mL)	0.80 ± 0.14^{b}	0.80 ± 0.14^{b}	$0.99 \pm 0.20^{b,c}$	1.35 ± 0.18^{b}	2.02 ± 0.42^{d}	2.27 ± 0.58^{d}	2.12 ± 0.64^{d}
n-3:n-6 ratio	1.44 ± 0.27^{b}	1.74 ± 0.29^{b}	1.61 ± 0.28^{b}	0.70 ± 0.10^{c}	0.31 ± 0.09^{d}	0.21 ± 0.03^{e}	0.11 ± 0.03

[a]Values are means ± SEM. Data were analyzed by ANOVA with post hoc comparisons by the method of Bonferroni. Values within a row with different superscripts (b, c, d, e) are statistically different from each other ($P < 0.05$). *N.D.* not detected

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Table 3.2 Effect of dietary n-3:n-6 ratio on cellular processes regulating cell proliferation and apoptosis

	Low	High	
Dietary n-3:n-6 ratio	0.6	14.6	P
Cell proliferation			
Ki-67 index (%)	34.9 ± 1.6	14.0 ± 0.9	<0.0001
Rb^{Ser780} ratio	0.41 ± 0.03	0.26 ± 0.02	<0.0001
Cyclin-D1	1302 ± 29	967 ± 29	<0.0001
p21	465 ± 31	664 ± 40	0.001
p27	326 ± 11	393 ± 10	<0.0001
Apoptosis			
Apoptotic index (%)	1.71 ± 0.05	3.92 ± 0.13	<0.0001
Bax	188 ± 8	242 ± 9	<0.0001
Bcl-2	589 ± 28	527 ± 26	0.117
Bax/Bcl-2	0.32 ± 0.01	0.47 ± 0.02	<0.0001
Apaf-1	392 ± 9	457 ± 17	0.005
PARP89	795 ± 26	577 ± 53	0.002
PARP116	547 ± 15	292 ± 28	<0.0001
PARP89/116 ratio	1.45 ± 0.02	1.99 ± 0.02	<0.0001

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Note: Values are means ± SEM (n = 11). Ki67 and apoptotic index were determined by immunohistochemistry whereas the remaining analysis was done by Western blotting quantitated by densitometry. The data were analyzed by Kruskal-Wallis rank test. Ratio is the ratio of phospho-protein (arbitrary units of optical density) to non-phospho-protein (arbitrary units of optical density)
Bax Bcl-associated X, *Bcl* B-cell leukemia oncogene

of Bcl2 was not significantly affected (Table 3.2). These changes are indicative of the induction of apoptosis via the intrinsic pathway. The data also show that the suppressive effect of n-3FA on proliferation was dominant over its effect on induction of apoptosis. Using western blotting followed by densitometry, we performed an extensive analysis of transcription factors, growth factor-related molecules and proteins involved in lipid metabolism in the attempt to identify the cellular mechanisms by which high n-3FA diet leads to inhibition of proliferation and induction of apoptosis. The results are reported in detail in Tables 3.3 and 3.4 and are summarized in Fig. 3.3. As described in detail in the figure legend, the predominant effect of high n-3FA diet was PPPARγ activation, resulting in suppression of lipogenesis primarily through downregulation of fatty acid synthase (FASN). In addition, the high n-3FA diet suppressed the mTOR pathway (well-established to be critical in carcinogenesis), both by suppressing IGF-I signaling and upregulating phospho-AMPK as a result of the reduction in leptin and the increase in adiponectin. Furthermore, the activation of phospho-AMPK also contributed to the inhibition of lipogenesis through its effect on key regulators of lipid synthesis (pACC, HMGCR, and SREBP1), thus potentiating the effect of PPARγ activation on this critical metabolic parameter. The fact that high ratios of n-3FA:n-6FA were required to achieve profound antitumor effects not only indicates that these biological activities are not likely to be achieved by dietary

Table 3.3 Effect of dietary n-3:n6 ratio on cellular processes regulating cell transcription factors and insulin signaling

Dietary n-3	Low	High	*P*
Transcription factors			
PPARα	84 ± 12	83 ± 14	0.980
PPARβ	446 ± 19	381 ± 15	0.015
PPARγ	1212 ± 42	1589 ± 62	<0.0001
GPR120	282 ± 4	333 ± 5	0.001
NF-kB $p65^{Ser536}$ ratio	5.2 ± 0.4	3.2 ± 0.3	0.001
$FOXO1^{Thr24}$ ratio	0.71 ± 0.07	0.38 ± 0.01	0.001
$FOXO3a^{Thr32}$ ratio	0.52 ± 0.03	0.35 ± 0.02	<0.0001
Hif-1α	799 ± 60	541 ± 88	0.026
SIRT-1	55 ± 7	43 ± 9	0.324
GADD153	71 ± 4	104 ± 5	<0.0001
Growth factor signaling			
IGF-1R	712 ± 56	561 ± 37	0.039
P13Kp110	106 ± 5	85 ± 4	0.005
$IRS1^{Ser636/639}$ ratio	0.59 ± 0.01	0.47 ± 0.02	<0.0001
$AMPK^{Thr172}$ ratio	0.031 ± 0.001	0.040 ± 0.001	<0.0001
Akt^{Ser473} ratio	0.41 ± 0.01	0.32 ± 0.01	<0.0001
$mTOR^{Ser2448}$ ratio	0.37 ± 0.01	0.31 ± 0.01	0.001
$Raptor^{Ser792}$ ratio	0.032 ± 0.003	0.180 ± 0.008	<0.0001
$PRAS40^{Thr426}$ ratio	1.38 ± 0.02	0.76 ± 0.04	<0.0001
$P70S6K^{Thr389}$ ratio	0.95 ± 0.03	0.74 ± 0.01	<0.0001
$4E\text{-}BP1^{Thr37/46}$ ratio	1.03 ± 0.03	0.76 ± 0.03	<0.0001

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Note: Values are means ± SEM (n = 11). Ratio is the ratio of phospho-protein (arbitrary units of optical density) to non-phospho-protein (arbitrary units of optical density). Ratio data were analyzed by the Kruskal-Wallis rank test
4E-BP1 eukaryotic translation initiation factor 4E-binding protein 1, *Akt* protein kinase B, *GADD153* growth arrest and DNA damage protein 153, *GPR120* G-protein-coupled receptor 120, *PRAS40* 40-kDa proline-rich protein, *P70S6K* 70-kDa ribosomal protein S6 kinase, *RAPTOR* regulatory associated protein of mTOR, *SIRT*-1 sirtuin 1

Table 3.4 Effect of dietary n-3:n6 ratio on proteins regulating lipid metabolism

Dietary n-3	Low	High	*P*
ACC^{Ser79} ratio	2.35 ± 0.13	3.12 ± 0.08	<0.0001
FASN	1714 ± 39	1372 ± 34	<0.0001
HMGCR	859 ± 25	746 ± 16	0.001
SREBP-1	385 ± 12	279 ± 5	<0.0001

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Values are means ± SEM ($n = 11$). Actin-normalized Western blot analysis data, which are semi-quantitative estimates of protein expression, and the ratio data were analyzed by Kruskal-Wallis rank test. Ratio is the ratio of phospho-protein (arbitrary units of optical density) to non-phospho-protein (arbitrary units of optical density)
ACC acetyl-CoA carboxylase, *HMGCR* 3-hydroxy-3-methyl-glutaryl-CoA reductase, *SREBP-1* sterol regulatory element-binding protein 1

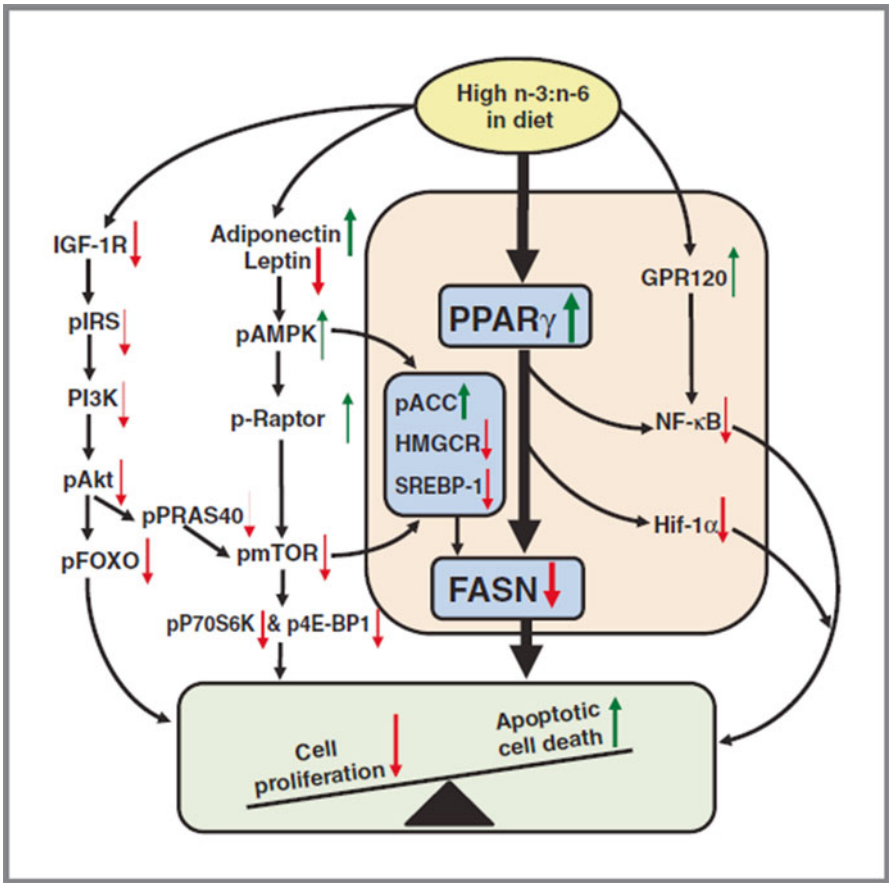

Fig. 3.3 Cellular processes regulating transcription factors, insulin signaling, and lipid synthesis that are likely to account for the effects on cell proliferation and apoptosis in mammary carcinomas of rats fed high versus low (control) dietary ratio of n-3:n-6 fatty acids. Diameter of *red* (decreased expression) and *green arrows* (increased expression) indicates magnitude of effect and font size of stated proteins indicates relative importance as determined by OPLS-DA. PPARγ and to a lesser extent, G-protein-coupled protein receptor 120 (GPR120) attenuate inflammation via direct or indirect effects on NF-kB and Hif-1a. PPARγ affects multiple targets in lipid metabolism including FASN. In addition, high dietary n-3:n-6 is accompanied by reduced activity of the mTOR as reflected in the reduced phosphorylation of its downstream targets including 70-kDa ribosomal protein S6 kinase (P70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), which in turn, exert effects on cell proliferation and cell survival. Mechanisms by which mTOR activity is downregulated include (1) downregulation of IGF-1R, phosphorylated insulin receptor substrate 1 (pIRS1), phosphoinositide 3-kinase (PI3K), phosphorylated Akt, phosphorylated Forkhead box O, and phosphorylated 40-kDa proline-rich protein (PRAS40) and (2) upregulation of pAMPK by increased adiponectin and decreased leptin, phosphorylated acetyl-CoA carboxylase (ACC), and phosphorylated regulatory-associated protein of mTOR (RAPTOR). Decreased phosphorylated mTOR and increased pAMPK further attenuate fatty acids synthesis via reduction of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) and of sterol regulatory element-binding protein-1 (SREBP-1) that results in decrease of FASN. The overall consequence of these changes in cell signaling is a decrease in cell proliferation and an increase in cell death by apoptosis. Reproduced with permission from [38]

consumption of fish oil but also that there are specific metabolites of n-3FA accounting for these effects which are likely to be endogenously synthesized. As mentioned above, the identification of these metabolites is currently under active investigation in our laboratories.

3.2.4 Omega-3 Fatty Acids and Breast Cancer Molecular Subtypes

A novel aspect of the data analysis used in the signaling studies reported above was the application of supervised and unsupervised clustering techniques in an effort to minimize interpretation bias and to characterize the heterogeneity of responses observed both within and between groups. This analysis indicated considerable heterogeneity in the nature of carcinoma responsiveness to n-3FA indicating a need for assessing how various molecular subtypes of breast cancer respond to the administration of n-3FA in the diet. We started addressing this issue focusing on DHA since evidence in the literature indicates that DHA is superior to EPA and the combination of EPA + DHA in suppressing mammary carcinogenesis [39, 40]. The anti-proliferative effects of DHA and its metabolite 4-OH-DHA as well as its putative metabolite 4-OXO-DHA were tested in five triple negative human breast cancer cell lines at different stages of transformation (MCF-10F, trMCF, bsMCF, MDA-MB-231, and BT-549) and three luminal breast cancer cell lines (MCF-7, T47D, and SK-BR-3) [41]. We observed that DHA and its oxidized derivatives significantly inhibited cell proliferation (20–90 % reduction) of both basal and luminal breast cancer cell lines. The inhibitory effect was more pronounced on triple negative breast cancer cell lines as compared to luminal breast cancer cell lines after 4-OXO-DHA treatment [41]. These preliminary results offer some promise that n-3FA may be helpful in preventing the development of estrogen receptor negative tumors for which currently there is no effective chemopreventive strategy available.

3.3 Combination of n-3FA and Antiestrogens

3.3.1 Rationale

A major focus of research in our laboratories has been to test the anti-tumor efficacy and safety of the combination of n-3FA and antiestrogens for breast cancer prevention. The rationale behind this approach is based on the multiplicity of the signaling pathways affected by n-3FA, several of which are well known to interact with the estrogen receptor pathway. For instance, it has been increasingly recognized that activation of the PI3 kinase and mTOR pathway contribute to the development of antiestrogens resistance [42]. Preclinical studies have shown that combined

treatment with antiestrogens and inhibitors of these kinases is effective in antiestrogens resistant cells and prevents the emergence of antiestrogens resistance in antiestrogens sensitive tumors [42]. In addition, clinical data suggest that the addition of the mTOR inhibitor everolimus to antiestrogens, is beneficial in patients with advanced/metastatic hormone-receptor positive tumors that acquire antiestrogens resistance [43]. Therefore, it is reasonable to expect that n-3FA which are potent inhibitors of the PI3 kinase mTOR pathway will potentiate the tumor protective effect of antiestrogens. In addition, there is a well-documented crosstalk between the estrogen receptor and the PPARγ receptor [44, 45], the latter being a major mediator of n-3FA effect in breast cancer cells [46]. There is experimental evidence that inhibition of estrogen receptors with antiestrogens and activation of PPARγ synergistically downregulates the PI3 kinase/AKT pathway and inhibits breast cancer cell proliferation [44]. In addition, because of the complementarity of their antitumor action and the well-established antiproliferative effects of n-3FA in estrogen receptor negative breast cancer cell lines demonstrated by us [41] and other investigators [31, 47], we believe that the chemopreventive effect of the combination of n-3FA and antiestrogens will not be restricted to ER positive tumors but will extend to ER negative tumors which are more aggressive and associated with shorter survival.

3.3.2 Antitumor Effects

In experiments conducted in a prepubertal model of MNU-induced rat mammary carcinogenesis, we demonstrated for the first time that the combination of a fish oil rich diet (calculated n-3FA:n-6FA ratio = 2.3) and Tamoxifen inhibited tumor incidence (Fig. 3.4), multiplicity and volume (Table 3.5) to a greater extent than the individual interventions [28]. The potential superiority of the combination was particularly evident at a suboptimal dose of Tamoxifen 50 μg/kg which, by itself, was unable to significantly decrease tumor development. Following these observations, we became interested in investigating which stages of mammary carcinogenesis are inhibited by n-3FA and Tamoxifen. To address this issue, we felt that the prepubertal model used in our previous experiments was not ideal because of the confounding influence of the concomitant physiologic changes of the mammary gland associated with puberty. Therefore, in those studies, we could not clearly determine which stages of breast cancer development were inhibited by the combination of n-3FA and Tamoxifen. Therefore, subsequent experiments were conducted in a postpubertal model of MNU-induced mammary carcinogenesis where the carcinogen was administered at day 50 of age (as opposed to day 21) when sexual maturation is complete [29]. In these experiments, following MNU administration, groups of female Sprague-Dawley rats were randomized to either a control diet (20 % corn oil [CO]) or a fish oil (FO) rich diet (10 % FO + 10 % CO) with or without the addition of Tamoxifen in the diet (0.6 ppm). Separate groups of rats were sacrificed at weeks 4 (before palpable tumors), 8 and 12 (when approximately 90 % of control rats had palpable tumors). In addition to removing palpable tumors, abdominal inguinal mammary fat pads were excised for full

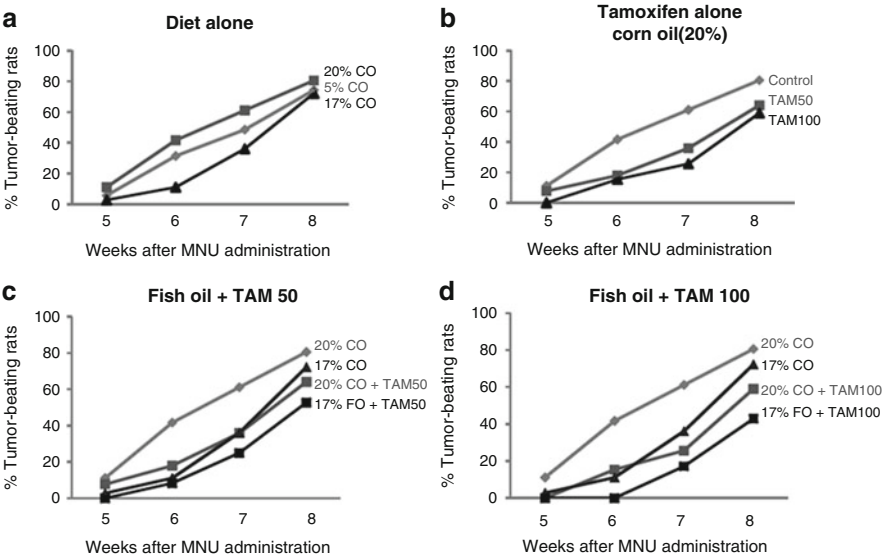

Fig. 3.4 Effects of dietary interventions and tamoxifen administration on mammary carcinogenesis. (**a**–**d**), groups of 21-day-old MNU-injected rats (n = 35–39 per group) were randomly assigned to the indicated experimental interventions *CO* corn oil, *FO* fish oil; *TAM50* 50 μg/kg tamoxifen, s.c., 5 days/week; *TAM100* 100 μg/kg tamoxifen, s.c., 5 days/week. Reproduced with permission from [28]

histologic analysis of preneoplastic lesions classified as mild hyperplasia, modest hyperplasia, and florid hyperplasia as well as ductal carcinoma *in situ* and invasive adenocarcinomas. As can be seen in Table 3.6, the mammary fat pads of control rats fed 20 % CO exhibited the expected progression of mammary carcinogenesis over time following MNU injection. Our data show a major highly statistically significant effect of Tamoxifen in reducing all histological parameters of mammary carcinogenesis. While the FO-rich diet had marginal effects on its own, it potentiated the protective effect of Tamoxifen. In particular, the combination treatment reduced lesion incidence by 50 % and multiplicity by 54 % at week 12 compared to week 8. In contrast, none of the other groups exhibited a reduction in either parameter over this time frame. This finding suggests that the combination treatment not only prevented carcinogenesis but also induced regression of established preneoplastic lesions. This effect is of particular translational relevance since it is likely that when a chemopreventive intervention is applied to women, preneoplastic lesions are already present. In these experiments, we also demonstrated the greater biological relevance of tissue level of n-3FA over plasma levels when with regard to tumor protective effects of n-3FA in experimental models of mammary carcinogenesis. In our experiments, we observed parallel consistent increases over time in plasma and mammary gland n-3FA levels in the groups of rats fed the 10 % FO rich diet. However, when we correlated plasma and mammary fat pad fatty acid content on an individual per rat basis, such correlation was either weak or absent (Fig. 3.5). This finding indicates that caution should be used when attempting to define the

Table 3.5 Treatment effects on tumor multiplicity and volume

Experimental groups	No. of rats	No. of tumors/rats (mean ± SE)				Tumor volume/rat (mm^3; mean ± SE)
		Week 5	Week 6	Week 7	Week 8	Week 8
1, 5 % CO	35	0.06 ± 0.04	0.37 ± 0.1	0.63 ± 0.13	1.60 ± 0.25	2163 ± 570
2, 20 % CO	36	0.11 ± 0.05	0.47 ± 0.1	0.83 ± 0.15	1.78 ± 0.23	2529 ± 627
3, 20 % CO + 50 μg/kg tamoxifen	39	0.08 ± 0.04	0.18 ± 0.06	0.41 ± 0.09	1.23 ± 0.21	992 ± 336
4, 20 % CO + 100 μg/kg tamoxifen	39	0	0.15 ± 0.06*	0.31 ± 0.09*	0.97 ± 0.18*	1183 ± 633
5, 17 % FO	36	0.03 ± 0.03	0.19 ± 0.1	0.69 ± 0.23	1.61 ± 0.03	1937 ± 700
6, 17 % FO + 50 μg/kg tamoxifen	36	0	0.08 ± 0.05+	0.31 ± 0.10*	0.86 ± 0.17*	572 ± 262
7, 17 % FO + 100 μg/kg tamoxifen	35	0	0‡	0.23 + 0.09†	0.60 ± 0.17‡	276 ± 145*

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Note: *P* values are from statistical comparisons to control (20 % corn oil)
CO corn oil, *FO* fish oil
*P < 0.05, †P < 0.01, ‡P < 0.001

Table 3.6 Individual and combined time dependent effects of FO and Tam on histologic parameters of MNU-induced mammary carcinogenesis[a]

		Experimental groups				P-values[b]	
		20 % CO	Tam 0.6 ppm	10 % FO	Tam 0.6 ppm + 10 % FO	Tam effect	FO effect
Lesion incidence							
4 week		0.90	0.90	0.80	0.90		
8 week		0.90	0.70	0.90	1.00		
12 week		0.89	0.76	0.89	0.50	<0.005	0.18
Lesion multiplicity (mean ± SE)							
4 week		1.30 ± 0.26	1.70 ± 0.30	1.40 ± 0.34	1.60 ± 0.3		
8 week		1.70 ± 0.45	1.00 ± 0.30	3.00 ± 0.58	1.40 ± 0.22		
12 week		3.60 ± 0.62	1.35 ± 0.27[3]	3.22 ± 0.46	0.61 ± 0.18[3]	<0.0001	0.11
Most severe lesion grade							
4 week	No lesion (%)	10	10	20	10		
	Grade 1 (%)	30	30	10	40		
	Grade 2 (%)	60	50	50	50		
	Grade 3 (%)	0	10	20	0		
	Grade 4 (%)	0	0	0	0		
	Grade 5 (%)	0	0	0	0		
8 week	No lesion (%)	10	30	0	10		
	Grade 1 (%)	20	30	20	50		
	Grade 2 (%)	0	30	0	20		
	Grade 3 (%)	0	0	10	10		
	Grade 4 (%)	10	0	0	0		
	Grade 5 (%)	60	10	70	10		

Table 3.6 (continued)

		Experimental groups				P-values[b]	
		20 % CO	Tam 0.6 ppm	10 % FO	Tam 0.6 ppm + 10 % FO	Tam effect	FO effect
12 week	No lesion (%)	11	23	11	50		
	Grade 1 (%)	0	12	6	17		
	Grade 2 (%)	0	6	6	5		
	Grade 3 (%)	0	0	6	0		
	Grade 4 (%)	0	6	0	0		
	Grade 5 (%)	89	53	72	28[3]	<0.001	<0.05
Composite lesion score (mean ± SE)							
4 week		1.90 ± 0.38	2.50 ± 0.45	2.30 ± 0.50	2.10 ± 0.38		
8 week		6.80 ± 2.32	1.70 ± 0.65	9.90 ± 2.69	2.50 ± 0.81		
12 week		15.6 ± 2.81	4.94 ± 1.11[4]	12.6 ± 2.29	2.00 ± 0.71[3]	<0.0001	0.071

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1. Following ip administration of MNU at day 50 of age, separate groups of rats within each experimental group were sacrificed at week 4 (n = 10), week 8 (n = 10), and week 12 (n = 18). One inguinal MFP per rat was excised and processed for histologic analysis. All lesions were histologically categorized as mild hyperplasia, moderate hyperplasia, florid hyperplasia, DCIS, and invasive adenocarcinomas and assigned a grade of 1–5 in a crescendo order of severity. MFPs containing normal breast tissue were assigned a grade of 0. For each rat, a composite lesion score was calculated by adding the products of the number of lesions in each histologic category by their corresponding assigned grade
2. These p values reflect the significance of the main effects of Tam (in the presence of 20 % CO and 10 % FO diet) and FO (in the presence and in the absence of Tam). Statistical comparisons between individual groups (after adjusting for multiple testing) are reported below
3. $p < 0.01$, vs. 20 % CO
4. $p < 0.05$, vs. 20 % CO

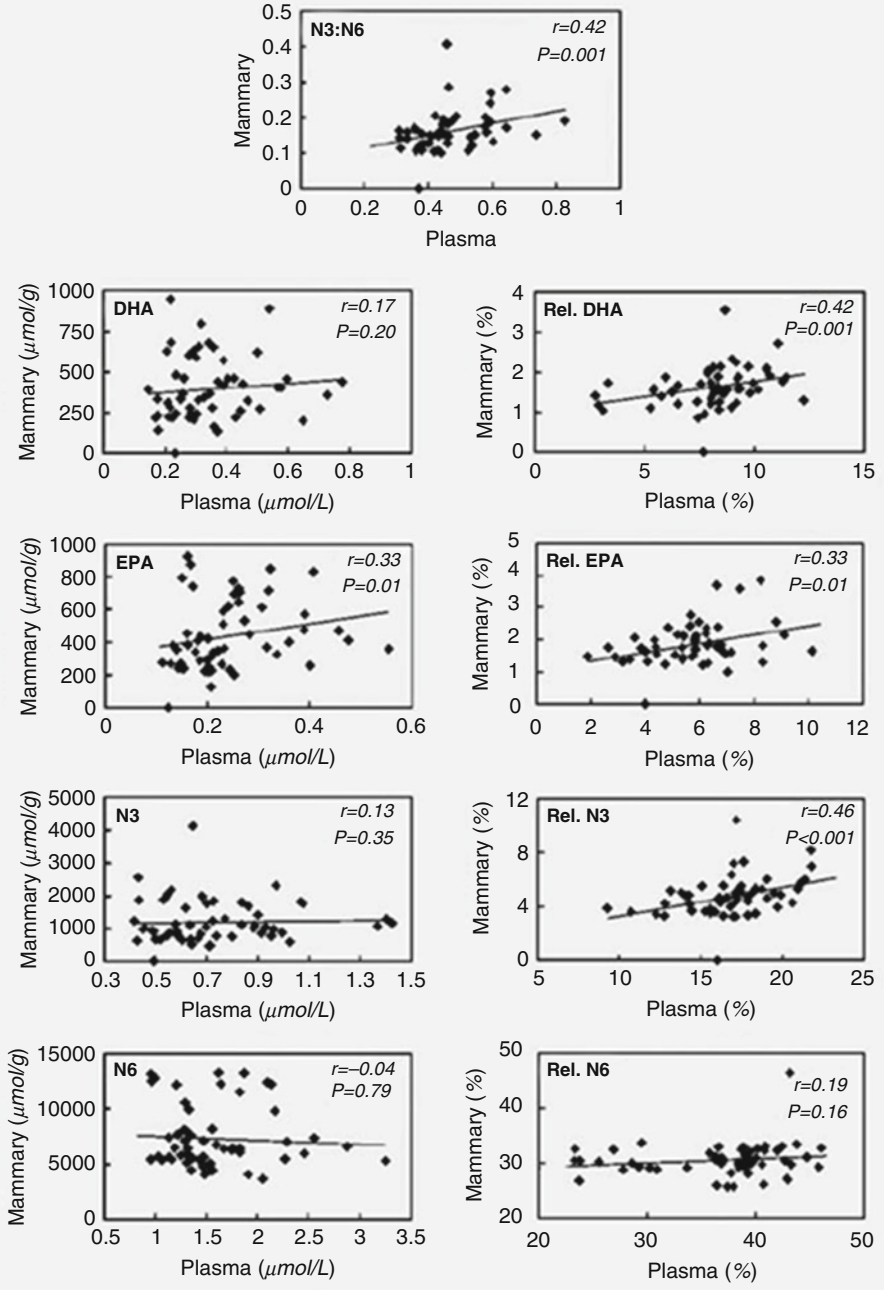

Fig. 3.5 Correlation between plasma and MFP levels of FA on an individual per rat basis. Only rats fed 10 % FO diet with and without Tamoxifen are included in this analysis. The data from the three time points are combined (n = 60 for each correlation)

role of n-3FA in a biological effect based upon plasma n-3FA level. We, indeed, observed that tissue (Table 3.7) but not plasma levels (data not shown) of n-3FA correlated with the expression of several possible biomarkers of carcinogenesis in the mammary gland in a way that was generally consistent with a protective effect.

3.3.3 Genomic Effects

To gain insight into the potential mechanisms underlying the superior chemopreventive efficacy of the combination, we have performed transcriptomic analysis (microarray followed by real time PCR validation of select genes of interest) in the tumors of control rats and Tamoxifen-treated rats each fed either a corn oil or fish oil rich diet [48]. We used gene ontology analysis and analysis of the relation of each differentially expressed gene with cancer-related processes. We identified alterations in genes directly related to the biologic features of breast cancer (such as tumor differentiation and progression) as well as genes related to the immune response. Gene ontology enrichment analysis showed that administration of a fish oil rich diet resulted in the differential expression of several genes that promote a more efficient immune response against tumor development (Fig. 3.6). In addition, tumors of fish oil fed animals that received Tamoxifen showed decreased mRNA for genes directly related to tumor growth and metastasis (Fig. 3.7), thus indicating that Tamoxifen treatment was more efficient in a fish oil rather than corn oil diet background. On the other hand, we observed that the expression of genes associated with immunity in animals in the fish oil + Tamoxifen group indicated a shift to the Th2 pattern of immune response which may favor tumor escape (Fig. 3.6). In conclusion, a FO rich diet resulted in the differential expression of several mRNAs that encode genes suggestive of more differentiated tumors and a more efficient immune response against tumorigenesis compared to a CO rich diet. While genes related to tumor growth and metastasis were downregulated by Tamoxifen in FO fed rats, our data also point to a potential immunologic mechanism of tumor escape from the combined intervention.

3.3.4 Proteomic Effects

We have also used a proteomic approach to gain insights into the mechanism of protection at the protein level by n-3FA in the absence and in the presence of Tamoxifen [49]. Using the isobaric tags for relative and absolute quantitation (iTRAQ) followed by confirmation by western blots, we found that increasing ratios of n-3FA:n-6FA in the diet induced dose-dependent changes in the plasma level of several proteins in a manner consistent with chemoprevention. Those included an increase in gelsolin and vitamin D binding protein, both shown to have tumor protective properties [50, 51]. A high ratio of n-3FA:n-6FA also increased the expression of 14-3-3 sigma, a well-known tumor suppressor gene [52]. In contrast,

Table 3.7 Correlation between FA levels and biomarker expression by mammary gland lesions [a]

	Fatty acids[b]															
	Absolute amount									Relative concentrations						
Biomarkers	ALA	AA	EPA	DPA	DHA	N3	N6	N3:N6	Total	ALA	AA	EPA	DPA	DHA	N3	N6
p-ERK	**−0.47**	−0.39	−0.12	−0.21	−0.32	−0.27	−0.09	−0.28	−0.22	**−0.57**	**−0.47**	−0.12	−0.26	−0.30	−0.20	0.16
p-AKT	−0.22	0.05	−0.11	−0.32	**−0.46**	−0.32	0.15	**−0.46**	−0.07	−0.36	0.05	−0.08	−0.29	**−0.45**	−0.31	**0.57**
p-mTOR	0.07	0.32	0.13	0.08	0.19	0.16	0.11	0.04	0.15	−0.15	0.32	0.01	−0.05	0.12	0.00	−0.15
p-S6	−0.27	−0.15	−0.05	−0.20	−0.28	−0.20	0.14	−0.36	−0.10	**−0.50**	−0.26	−0.07	−0.33	−0.35	−0.18	**0.50**
p-nuclear NFkB	−0.29	−0.32	**−0.52**	**−0.55**	**−0.50**	**−0.50**	**−0.57**	−0.27	**−0.61**	−0.09	−0.04	−0.25	−0.30	−0.23	−0.24	0.13
Ki-67	**−0.44**	−0.38	−0.27	−0.37	**−0.40**	−0.38	−0.04	**−0.42**	−0.13	**−0.55**	−0.34	−0.21	−0.34	**−0.44**	−0.35	−0.36
CC3	−0.33	−0.17	−0.28	−0.36	−0.33	−0.33	0005	**−0.43**	−0.15	−0.34	−0.32	−0.25	−0.25	−0.33	−0.27	**0.46**

[a]Tissue levels (absolute and relative amounts) of the indicated FA was correlated with the expressions of the indicated biomarkers in the contralateral mammary gland of the same rat as determined by immunohistochemistry

[b]Values are correlation coefficients (r) n = 26–33; values in **bold** are statistically significant ($p<0.05$)

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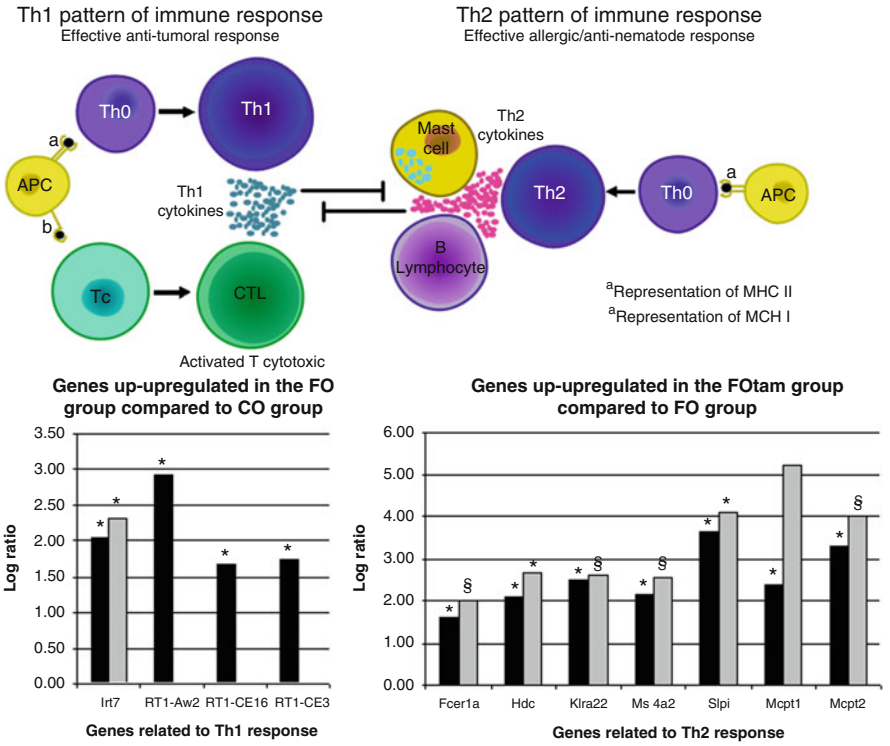

Fig. 3.6 Diagram depicting the patterns of immune responses found represented among the genes up-regulated by fish oil (FO) and tamoxifen in a FO rich diet (FOtam). Graphs show side-by-side log2 values of gene expression in microarray (*black bars*) and real time PCR (*grey bars*) of genes related to the immune response. *$p<0.05$; §$0.05<p<0.20$, with fold change >3.0 (log2 > 1.58). Figure adapted from [48]

alpha-1β-glycoprotein, shown to be increased in a variety of cancers [53–55] was reduced by a high n-3FA diet. We also observed that the combined administration of Tamoxifen with a high ratio of n-3FA:n-6FA altered additional proteins also in a manner consistent with chemoprevention (Fig. 3.8) [49]. These changes included a reduction in apolipoprotein E, haptoglobin, and inter-alpha inhibitor H4 heavy chain, all shown to have tumor promoting properties [56–58]. Measurement of these differentially regulated proteins could be useful for monitoring the efficacy of n-3FA and Tamoxifen as chemopreventive agents in clinical trials.

3.4 Omega-3 Fatty Acids and Obesity-Associated Risk of Breast Cancer

There is strong evidence that obesity is linked to risk of postmenopausal breast cancer [59]. There are multiple mechanisms by which obesity predisposes to breast cancer such as altering production and bioavailability of critical mitogens such as

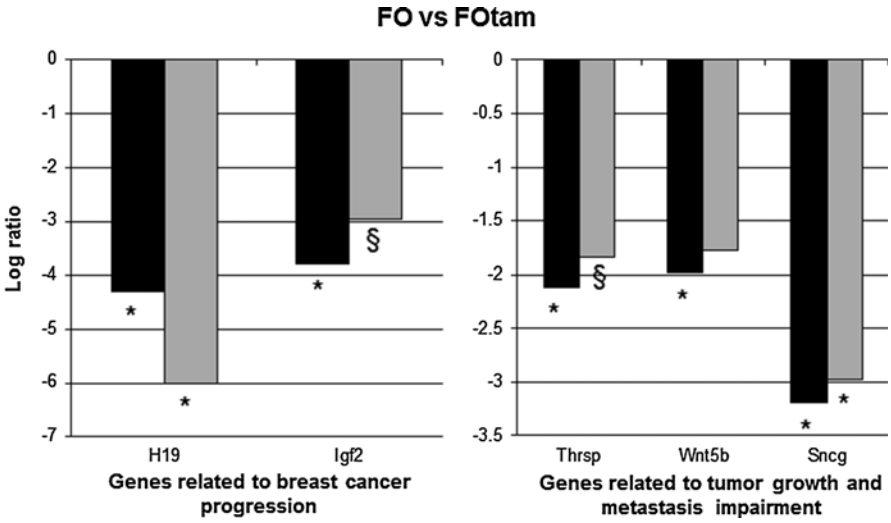

Fig. 3.7 Side-by-side log2 values of gene expression in microarray (*black bars*) and real time PCR (*grey bars*) of genes related to tumor profile. *$p < 0.05$; §$0.05 < p < 0.20$, with fold change >3.0 (log2 > 1.58). Figure adapted from [48]

estradiol [60] and IGF-I [61]. Insulin resistance associated with obesity can also contribute to mammary carcinogenesis as a result of the high circulating level of insulin acting as a growth factor [62]. Recent interest has focused on the role of adipokines, primarily leptin [63] and adiponectin [64] and inflammatory markers [65]. Leptin synthesis and plasma levels increase with obesity and recent work has shown that higher leptin levels were significantly associated with an increase in breast cancer [66]. In contrast, adiponectin levels in the serum decrease with increased obesity and three epidemiological studies have shown an inverse association between serum adiponectin levels and breast cancer risk [67].

In addition to the role of obesity in altering adipokines, steroid hormones, and growth factors, obesity has also been shown to create a pro-inflammatory milieu systemically in the visceral and subcutaneous fat [68, 69] and locally in the breast [70]. In breast tissue saturated fatty acids released from necrosed adipocytes cause increased production of inflammatory cytokines such as TNF-α, IL-1β and PGE_2 by macrophages in response to NFkB activation. These cytokines in turn increase aromatase activity in neighboring adipocytes, thus inducing a local hyperestrogenic milieu [68, 70, 71]. Obesity may also alter fatty acid (FA) metabolism in a way which may favor tumor development and progression [72]. It has been shown to be associated with increased adipose soluble epoxyhydrolase (sEH) which would be expected to result in hydrolysis and thus inactivation of tumor protective DHA metabolites produced by the cytochrome P450 (CYP) epoxygenase pathway.

We believe that omega-3 fatty acids may be particularly effective in reducing obesity associated breast cancer risk and that their tumor protective effects may be potentiated by weight reduction induced by dietary energy restriction.

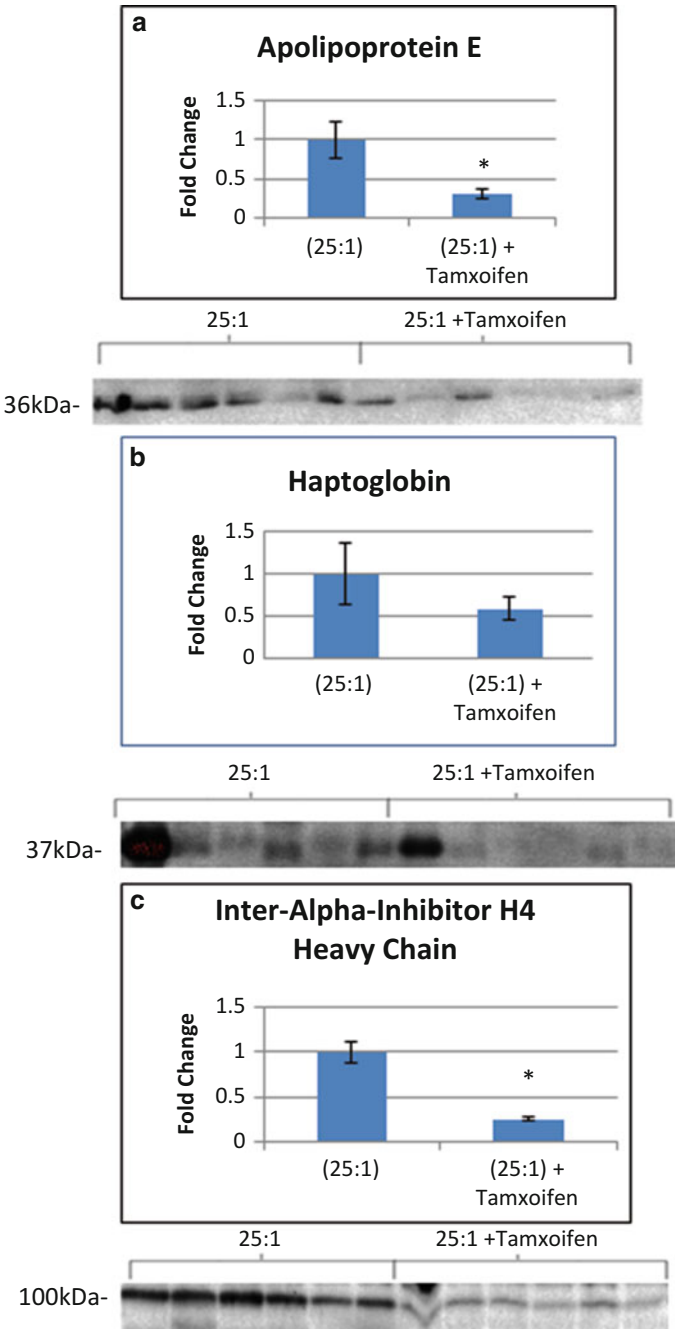

Fig. 3.8 Western blot analysis of specific proteins for validation of iTRAQ analysis (comparison of 25:1 n-3:n-6 with 25:1 n-3:n-6 plus Tamoxifen). (**a**), lipoprotein E expression, (**b**) haptoglobin expression, (**c**) inter-α-inhibitor H4 heavy chain expression; $^*P \leq 0.05$. Reproduced with permission from [49]

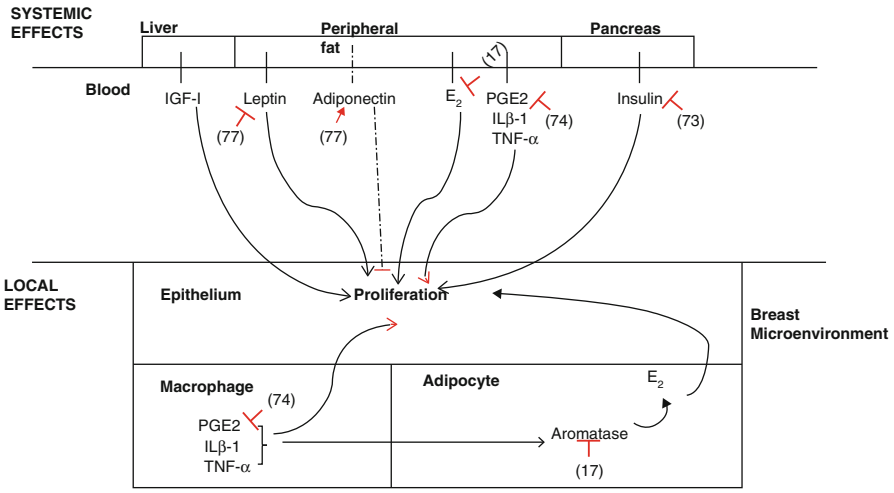

Fig. 3.9 Systemic and local mechanisms postulated to be involved in obesity-related breast cancer development which may be reversed by calorie restriction. n-3FA may potentiate several of the chemopreventive effects of calorie restriction as indicated by the *icons* inserted in the figure (supporting references indicated in *parenthesis*)

The experimental and clinical evidence supporting our hypothesis is schematically summarized in Fig. 3.9.

Preclinical studies have indicated that n-3FA ameliorate obesity-linked inflammation and insulin resistance [73, 74]. A link has recently been reported between a higher intake of n-3FA and decreased inflammation among breast cancer survivors [75]. Most relevant is the recent report that dietary n-3FA and mild DER in obese mice synergistically reduce the degree of inflammation of the white adipose tissue by synergistic induction of mitochondrial oxidative capacity, lipid catabolism, and specific anti-inflammatory lipid mediators [76]. In addition, fish oil rich in n-3FA has been shown to increase plasma level of adiponectin in rodents and in human subjects and to decrease plasma leptin concentrations [77]. Finally, it has been suggested that a high intake of n-3FA relative to that of n-6FA may decrease endogenous estrogen production via inhibition of aromatase activity/expression [17]. Clearly the above findings suggest that n-3FA may be particularly effective in reducing breast cancer risk in overweight and obese women. The possible preferential protective effect of n-3FA in obese subjects has also been suggested by a recently published epidemiologic study [78]. These data also provide a strong rationale for testing the combination of DER and n-3FA in reversing the proinflammatory and procarcinogenic milieu induced by obesity. Furthermore, the rationale for combining DER specifically with DHA is strengthened by recent finding that obesity may diminish the antitumor effect of DHA against breast cancer by increasing soluble epoxyhydrolase (sEH) which catabolizes tumor protective CYP-derived oxylipins (epoxides) to inactive compounds (vicinal diols) [72].

Therefore, we anticipate that DER will potentiate the antitumor action of DHA by reversing the effects of obesity on sEH and thus restoring the levels of CYP-derived oxylipins which have been shown to have major antitumor effects [79].

3.5 Future Directions

Considerable evidence primarily derived from preclinical studies supports a protective effect of n-3FA against breast cancer as summarized in this chapter as well as other recently published reviews [24]. Data in humans are also in general supportive. However, significant inconsistencies and gaps in knowledge still remain which prevents us from providing specific evidence-based guidelines on the use of n-3FA for breast cancer prevention in women. As it is the case for other prevention and treatment strategies, it is likely that not all women will be equally responsive but specific subsets may benefit more than others from the tumor protective effect of n-3FA. Future research should be directed at identifying these target populations so that a personalized approach can be developed. Evidence in the literature is emerging which can help us in this task. As we review in this chapter, we believe that obese women may preferentially benefit from n-3FA because they frequently (but not always) harbor a pro-inflammatory and pro-tumorigenic milieu which can be ameliorated by n-3FA. However, a metabolically unhealthy state does not necessarily equate with obesity, as a metabolically healthy obese phenotype has been identified at least with regard to cardiovascular disease risk [80–82]. A recent provocative paper has shown that insulin resistance which may be present in normal weight individuals may be more biologically relevant and more useful for breast cancer risk stratification than adiposity [83]. Epidemiologic studies have frequently found an association between fasting insulin levels and breast cancer incidence [84, 85]. A recent study has also reported an association between serum insulin and the risk of benign proliferative breast disease which confers an increased risk of breast cancer [86]. Our experimental data indicate that the IGF-I pathway (activated by high insulin levels) is downregulated by n-3FA [38]. Therefore, the presence of insulin resistance may be superior to obesity *per se* as a selection criteria for testing the preventive effect of n-3FA against breast cancer in future clinical trials.

We believe that future research should also be aimed at identifying the specific compounds present in fish oil which account for the tumor protective effect. The composition of fish oil is very heterogeneous and highly variable from lot to lot. Therefore, research using fish oil is likely to continue giving inconsistent results since the biological actions of the n-3FAs present in fish oil are likely to be different and probably tumor-specific. Based on our experience and other reports in the literature [39, 40], we believe that DHA is the most active n-3FA against breast cancer and should be preferentially tested in future trials. We also believe that attention should be focused on metabolism of DHA as metabolites of DHA derived from the LOX and CYP pathways may play a major role in the antitumor action of DHA [41, 79]. Furthermore, the activity of the enzymes involved in the synthesis

and catabolism of the DHA-derived metabolites is likely to be affected by genetic [87] and environmental factors [72]. Further information in this regard will help us to optimize subject selection based on genetic polymorphism and lifestyle modification which would augment the protective effect of DHA.

Finally, we believe that n-3FA and DHA in particular can best be exploited for breast cancer prevention in conjunction with other safe interventions with complementary mechanism of action. Our preclinical data suggest to combine n-3FA with antiestrogens, particularly if the latter can be used at less than conventional doses which may be less toxic without losing anti-tumor efficacy. Evidence reviewed in this chapter also provides the rationale for combining DHA with weight loss induced by dietary energy restriction since n-3FA inhibits many of the pro-tumorigenic pathways activated by obesity.

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Chapter 4
Is There a Role for Raloxifene and Tamoxifen for the Prevention of Breast Cancer?

Philipp Y. Maximov and V. Craig Jordan

Abstract Breast cancer is the leading oncologic disease in women in the world and is the second leading cause of death in Western women. A major advance in breast cancer chemoprevention in healthy high risk women occurred with the pioneering studies of the Selective Estrogen Receptor Modulators (SERMs) tamoxifen and raloxifene. Tamoxifen, the first targeted therapy for the treatment of breast cancer approved by the Food and Drug Administration (FDA), was subsequently successful in significantly reducing the incidence of breast cancer in high risk women. Nevertheless, tamoxifen has some adverse effects in the uterus. Raloxifene, that is also FDA approved for breast cancer prevention in high risk postmenopausal women and separately for osteoporosis, has a better safety profile than tamoxifen. In this chapter we will describe the history, the current role and deficiencies of tamoxifen and raloxifene in the prevention of breast cancer. The potential of other SERMs and new approaches to hormone replacement to improve women's health but to reduce the risk of breast cancer are illustrated.

Keywords Tamoxifen • Raloxifene • Selective estrogen receptor modulators • SERM action • Breast cancer prevention • Estrogen replacement therapy

4.1 Introduction

Breast cancer continues to have the highest incidence of any cancer in women, the top cause of death from cancer in the world and the second highest cause of mortality from cancer in women in the US. Annually there are 22.07 cases per 100,000 women in the People's Republic of China, 45.64 in the Russian Federation, 59.46 in Brazil, 94.99 in the United Kingdom and 92.93 in the United States of America [1]. Nevertheless, significant advances have been made in the reduction of mortality as well as treatment and prevention of recurrence of breast cancer in the past 30 years with new medicines and their application with novel therapeutic strategies. Antihormonal therapy remains the most prescribed therapy for breast cancer as the majority (≈70 %) of breast cancers are

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estrogen receptor (ER) positive. Antihormonal therapy of breast cancer is a long-term therapy and includes treatment with antiestrogenic agents such as tamoxifen (Fig. 4.1) that targets the tumor ER, and aromatase inhibitors (AIs) such as anastrozole, letrozole and exemestane, which target the aromatase enzyme that synthesizes estrogens. Both approaches abrogate the proliferative effect of estrogens on breast cancer cells and both are approved for the treatment of breast cancer. However, tamoxifen is approved to treat breast cancer in pre- or postmenopausal patients, and AIs are used only to treat postmenopausal patients. Currently, only tamoxifen, of all breast cancer drugs used for treatment, is also approved for chemoprevention of ER-positive breast cancer in both pre- and postmenopausal women at high risk of developing breast cancer.

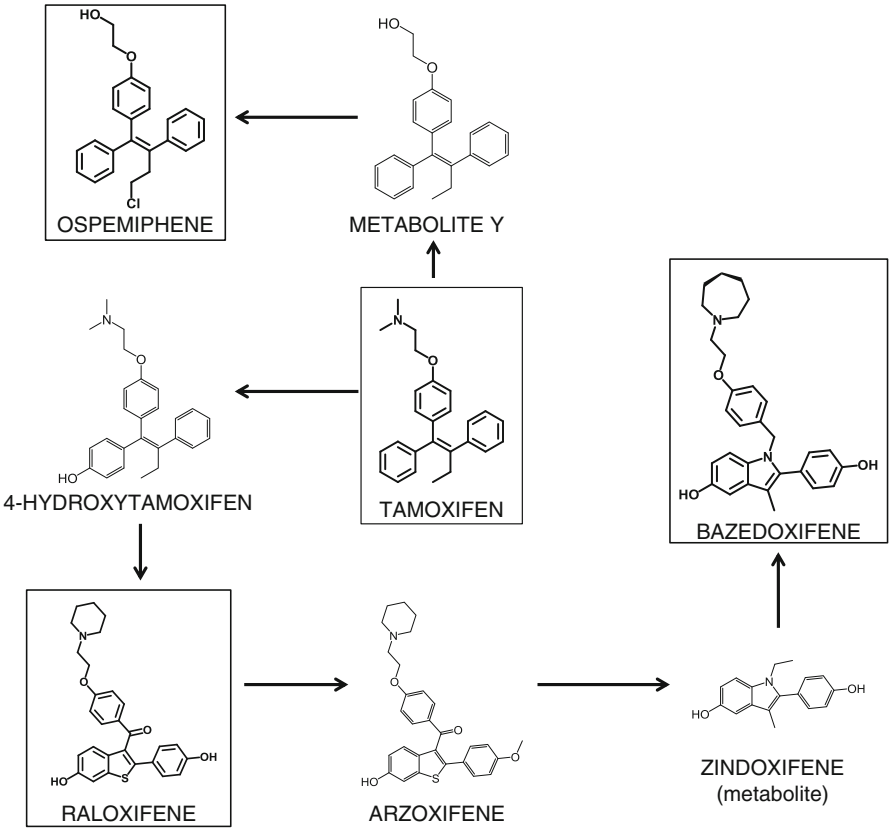

Fig. 4.1 Structure of presently FDA approved SERMs (boxed) for the treatment and prevention of breast cancer (tamoxifen), prevention of breast cancer while treating or preventing osteoporosis (raloxifene) and presently marketed drugs for the treatment of osteoporosis (bazedoxifene) and vaginal dryness in postmenopausal women (opemiphene) that could potentially also prevent breast cancer. Many of the currently developed SERMs had their origins from previously developed failed breast cancer drugs or either tamoxifen metabolites, such as 4-hydroxytamoxifen or metabolite Y. Zindoxifene is the acetylated derivative of the metabolite shown but only has estrogenic activity [97]

The idea of chemoprevention of breast cancer is not new. Professor Antoine Lacassagne expressed his idea of chemoprevention of breast cancer at the annual meeting of the American Association for Cancer Research in 1936 [2]: *"If one accepts the consideration of adenocarcinoma of the breast as a consequence of a special hereditary sensibility to the proliferative action of oestrone, one is led to imagine a therapeutic preventive for subjects predisposed by their heredity to this cancer, to stop the congestion of oestrone in the breast."*

Unfortunately, there were at that time no agents that could "…stop the congestion of oestrone in the breast" to prevent breast cancer, until the development of tamoxifen [3, 4]. Tamoxifen was a logical choice for prevention as there was a large body of evidence about the value of the medicine to treat breast cancer in both animal models and as long-term adjuvant therapy in breast cancer patients. Indeed, the fact that tamoxifen could prevent contralateral breast cancer during adjuvant therapy [5] already was a proof of principle for chemoprevention. Additionally, experiments on rat models demonstrated that continuous tamoxifen administration 1 month after the carcinogenic insult with 7,12-dimethylbenz[α] anthracene (DMBA) can completely prevent the formation of mammary tumors [6, 7]. Once the administration of tamoxifen was stopped prematurely the preventive actions of tamoxifen were reduced and microfoci of tumor cells developed into palpable tumors. Tamoxifen also performed better than oophorectomy in the DMBA-induced mammary carcinoma model.

The possibility of the long-term tamoxifen use in healthy women created a concern in the clinical community. If estrogen is important to maintain bone density and lower Low Density Lipoprotein (LDL) and prevent Coronary Heart Disease (CHD) (the clinical position at that time in the 1980s before the Woman's Health Initiative trial) then tamoxifen an antiestrogen may put the majority of healthy women at risk for osteoporosis and CHD, whilst preventing a few breast cancers. However, animal models demonstrated that tamoxifen, and keoxifene (Fig. 4.1) (that was later reinvented as raloxifene) maintain bone density in overiectomized rats and have a synergistic effect with estradiol [8, 9]. Tamoxifen, however, was shown, first in the laboratory [10] and then clinically [11] to increase the risk of endometrial cancer. Be that as it may, tamoxifen did maintain bone density in postmenopausal women and reduced LDL in postmenopausal breast cancer patients [12, 13]. The concept of Selective ER Modulation (SERM) was born, but a safer SERM was needed. Raloxifene (then called keoxifene), a failed breast cancer drug also demonstrated paradoxal agonist/antagonist properties (like prevention of breast cancer and maintenance of bone density) like tamoxifen [7], however, it did not show any estrogenic properties in the uterus [10]. This discovery opened the door for the development of multiple strategies to prevent breast cancer in high risk and low risk postmenopausal women. The idea was simple; either focus clinical effects on select high risk populations or treat a major disease like osteoporosis and prevent breast cancer at the same time. We will advance this argument with clinical evidence, but first it is valuable to describe how SERMs function.

4.2 Mechanisms of SERM Action

Selective ER Modulators exert their action through their interaction with the ER, and depending on the structure of the ligand the ER conformation changes, and so do the properties and the activity of the SERM:ER complex, e.g.: estrogenic properties of tamoxifen in the uterus and antiestrogenic properties of raloxifene, both being antiestrogens in the breast. The ER protein consists of six domains labeled A-F [14]. The N-terminal region of the ER contains a ligand-independent and transcriptionally minor activating function region AF-1 and is referred to as the A/B domains. Domain C contains the zinc-finger DNA-binding region necessary for binding to the promoters of the target genes. The D domain contains the nuclear localization signal region and also doubles as a hinge to the C domain. The E domain is also referred to as the Ligand Binding Domain (LBD). The LBD contains 12 α helices, where helices H3-12 constitute a ligand-binding pocket with H12 acting as a cover for the agonist ligand, once it is bound to the receptor. The E domain also contains a major transcription activation region called AF-2 and recruits co-activators via an LXXLL-motif. The F domain located at the C-terminus of the protein modulates the functions of the ER in a ligand, promoter and cell specific fashion [15–18]. The interaction of tamoxifen and raloxifene were resolved by X-ray crystallography [19, 20]. The SERMs 4OHT and raloxifene use the same amino acids Glu353 and Arg394 in the LBD that position and bind the 17β-estradiol (E_2) via the 3 phenolic hydroxyl. The bulky antiestrogenic side chain (alkylaminoethoxyphenyl) does not permit the closure of Helix 12 to seal the LBD, like a "stick in the jaws of the crocodile" [21, 22], whereas the agonist ligands, like E_2 or diethylstilbestrol (DES) induce closure of H12 ("closed crocodile jaws"). However, the major role is now played by Asp351, and its ability to interact with the alkylaminoethoxy side chain of the SERM and govern the intrinsic estrogenic activity of the SERM:ER complex. The piperidine ring of raloxifene is closer to Asp351 and shields and neutralizes it from further interaction. By contrast, the dimethylalkylaminoethoxy side chain of tamoxifen, is further from Asp351, which remains exposed. As a result, there is an interaction with Helix 12 and transient closure of the LBD. Thus 4OHT is more estrogen-like than raloxifene as noted from the reported differences of the two SERMs in the Study of Tamoxifen and Raloxifene (STAR). This description of the interaction between SERMs and the ER is supported by molecular pharmacology studies of the 4OHT:ER and raloxifene:ER complexes in the TGFα reporter gene assay [23–27]. This mechanism is, however, only one part of the SERM story. This is summarized in Fig. 4.2, which illustrates the complexity of SERM action in different target tissues. It is a complex decision-making network dependent upon the external shape of the SERM:ER complex that regulates co-regulator binding. This, is turn, modulates gene function through receptor at target gene promoter sites.

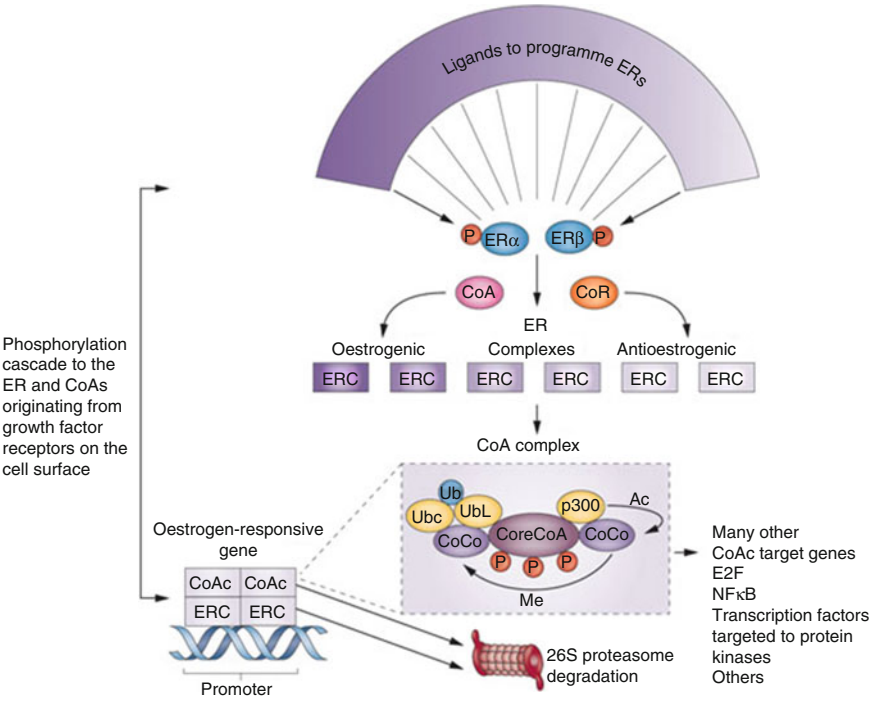

Fig. 4.2 The shape of the ligands that bind to the estrogen receptors (ERs)alpha and beta programs the complex to become an estrogenic or antiestrogenic signal. The context of the ER complex (ERC) can influence the expression of the response through the numbers of co-repressors (CoR) or co-activators (CoA). In simple terms, a site with few CoAs or high levels of CoRs might be a dominant antiestrogenic site. However, the expression of estrogenic action is not simply the binding of the receptor complex to the promoter of the estrogen-responsive gene, but a dynamic process of CoA complex assembly and destruction [98]. A core CoA, for example, steroid receptor coactivator protein 3 (SRC3), and the ERC are influenced by phosphorylation cascades that phosphorylate target sites on both complexes. The core CoA then assembles an activated multiprotein complex containing specific co-co-activators (CoCo) that might include p300, each of which has a specific enzymatic activity to be activated later. The CoA complex (CoAc) binds to the ERC at the estrogen-responsive gene promoter to switch on transcription. The CoCo proteins then perform methylation (Me) or acetylation (Ac) to activate dissociation of the complex. Simultaneously, ubiquitiylation by the bound ubiquitin-conjugating enzyme (Ubc) targets ubiquitin ligase (UbL) destruction of protein members of the complex through the 26S proteasome. The ERs are also ubiquitylated and destroyed in the 26S proteasome. Therefore, a regimented cycle of assembly, activation and destruction occurs on the basis of the preprogrammed ER complex [98]. However, the co-activator, specifically SRC3, has ubiquitous action and can further modulate or amplify the ligand-activated trigger through many modulating genes [99] that can consolidate and increase the stimulatory response of the ERC in a tissue. Therefore, the target tissue is programmed to express a spectrum of responses between full estrogen action and antiestrogen action on the basis of the shape of the ligand and the sophistication of the tissue-modulating network. The figure legend and the figure are reproduced with the permission of Nature Publishing Group from reference [3]

4.3 Prevention Trials with Tamoxifen and Raloxifene

Women at high risk for breast cancer are healthy women with certain risk factors such as obesity, a relative with breast cancer, menarche before the age of 12, no children, pregnancy after 35, atypical hyperplasia, breast biopsy in the past and the use of hormone replacement therapy (HRT). All these factors are taken into consideration when estimating the risk level for breast cancer. If the 5-year breast cancer risk is above 1.67 %, the woman is at risk of developing breast cancer and should be offered chemoprevention as an option, unless preexisting conditions, like cardiovascular pathologies, prevent an intervention.

Since tamoxifen, as noted previously, was first to be approved by the FDA for long-term adjuvant therapy of ER-positive breast cancer, it was also the first to be tested as a breast cancer chemoprevention drug. Multiple trials tested tamoxifen in high-risk women. The major results from these trials are summarized in Table 4.1.

The first placebo-controlled prevention study to evaluate the worth of tamoxifen as a chemopreventive agent for breast cancer was launched in 1989 at the Royal Marsden Hospital [28]. High-risk women took 20 mg of tamoxifen daily for up to 8 years. The results of the trial were published in 1994 and demonstrated low acute toxicity in women taking tamoxifen, but significantly higher incidence of side effects, such as hot flashes, vaginal discharges and menstrual irregularities in mainly premenopausal women [29]. However, compliance was similar in women taking tamoxifen compared

Table 4.1 Results from the tamoxifen breast cancer prevention trials in high-risk women

	Royal Marsden	NSABP P-1	Italian	IBIS-1
Participants	2471	13,388	5408	7152
Premenopausal	62 %	40 %	36 %	52 %
Postmenopausal	38 %	60 %	64 %	48 %
Women years of follow up	12,355	46,858	5408	29,800
Breast cancer incidence/1000 during treatment				
Placebo	5.5	6.7	2.3	6.7
Tamoxifen	4.7	3.4	2.1	4.7
Side effects				
Endometrial carcinomas				
Placebo	5 total	8 premen.	n/a	2 premen.
		7 postmen.		9 postmen.
Tamoxifen	13 total	9 premen.	n/a	1 premen.
		27 postmen.		16 postmen.
Deep vein thrombosis				
Placebo	9	22	n/a	38
Tamoxifen	14	35		64
Pulmonary embolism				
Placebo	None reported	6	n/a	32
Tamoxifen		18		44

n/a not applicable

to women on placebo (77 % vs. 82 %, respectively). No thromboembolic effects were observed (Table 4.1), and tamoxifen significantly lowered LDL within 3 months after the start of tamoxifen administration [30]. Tamoxifen was also able to maintain and increase bone density in the spine and hip in postmenopausal women, however tamoxifen did cause a small, but significant, decrease in bone density in both the spine and hip at 3 years in premenopausal women [31].

A much larger trial to evaluate tamoxifen's potential as a preventive for breast cancer was launched in 1992 by the National Surgical Adjuvant Breast and Bowel Project (NSABP), designated as P-1 (P for prevention). The first results for the NSABP P-1 study were published in 1998 after a median of 47.7 months follow up [11]. The results demonstrated a 49 % reduction in invasive and a 50 % reduction in noninvasive breast cancer incidence. Also 56 % reduction in incidence was observed in women who were diagnosed with Lobular Carcinoma In Situ (LCIS) prior to the trial, and an 86 % reduction in women with atypical hyperplasia. It was also demonstrated that tamoxifen was able to reduce the incidence of only ER-positive breast cancers by 69 % per year, with no correlation with ER-negative disease. Tamoxifen reduced the incidence of bone fractures in the spine, hip and radius by 19 %, however, the decrease in fractures never reached statistical significance. The greatest bone protecting benefit was observed in the postmenopausal group.

Another two smaller trials that were designed to evaluate the worth of tamoxifen for breast cancer chemoprevention were the Italian study [32] and the International Breast Cancer Intervention Study (IBIS-I) [33]. Out of 5408 recruited women in the Italian study, only 149 completed a 5 year treatment with 20 mg/day of tamoxifen. The results demonstrated a significant reduction in the incidence of breast cancer in the tamoxifen vs. the placebo group; however, a significant increase in superficial thrombophlebitis was noted [32].

The double-blind placebo-controlled IBIS-I trial recruited a total of 7152 high-risk women, which were randomized to either take 20 mg/day tamoxifen for 5 years or placebo [33]. The results of the study demonstrated that tamoxifen was able to reduce the incidence of breast cancer by 32 % after a 50 month follow up. The endometrial cancer events were not increased significantly, however, thromboembolic events were considerably increased in the tamoxifen group compared to the placebo group.

The results obtained from tamoxifen prevention trials, created new concerns and new opportunities for breast cancer prevention. Tamoxifen demonstrated excellent efficacy in preventing breast cancer in high-risk women, however with a much better safety profile for premenopausal women, than for postmenopausal. Despite concerns tamoxifen remains to this day an option for breast cancer prevention and treatment in premenopausal women; however a safer agent was needed. The possibility to treat osteoporosis [9] with a drug that is an antiestrogen in the breast [7] and have minor uteroptropic properties [34] created new opportunities for testing another SERM for breast cancer prevention. A failed breast cancer drug keoxifene entered the stage and became raloxifene.

The first trial to evaluate raloxifene for treatment and prevention of osteoporosis was the Multiple Outcomes of Raloxifene Evaluation (MORE) trial [35]. The trial was a three-arm double-blind trial with 7705 recruited postmenopausal women

with osteoporosis. Participants were randomized to take placebo or either 60 or 120 mg/day of raloxifene. The results demonstrated that raloxifene reduced the number of bone fractures by about 30–40 %, and was also able to increase bone density by 1–2 %, i.e.: similar to tamoxifen. In addition to the osteoporosis evaluation, the MORE trial evaluated other outcomes such as breast cancer incidence, gynecological evaluation, and DVT and pulmonary embolism incidence. The treatment of women with raloxifene reduced the risk of ER-positive breast cancer incidence by 90 % after 3 years of treatment [36], and incidence by about 72 % after 4 years of treatment in the MORE trail [37]. There were less endometrial cancers with raloxifene than previously noted with tamoxifen; however, raloxifene caused a few gynecological, vascular and pulmonary side effects. Strangely enough, raloxifene did not decrease DCIS as effectively as previously observed using tamoxifen in the NSABP P-1 trial.

Since the overall results of the trial were positive, it was decided to extend the trial for another 4 years of treatment with raloxifene as Continued Outcomes Relevant to Evista (CORE) trial. After an 8 year follow up is was shown that raloxifene reduced the incidence of both invasive and non-invasive ER-positive breast cancers by about 66 % and 72 %, respectively overall for both the MORE/CORE trials [38]. Since raloxifene was able to prevent osteoporosis, for which it was marketed, and effectively reduced the incidence of breast cancer at the same time, it was important to compare the only FDA-approved drug for prevention of breast cancer tamoxifen head-to-head in the Study of Tamoxifen and Raloxifene (STAR) trial.

The STAR trial was an unprecedented phase III, double blind clinical trial that recruited 19,747 eligible postmenopausal women with high risk of developing breast cancer. The breast cancer risk had to be above 1.65 % or women had to be over 60 years old, irrespective of their risk, postmenopausal women between ages 35–59 with a high risk of developing breast cancer, or either postmenopausal with previously diagnosed LCIS. The risk factors were calculated using a modified Gail model [39], similar to the one used to establish the risk for women in the NSABP P-1 trial. After a year of screening, women were randomized into two cohorts: taking 20 mg/day tamoxifen or 60 mg/day raloxifene for 5 years. The first results reported from the STAR trial were after a 77 month follow up [40]. The results demonstrated that raloxifene was able to reduce the incidence of invasive breast cancer just as effectively as tamoxifen. There were less non-invasive breast cancers in the tamoxifen group that in the raloxifene group, but the difference was not significant. An important aspect of the STAR trial is that no placebo control was used to compare the effectiveness of the two drugs. It was considered unethical to use a placebo control for the study, considering that at that point tamoxifen was already an established and FDA approved medicine for breast cancer prevention. Instead, an estimate number of breast cancers, based on the Gail model [39], was used as a hypothetical placebo control. The projected number of breast cancers in the same population of women without prevention treatment was established as 312, thus both tamoxifen and raloxifene reduced the incidence of breast cancer by approximately 50 %. A very important outcome of the STAR trial was the safety comparison

of the two drugs. It was shown that there were 36 % fewer endometrial cancers in the raloxifene group compared to tamoxifen, however, this result did not reach a statistical significance. Interestingly, twice as many women taking tamoxifen chose to have hysterectomies, compared to the raloxifene group. Also there were fewer thromboembolic effects, cataracts and cataract surgeries in women taking raloxifene. It seemed like both tamoxifen and raloxifene were equivalent in reducing the incidence of breast cancer in high risk women, but raloxifene has a better safety profile than tamoxifen in postmenopausal women.

Based on these prevention trials both tamoxifen and raloxifene were FDA approved: tamoxifen for prevention of breast cancer in high risk pre- and postmenopausal women in 1998, and raloxifene for the reduction of breast cancers in high-risk postmenopausal women with osteoporosis in 2007. However, this was not the final answer on the position of tamoxifen and raloxifene in breast cancer prevention.

4.4 Tamoxifen the "Gift That Keeps on Giving"

Interestingly, the prevention of breast cancer after 5 years of treatment with raloxifene or tamoxifen did not remain equivalent after a 90 month follow up of the STAR trial [41]. Raloxifene retained only 76 % reduction of breast cancer than that of tamoxifen in the first 2–3 years of the post-treatment period. Antiestrogens, that prevent binding of the estrogens to the ER, will be cleared from the organism after therapy termination, and will not be blocking estrogen action anymore, and tumor cells in the micrometastases should grow. Paradoxically, they do not. Curiously, tamoxifen also was able to retain cancer preventive properties in rats after the cessation of the drug, and tumor occurrences never reached the control levels [42]. The 5 years of treatment with tamoxifen somehow changed the tumor environment, and there seems to be a "carry over" preventive effect, that raloxifene did not have to the same extent as tamoxifen. It is important to note that osteoporosis treatment with raloxifene is approved to be indefinite, as the adverse effects are well tolerated by patients and benefits outweigh the risks [41], which is in concordance with that raloxifene is safe even after at an 8 year follow up in the MORE trial [43]. This difference between tamoxifen and raloxifene in the "carry over" effect after the termination of therapy after the STAR trial could be explained simply by the fact that tamoxifen is a prodrug. Tamoxifen is accumulated in the fatty tissue of the patient and is metabolized in to active hydroxilated metabolites that have high affinity to the ER. Tamoxifen itself is a slow releasing drug, and missing a few doses will not make a significant difference in the circulating levels of the active metabolites in the patient. Tamoxifen will continue in creating an antiestrogenic pressure on the tumor cells. Raloxifene, on the other hand, is a drug with a lower bioavailablity (2 %) and is excreted rapidly. Missing a few doses of raloxifene can reduce the effectiveness of the drug. However, this does not explain why is there a reduction of the incidence of breast cancers after 5 years of preventive therapy.

Estrogen-induced apoptosis in long-term estrogen deprived breast cancer cells [44, 45] was proposed as an explanation for the "carry over" phenomenon. Estrogens killing breast cancer cells has been first observed in the 1940s, during the high dose estrogen clinical trials of Sir Alexander Haddow [46]. The high dose estrogen therapy later became the standard of treatment for advanced breast cancer. However, Haddow in 1970 postulated that a 5 year period of estrogen deprivation is necessary after the menopause, as he found that women after 55 have a higher success rate with high dose estrogen therapy than women before 55 [47]. Long-term estrogen deprivation (LTED) sensitizes breast cancer cells to undergo apoptosis with physiological estrogen. Laboratory studies confirm this hypothesis in LTED breast cancer cells [48, 49], but also in breast cancer cells that have been selected under antiestrogenic pressure [50]. This phenomenon of estrogen-induced apoptosis has been recently reviewed [44, 51].

4.5 Acceptance, Compliance and Side Effects

Despite the fact that both tamoxifen and raloxifene are FDA approved for breast cancer chemoprevention, and that the estimated number of women between ages 36 and 79, that are eligible for the prescription of these drugs, is approximately 10 million in the US (or 15 %) [52], the number of women accepting therapy is very limited [53]. It is estimated that less than 5 % of eligible women have a favorable risk-benefit profile, and less than 1 % agree to take SERMs for chemoprevention [54]. The main reasons for such low acceptance of preventive therapy for breast cancer are side effects, which include: hot flashes, vaginal bleeding, vaginal dryness, vasomotor symptoms, mood swings, pulmonary embolism, DVT, stroke, cataracts, and, with tamoxifen, endometrial cancer. A meta-analysis of five studies [55] have shown that the acceptance rates for chemoprevention were approximately 15 % or even less (10 %) in another analysis study [56].

In the 1990s it was found that tamoxifen induces liver carcinogenesis in various strains of rats, in particular the Sprague-Dawley rats [57–60]. The formation of DNA adducts with tamoxifen (later it was identified that it was α-hydroxytamoxifen metabolite) was identified as a mechanism of liver carcinogenesis [61, 62]. It was, however, shown that tamoxifen in therapeutic doses was not able to induce the necessary amount of DNA adducts to induce liver carcinogenesis in human hepatocytes in vitro, as the rat hepatocytes were significantly more susceptible to tamoxifen as a carcinogen [63–65]. Nevertheless, this phenomenon was used to explain the carcinogenic properties of tamoxifen in a woman's uterus during the prevention trials. It was not yet widely accepted that tamoxifen is a SERM and has simply estrogenic properties in the uterus. However, this led the FDA to mandate the addition of information about tamoxifen as a carcinogen in the uterus on the drug's packaging. This created a concern within the women's breast cancer advocate groups and a negative image of tamoxifen. This factor probably is one of the major contributors to the low acceptance of tamoxifen for breast cancer chemoprevention.

Asides the acceptance issues of SERMs for chemoprevention of breast cancer, there is also an issue of compliance with the 5 year therapy. Two recent studies [66, 67] have analyzed both the acceptance and the compliance of high risk women with SERM breast cancer preventive therapy in a non-trial setting. One study was performed at the H. Lee Moffitt Cancer Center Breast Surveillance Clinic [67] that has selected 260 high-risk women with a 5-year Gail model risk above 1,7 % or Lobular Carcinoma in Situ (LCIS), which cannot be incorporated into the Gail model. Out of 260 women 219 were offered chemoprevention with SERMs, but only 118 (54.4 %) accepted the intervention. The analysis of patient information showed that women that had a high lifetime breast cancer risk, were diagnosed with osteoporosis, LCIS or atypical ductal hyperplasia were statistically more likely to accept chemoprevention. The authors hypothesized that words like carcinoma, e.g.: LCIS might influence women to accept the therapy; as well as the fact that their study was performed in a clinic for women with high risk of developing breast cancer might have influenced the results. Other criteria were not significantly associated with therapy acceptance. Out of 118 women that have accepted chemoprevention 58 (49.2 %) have discontinued prevention at least temporarily, out of which 37 (28.8 %) have discontinued therapy permanently. Out of the women who permanently discontinued therapy 29 (85.3 %) did so in the first 2 years of therapy. Most reasons for discontinuing therapy were side effects. Some women had an intervention to treat side effects, like hot flashes and vaginal dryness, or switch to another SERM. Those who attempted to address the side effects were more likely to return to chemoprevention. The authors of this study, however, did project that 60 % of women who accepted chemoprevention were most likely to complete full 5 years of therapy and benefit from it.

Similar acceptance and compliance rates were acquired during another study that was conducted within The Sister Study with tamoxifen for chemoprevention [66]. The Sister Study is a cohort of 50,884 healthy women at high-risk of developing breast cancer and had a sister with breast cancer. The authors have identified 788 tamoxifen users with no contraindicating preexisting conditions. The results demonstrate that 74 % of identified tamoxifen users had a favorable risk-benefit profile, while 20 % had unfavorable risk-benefit profile, and for 6 % of tamoxifen users this index was undetermined. Younger women and women with prior hysterectomy have a more favorable risk-benefit index, and African-American women had a lower risk-benefit profile than non-Hispanic white women. Out of all tamoxifen users in the study 46 % have discontinued tamoxifen use before 5 years with median 3 years duration of therapy. Interestingly, women who used raloxifen after discontinuing tamoxifen were 55 % less likely to complete the 5 years of therapy. The main reasons for discontinuation of chemoprevention with tamoxifen were again side effects. Women taking tamoxifen experienced a threefold higher incidence of strokes than non-users, 2.5-fold higher incidence of endometrial cancer, a 40 % increase in transient ischemic attacks, and a 23 % increase in cataracts. The discontinuation of tamoxifen in this study was significantly higher than in the prevention studies [68].

Taking into consideration the side effects of tamoxifen and raloxifene, acceptance and compliance with the chemoprevention therapy, new SERMs with a better safety profile, higher efficacy and more multifunctionality are going to be beneficial

for prevention of breast cancer in high-risk women. In the next section we will describe novel SERMs that are being currently tested for treatment of various pathological conditions in women and breast cancer at the same time.

4.6 The New Generation of SERMs

The side effects observed with currently prescribed tamoxifen and are the main culprits in low therapy acceptance and compliance rates. Higher rates of treatment take up and compliance in eligible high-risk women probably would result in higher reduction of breast cancer incidence in the US and the world. Hence, safer SERMs are needed that would be efficient in the treatment of hormone-dependent pathological conditions, such as osteoporosis, and prevent breast cancer at the same time. This strategy was first articulated in 1990 [69] and raloxifene was the pioneering drug. Various drug companies have now developed alternatives to tamoxifen and raloxifene. The new generation of SERMs that are going to be described in this section are: lasofoxifene, arzoxifene, bazodoxifene and ospemifene. Interestingly, all of these drugs have their origins from previously developed, but failed breast cancer drugs, or tamoxifen metabolites. The worth of the new SERMs described here were evaluated through Phase III clinical trials and some have undergone FDA reviews. However, success in clinical development is not assured.

Lasofoxifene is a naphthalene derivative structurally very similar to tamoxifen and has its origins from Nafoxidine, a failed breast cancer drug and "morning after pill" in rodents. Lasofoxifene binds to both forms of the ER (α and β) with a higher affinity than 4OHT or raloxifene. Lasofoxifene inhibited osteoclastogenesis, reduced bone turnover markers and prevented bone loss in laboratory animals [70, 71]. Multiple studies tested lasofoxifene as an anti-osteoporotic drug [72–77]. The results from all trials demonstrated that lasofoxifene, at doses much lower than raloxifene and tamoxifen, was able to maintain or increase vertebral and hip bone mineral density more efficiently than calcium and vitamin D supplementation, and decrease bone turnover superior to raloxifene. Lasofoxifene reduced LDL and, as a result, the incidence of CHD and strokes. However, only the PEARL study included the prevention of ER-positive breast cancer as one of the endpoints of the clinical trial [78]. The result of the PEARL trial demonstrates that lasofoxifene is able to decrease the incidence of ER-positive breast cancer at a dose of 0.5 mg/day, however this dose also increased the risk for DVT. Long-term data confirm the safety of this drug for long-term therapy [79]. Lasofoxifene was also shown to improve vaginal dryness, but over the 5 year treatment some women developed endometrial hypertrophy [79]. It is the most promising breast cancer chemopreventive agent for the future; however, more long-term clinical data is needed at this point.

Arzoxifene is a drug developed by Eli Lilly that is a derivative of raloxifene, with a protective methyl group on one of the phenolic hydroxyls and an ether hinge instead of the carbonyl hinge in raloxifene. These alterations to the structure of the compound have led to a better bioavailability and an increased affinity for the ER. Both preclinical

[80] and clinical [81] data suggest that arzoxifene has anti-breast cancer properties and is able to reduce the risk of invasive breast cancer in postmenopausal women. However, the drug was developed primarily as an anti-osteoporotic drug. Arzoxifene reduces the number of vertebral fractures in postmenopausal osteoporotic women, reduces bone turn-over markers and increases bone mineral density [82, 83]. Unfortunately, the drug was not able to reduce the incidence of non-vertebral fractures and increased the incidence of DVT, so its further development was abandoned by the company.

Bazedoxifene is another SERM that is being developed, and was shown to have a superior safety profile over raloxifene, in particular in the reduced incidence of endometrial events, venous thromboembolism, vasomotor symptoms, hot flashes that led to a reduced cessation of the drug. It is able to build bone by reducing bone turn-over [84]. However, despite the favorable effects on the bone and the uterus, the drug did not demonstrate any reduction of risk or incidence of breast cancer compared to placebo in osteoporotic postmenopausal women [85].

Ospemifene evolved from a tamoxifen metabolite called Metabolite Y [86], which is a weak antiestrogen. Ospemifene binds with higher affinity for the ERα than for ERβ, similar to E_2 and 4OHT. It induces osteoblast proliferation in the bone and not osteoclast apoptosis, like raloxifene [87]. In several clinical trials ospemifene showed its low toxicity profile with high tolerability [88]. Endometrial effects were comparable with those documented with raloxifene but with additional estrogenic effects in the vagina in postmenopausal women, thereby improving the dryness more effectively than raloxifene or tamoxifen [89]. The drug was able to reduce the levels of circulating LDLs, as well [89]. Though it was suggested that ospemifene could be efficient in preventing breast cancer [90] none of the clinical trials were designed to confirm this claim.

As it can be seen, multiple SERMs have been developed over the past 15 years, however, at this point tamoxifen and raloxifene are the first and the only drugs specifically approved for prevention of breast cancer in high-risk women.

4.7 Summary and Conclusions

The question to be addressed was: “what is the role of tamoxifen and raloxifene in breast cancer prevention?” The answer is simple. Tamoxifen is the pioneering medicine that was first to be FDA approved for breast cancer prevention. The concept has scientific merit in medicine. However, despite that fact that tamoxifen is a cheap and life-saving adjuvant therapy that continues to be used successfully world-wide, concerns about side effects lessens its value to prevent breast cancer. There are two principal problems with the current strategy to achieve chemoprevention of breast cancer. Firstly, the inaccuracy and impression of identifying who will develop breast cancer means that only populations can be identified at hypothetical high risk. In real terms this translates into a few dozen women at most per thousand who develop breast cancer per year. Regrettably, all 1000 women are exposed to the possibility of side effects so that half of the few dozen women per year who would have developed

breast cancer do not. These women do not know who these are in the hundreds, who would not have developed breast cancer in the high risk group anyway. This is not an effective public health strategy. The second problem is that not all women who will develop breast cancer each year will have their breast cancer prevented. The best result is 50 % reduction in incidence. So the concept of chemoprevention fails to be perfect. By contrast vaccination prevents the fatal infection completely.

The failed breast cancer drug keoxifene was reinvented as raloxifene with a new strategy to prevent breast cancer [69]. Raloxifene would go on to be developed for the treatment and prevention of osteoporosis in postmenopausal women with the simultaneous prevention of breast cancer. This was the beginning of a new class of medicines called SERMs. The commercial success of tamoxifen and raloxifene as SERMs prompted companies to develop new SERMs with better safety profiles. Unfortunately, not all were successful. Both tamoxifen and raloxifene remain the only FDA approved drugs for breast cancer prevention. Research and development of safer and more efficient SERMs to prevent multiple diseases remains a priority. Alternatively, the fact that it is now possible to recreate and test a safer Hormone Replacement Therapy (HRT) that prevents breast cancer in women without disease, provides another population based-chemoprevention strategy [91].

The unanticipated results of the Women's Health Initiative with estrogen replacement alone (ERT) that reduced the risk of breast cancer proved to be a paradox as HRT, i.e.: ERT plus medroxyprogesterone acetate (MPA) produced an increase in breast cancer incidence [92]. The decrease in breast cancer with ERT is explained by estrogen-induced apoptosis of estrogen-deprived occult breast cancer cells in the breasts of 60 year old women [93]. But if this is the case, how does MPA, a progestin, block estrogen-induced apoptosis? The answer is that MPA is not only a synthetic progestin, but also is a synthetic glucocorticoid. Glucocorticoids, as anti-inflammatory agents block estrogen-induced apoptosis [94, 95] and MPA, a weak glucocorticoid, will modulate estrogen-induced apoptosis successfully so that over prolonged periods, new populations of cells eventually grow back into tumors in 60 year old HRT treated women [91, 92]. New approaches to HRT using a synthetic 19-nortestosterone derivative in the oral contraceptive with predominantly progestin activity, but also estrogenic activity will potentially act as a new HRT for women over the age of 55. This will decrease breast cancer incidence as a public health strategy. But one can argue: "What about women around the menopause who had HRT to prevent menopausal symptoms?" A combination of bazedoxifene plus conjugated estrogens (CE) is approved for the control of hot flashes at menopause so a judicial sequential strategy of chemoprevention can now be deployed to hold and prevent breast cancer [96]. As a result, several years of bazedoxifene plus CE can be used around the menopause to create breast cancer cell populations that are estrogen-deprived and are vulnerable after about 5 years to estrogen alone to trigger apoptosis. That could be where the second generation (estrogen plus a 19-nortestosterone derivative) HRT comes into play.

In 1970 there was no tamoxifen, no SERMs and no raloxifene. Regrettably there was ERT plus MPA called HRT. Millions of women were seduced by the proposal of prolonging life and maintaining youth. Today, translational research has transformed

women's health to provide well tested and safer options for select women with high risk factors for multiple diseases of the menopause. The beneficial side effect is a reduction of breast cancer, a benefit that has the prospect of creating healthier and longer lives.

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Chapter 5
Aromatase Inhibitors for Breast Cancer Prevention

Saranya Chumsri, Stephen Yu, Amanda Schech, Gauri Sabnis, and Angela Brodie

Abstract Aromatase inhibitors (AIs) offer a new treatment option for breast cancer prevention without increased risks of venous thromboembolism and endometrial cancer. Compared to placebo, both exemestane and anastrozole significantly reduced the risk of not only invasive breast cancer but also non-invasive lesions. AIs are associated with unique side effects, particularly musculoskeletal symptoms, vasomotor symptoms, and bone loss. However, these side effects are manageable. There appeared to be no difference in the incidence of cardiovascular disease and the difference in quality of life is numerically small.

Keywords Aromatase inhibitors • Breast cancer • Chemoprevention

5.1 Introduction

Aromatase is a member of the cytochrome P450 superfamily and is involved in the last step of steroid biosynthesis estrogen production. Unlike other CYP450 enzymes, aromatase is highly specific and has an androgen-specific cleft. Using the process termed aromatization, aromatase specifically converts testosterone to estradiol, androstenedione to estrone, and hydroxytestosterone to estriol [1] (Fig. 5.1). Aromatase is highly expressed in the granulose cells of ovarian follicles and in the placenta. Other tissues including subcutaneous fat, muscle, brain, liver, normal breast, and breast cancer also express aromatase but at lower levels. These tissues serve as main sources for estrogen production in postmenopausal women after cessation of ovarian function. Multiple population-based studies have observed that obesity is associated with

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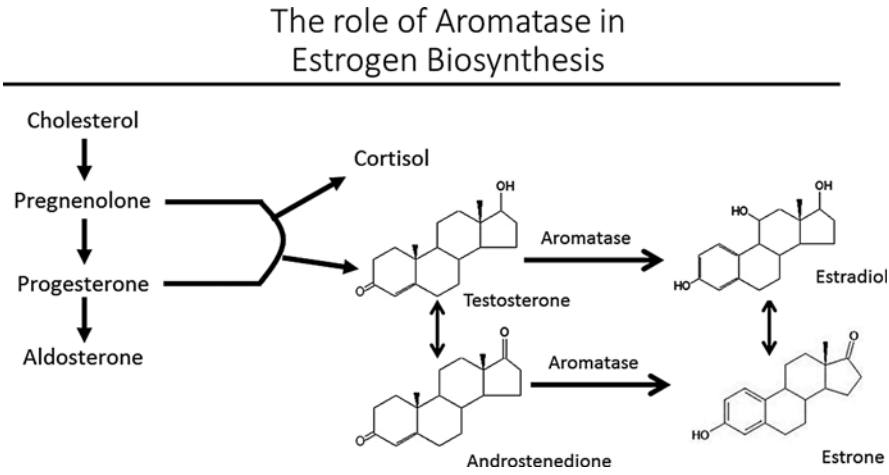

Fig. 5.1 "Aromatase regulates the last step in the biosynthesis of steroids from cholesterol. The enzyme mediates the conversion of androgens to estrogens

increased risk of breast cancer in postmenopausal women [2, 3], which is likely to be due to higher estrogen production from the aromatase enzyme which resides in the subcutaneous fat tissue of obese women.

Given that estrogens are the main driving force in the majority of breast cancer, inhibition of their production provides a logical target for the treatment of breast cancer. Furthermore, as the aromatase enzyme is specific for the conversion of androgens to estrogens, inhibition of its function would not be accompanied by disturbance in other steroid biosynthesis. Since the discovery of aromatase inhibitors (AIs) in 1970s [4], AIs have revolutionized the treatment for hormone receptor-positive (HR+) breast cancer. Due to their superiority over tamoxifen, AIs are now considered the standard treatment for postmenopausal women with HR+ breast cancer [5]. Currently, there are three oral AIs approved for use by the US Food and Drug Administration (FDA): exemestane (Aromasin® anastrozole (Arimidex® and letrozole (Femara®). These AIs can be categorized into two subclasses, steroidal and non-steroidal AIs. Type I or steroidal AIs are referred to as substrate analogs due to their resemblance to androgens. Steroidal AIs bind to the substrate-binding site of the aromatase enzyme and are converted to a reactive intermediate that binds irreversibly or covalently to the enzyme, causing permanent inactivation. This type of inhibitor is also known as an "inactivator" or "suicide inhibitor" because the enzyme is inactivated by its own mechanism of action [6, 7]. Due to the structural similarity between steroidal AIs and androgens, it has been speculated that steroidal AIs may exert androgenic effects which can mitigate some of the side effects of AI. However, this has not been uniformly proven in the clinical setting [7]. This type of AI currently in clinical use includes exemestane (Aromasin®). In contrast, type II or non-steroidal AIs exert their activity by binding non-covalently to the heme moiety of the aromatase enzyme, which prevents binding of androgens by occupying the substrate-binding site.

Table 5.1 Classification of AIs

Generations	Type 1 Steroidal inhibitors	Type 2 Non-steroidal inhibitors
Nonspecific inhibitor		Aminoglutethimide
Previous selective inhibitors not currently in clinical use	Formestane	Fadrozole
		Rogletimide
		Vorozole
Selective oral inhibitors currently in clinical use	Exemestane (Aromasin®)	Anastrozole (Arimidex®)
		Letrozole (Femara®)

This process is reversible via competitive inhibition of endogenous substrate, such as androstenedione [7, 8]. This type of AIs includes anastrozole (Arimidex®) and letrozole (Femara®). The classification of AIs according to their mechanism of action as well as their development timeline is summarized in Table 5.1.

To date, there are two large randomized control trials [9, 10] which have demonstrated the benefit of AIs when used as chemoprevention for breast cancer. These two trials, namely NCIC CTG MAP.3 and IBIS-II, provide level 1 evidence which support the use of AIs for breast cancer prevention. AIs are presently considered to be one of the standard of care options for the prevention of breast cancer in postmenopausal women with high risk of breast cancer [11]. Here we discuss the rationale supporting the use of AI as chemoprevention for breast cancer by reviewing recent supporting clinical data and the side effect profiles of these agents.

5.2 Rationale for Using AIs as a Breast Cancer Chemoprevention

Traditionally, selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene have been used for breast cancer prevention. There are concerning side effects associated with these agents, particularly the risk of endometrial cancer and venous thromboembolism. In contrast to SERMs, AIs do not act as partial estrogenic agonists and subsequently not associated with these risks. Therefore, AIs serve as a more appealing option for physicians and patients pursuing chemoprevention for breast cancer. Prior studies from 2000 and 2005 National Health Interview Surveys (NHIS) reported extremely low prevalence of tamoxifen use among U.S. women for primary chemoprevention of breast cancer (0.08–0.2 %) [12]. This may be due to the reluctance of both treating physicians to prescribe tamoxifen and patients to take tamoxifen. One of the surveys among 345 women who were evaluated for a breast lump showed an extremely low acceptance rate regarding the use of tamoxifen as a primary chemoprevention for breast cancer [13]. In this study, only 1 out of 89 high risk patients (0.01 %) who were recommended to take tamoxifen for chemoprevention decided to take tamoxifen. More

intriguingly, 42 % of the treating physicians actually recommended against the use of tamoxifen in these high risk women who already had a breast lump. The main reasons for not taking tamoxifen were a fear of adverse events (46.8 %).

AIs have consistently been shown to be equally or more effective than tamoxifen in both the metastatic and adjuvant setting [14], independent of toxicity concerns. In the adjuvant setting, it has been observed that AIs not only improved disease free survival but also significantly reduced the risk of second primary tumor in the contralateral breast. In the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial, which compared 5 years of adjuvant tamoxifen vs. anastrozole in postmenopausal women with localized disease, there was a 42 % reduction in contralateral breast cancer among patients taking anastrozole compared to tamoxifen (35 vs. 59, $p=0.01$) [15]. These data support the rationale of exploring AIs as a chemoprevention in breast cancer.

5.3 Clinical Experience of AIs for Chemoprevention

Based on the rationale above, AIs were investigated as chemopreventive agents in two large randomized phase III trials, including the NCIC CTG MAP-3 trial in Canada and IBIS-II trial in Europe. The results of these two trials are summarized in Table 5.2.

The NCIC CTG MAP-3 trial[16] was reported in 2011 and is a randomized, placebo-controlled, double-blind phase III trial of exemestane vs. placebo. Eligible patients included postmenopausal women 35 years of age or older with at least one of the following risk factors: 60 years of age or older, Gail 5-year risk score greater than 1.66 % (chances in 100 of invasive breast cancer developing within 5 years); prior atypical ductal (ADH), lobular hyperplasia (ALH) or lobular carcinoma in situ (LCIS); or ductal carcinoma in situ (DCIS) with mastectomy. The exclusion criteria include premenopausal, history of prior invasive breast cancer or prior DCIS with lumpectomy, known BRCA1 and BRCA2 carriers, history of other malignancies, uncontrolled hypothyroidism, hyperthyroidism, and chronic liver disease. There were 4,560 postmenopausal women enrolled in this study, with a median age of 62.5 years and a median Gail risk score of 2.3 %. With a median follow-up of almost 3 years, there were a total of 11 invasive breast cancer cases in the exemestane arm compared to 32 cases in the placebo arm, which corresponds to a 65 % reduction in the annual incidence of breast cancer (HR 0.35, $p=0.002$). All of the prespecified subgroups of patients, including concurrent use of low-dose aspirin, Gail risk score, age, body mass index, prior ADH, ALH, LCIS, and DCIS with mastectomy appeared to benefit with exemestane. Unlike raloxifene, which has been shown to only reduce invasive breast cancers but not non-invasive lesions, exemestane also appeared to reduce LCIS, ADH, and ALH (4 cases in exemestane arm and 11 cases in placebo arm; HR 0.36; 95 % CI 0.11–1.12).

Another phase III trial that evaluated a different AI for chemoprevention is the IBIS-II trial [17]. This trial also compared placebo but to a different non-steroidal

Table 5.2 Results from clinical trial experience of aromatase inhibitors for chemoprevention

Trials	Trial design	*N*	Patient population	Endpoints	Results
NCIC CTG MAP.3	Double-blind, randomized placebo controlled	4560	Postmenopausal women age ≥ 35	■ Incidence of invasive breast cancer	■ 11 vs. 32 women with invasive breast cancer (HR 0.35, p = 0.002)
Goss et al. [16]	Exemestane vs. Placebo		And	■ Combined invasive or noninvasive breast cancer	■ Annual incidence of invasive plus noninvasive breast cancer 0.35 % vs. 0.77 % (HR 0.47, p = 0.004)
	For 5 years		At least one of risk factors:	■ Incidence of receptor negative breast cancer	■ No significant difference in adverse event (88 % vs. 85 %)
			■ Age ≥ 60	■ Incidence of combined ADH, ALH, and LCIS	
			■ Gail risk score ≥ 1.66 %	■ Clinical fractures	
			■ Prior ADH or ALH	■ Adverse cardiovascular events	
			■ LCIS	■ Overall incidence of other cancers	
			■ Prior DCIS treated with mastectomy	■ Side-effect profile and safety	
				■ Health-related and menopause-specific qualities of life	
IBIS-II	Double-blind, randomized placebo controlled	3864	Postmenopausal women aged 40–70 years	■ Histologically confirmed breast cancer	■ 40 (2 %) vs. 85 (4 %) with breast cancer (HR 0.47, p < 0.0001)
Cuzick et al. [17]	Anastrozole vs. Placebo		And	■ ER+ breast cancer	■ ↓ Other cancer with anastrozole (gastrointestinal cancer p = 0.05; skin cancer p = 0.06)
	For 5 years		At least one of risk factors:	■ Breast cancer mortality	■ Anastrozole reduced more high-grade tumors compared to low-grade tumors
			■ Women with higher RR of breast cancer	■ Other cancer	
			■ 10-year risk of Tyrer-Cuzick model ≥ 5 %	■ Breast cancer mortality	
				■ Other cancer mortality	
				■ Cardiovascular disease	
				■ Fractures	
				■ Adverse events	
				■ Deaths not due to breast cancer	

ADH atypical ductal hyperplasia, *ALH* atypical lobular hyperplasia, *LCIS* lobular carcinoma in situ, *DCIS* ductal carcinoma in situ, *RR* relative risk, *ER+* estrogen receptor positive, *HR* hazard ratio

AI, anastrozole. This trial enrolled a total of 3864 postmenopausal women who had an increased risk of breast cancer (Relative Risk [RR] ≥ 4 for women aged 40–44 years, ≥2 for women aged 45–60 years, and ≥1.5 for women aged 60–70 years), any woman with LCIS, ADH, ALH, DCIS within the last 6 months with completed adequate local therapy, or >5 % risk of breast cancer in 10 years based on the Tyrer-Cuzick model. This trial excluded patients who were premenopausal, had previous diagnosis of breast cancer, were previously on SERM for more than 6 months, intended to continue hormone replacement therapy, had prophylactic mastectomy, had severe osteoporosis, had <10-year life expectancy, had psychological or physiological unfit reason for the study, or had a history of gluten and/or lactose intolerance. The median age of patients in this trial was 59.5 years. After a median follow up of 5.0 years, there were 32 (2 %) cases of invasive breast cancer in anastrozole arm and 64 (3 %) cases in the placebo arm, which corresponds to a 50 % risk reduction for invasive breast cancer (HR 0.5, 95 % CI 0.32–0.76, p = 0.001). These results compare well with the results of the MAP3 trial. Furthermore, patients who received anastrozole also had lower grade tumors. All of the subgroups according to age, body-mass index, previous use of hormone replacement therapy, and DCIS appeared to benefit from anastrozole. As expected from its mechanism of action, anastrozole appeared to be only effective in reducing ER-positive breast cancer. There was no significant difference in the incidence of ER-negative breast cancer among two arms (11 cases in anastrozole group and 14 cases in placebo group, HR 0.78, p = 0.538). Overall, patients who received anastrozole had reduced incidence of cancers other than breast cancer (40 cases in anastrozole group and 70 cases in placebo group, HR 0.58, p = 0.005). Interestingly, there were statistically less gastrointestinal and skin cancers (both p = 0.05) among patients who received anastrozole. However, similar findings was not observed in the MAP3 trial with exemestane (50 [2.2 %] vs. 40 [2.0 %] cases).

5.4 Side Effects of AIs

One of the major concerns for any chemopreventative agent is the side effect profile of the specific agent. This is particularly important as chemopreventative agents are prescribed to healthy individuals without disease and, thus, the risk-benefit ratio between side effects and chemopreventative benefit has to be justified. Compared to SERMs, AIs are not associated with increased risk of endometrial cancer and thromboembolism. Common side effects of AI include vasomotor symptoms, musculoskeletal symptoms, and bone loss. However, these symptoms are generally associated with menopausal symptoms and aging. Interestingly, in the MAP3 trial, symptoms and all grade adverse events (AEs) were recorded as high as 85 % in the placebo arm. Although patients who received exemestane had statistically higher AEs (88 %, p = 0.003), the absolute difference is modest. Similar findings were also observed in the IBIS-II trial where AEs were observed in 89 % of patients taking anastrozole and also 89 % in placebo group (HR 1.0, 95 % CI 0.98–1.03). In both trials, there was no death related to treatment.

5.4.1 Musculoskeletal Symptoms

Specifically, there were more all grade musculoskeletal symptoms in the exemestane arm compared to the placebo arm (11 % vs. 9 %, p=0.01). However, these symptoms were mostly grade 1 and 2, and the absolute difference between grade 2 or higher arthritic symptoms was quite small (6.5 % vs. 4.0 %). In the IBIS-II trial, there was significantly more overall musculoskeletal symptoms reported in the anastrozole arm compared to the placebo arm (64 % vs. 58 %, HR 1.1, p=0.0001). However, when the severity of symptoms was evaluated, there was no significant difference seen in mild (p=0.9) and severe (p=0.06) arthralgia. Furthermore, while moderate arthralgia was more prevalent in the anastrozole arm, the absolute difference in moderate arthralgia between the anastrozole and placebo arms was small (20 % vs. 17 %, HR 1.2, p=0.01). Of note, carpal tunnel syndrome (3 % vs. 2 %) and joint stiffness (7 % vs. 5 %) were more common in the anastrozole group but these symptoms were infrequent.

5.4.2 Vasomotor Symptoms

In both trials, hot flashes were reported frequently in both the AI and placebo arms. In the MAP3 trial, there were significantly more hot flashes reported in exemestane arm compared to the placebo arm (40 % vs. 32 %, p<0.001). This result is comparable to the IBIS-II trial with anastrozole which also showed significant increase in vasomotor symptoms in patients who received anastrozole compared to placebo (57 % vs. 49 %, HR 1.15, p<0.0001).

5.4.3 Osteoporosis and Fracture

Given the similar structure of the steroidal AI, exemestane, and androstenedione, it has been previously shown in both preclinical and clinical studies that exemestane exerts mild androgenic activity which reduces bone resorption [18, 19]. Based on this data, patients with a current diagnosis of osteoporosis were allowed to enter the MAP3 trial. At baseline, 13.3 % of patients in the exemestane arm had a history of osteoporosis and 12.9 % in the placebo arm. There was no significant difference in either new diagnosis of osteoporosis (1.7 % vs. 1.3 %, p=0.39) or clinical fracture rates (6.7 % vs. 6.4 %, p=0.72), and the proportion of women who were prescribed bisphosphonates was comparable in both arms (24.5 % vs. 24.1 %). Nevertheless, these new osteoporotic cases were self-reported and there was no formal serial assessment of bone density performed in this trial. In contrast, patients with a previous history of osteoporosis were excluded from an enrollment in the IBIS-II trial. There was also no difference in the total number of fractures (9 % vs. 8 %) and the number of fractures in specific sites in both anastrozole and placebo arms. The number of women taking bisphosphonates during the trial was well balanced between both arms (17 % in anastrozole arm and 15 % in placebo arm).

5.4.4 Cardiovascular Systems

Unlike SERMs, which have partial estrogenic effects, AIs are not associated with increased risk of venous thromboembolism. In contrast to tamoxifen that has cardioprotective effects due to favorable effects on the lipid profile [2, 21], AIs have been associated with increased risk of cardiovascular disease (OR 1.26, $p<0.001$) in meta-analyses of seven adjuvant clinical trials in patients with early-stage breast cancer. Notably, the incidence of cardiovascular disease of AI was compared to tamoxifen as there was no placebo arm in these adjuvant clinical trials. When compared to the placebo arms in both MAP3 and IBIS-II trials, there was no significant increase in cardiovascular events. In the MAP3 trial, the incidence of cardiovascular events was 4.7 % in the exemestane arm and 4.9 % in the placebo arm ($p=0.78$). In the IBIS-II trial, there was no significant difference in either cerebrovascular events or myocardial infarction. However, hypertension was significantly increased with anastrozole (5 % vs. 3 %, HR 1.64). Therefore, longer followup may be needed to fully evaluate the cardiovascular effects of anastrozole compared to placebo.

5.4.5 Quality of Life

Systematic assessment of quality of life was performed in the MAP3 trial [22]. The health-related quality of life was assessed using the Medical Outcomes Study 36-Item Short-Form Health Survey (SP-36) and the menopausal-specific quality of life was assessed by the Menopause-Specific Quality of Life (MENQOL questionnaire). These assessments were performed at baseline, 6 months and yearly thereafter. Overall, there were significant increases in vasomotor symptoms, sexual symptoms, and pain. Notably, the vasomotor symptom scores were highest at 6 months but appeared to decrease over time. There was only an 8 % increase in the vasomotor symptoms and 4 % increase in sexual symptoms and pain.

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Chapter 6
Inhibition of Depurinating Estrogen-DNA Adduct Formation in the Prevention of Breast and Other Cancers

Ercole L. Cavalieri and Eleanor G. Rogan

Abstract One problem in the efforts to prevent breast cancer has been the lack of recognition that estrogens can initiate cancer by acting as chemical carcinogens and reacting with DNA. Specific metabolites of endogenous estrogens, the catechol estrogen-3,4-quinones, react with DNA to form depurinating estrogen-DNA adducts. Loss of these adducts leaves apurinic sites in the DNA, generating mutations that can lead to the initiation of cancer. If estrogen metabolism becomes unbalanced and generates excessive catechol estrogen-3,4-quinones, formation of depurinating estrogen-DNA adducts and the risk of initiating cancer increase. Inhibiting formation of depurinating estrogen-DNA adducts can, therefore, prevent cancer.

Levels of the estrogen-DNA adducts are high in women diagnosed with breast cancer and those at high risk for the disease. The finding that high levels of depurinating estrogen-DNA adducts precede the presence of breast cancer indicates that formation of these adducts is a critical factor in breast cancer initiation. Women with thyroid or ovarian cancer also have high levels of estrogen-DNA adducts, as do men with prostate cancer or non-Hodgkin lymphoma.

The findings summarized above and other discoveries led to the recognition that reducing the levels of estrogen-DNA adducts would prevent the initiation of breast and other types of human cancer. We have found that the dietary supplements *N*-acetylcysteine and resveratrol inhibit estrogen-DNA adduct formation in both cultured human breast cells and in women. These results suggest that these two supplements offer an approach to reduce risk of developing breast and other types of human cancer.

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6.1 Introduction

To date, efforts to prevent breast cancer have been unsuccessful. A major contributing factor has been a lack of recognition that estrogens can become chemical carcinogens and initiate cancer. Understanding the etiology of various types of cancer is necessary to devise strategies for prevention. We have developed an approach to prevent breast and other types of cancer based on their etiology

6.2 The Complexity and Simplicity of Cancer

One of the major obstacles in cancer research is related to the concept that cancer, due to the characteristics of different expression, is considered a problem of 200 diseases. This viewpoint has impeded researchers from looking at the etiology of cancer because the research would be prohibitively complex. For this reason, the etiology of various cancers remains virtually unknown. However, if the most prevalent cancers have a common initiation, the problem would become much simpler.

A second barrier to the progress of cancer research is related to the reluctance of the scientific community to recognize that the natural estrogens, estrone (E_1) and estradiol (E_2), can become true chemical carcinogens via their metabolism, and initiate cancer in various hormone-dependent and hormone-independent organs.

The lack of acceptance of estrogens as carcinogens has been dictated by the incapacity of these compounds to induce mutations in bacterial and mammalian test systems [1–5]. These results have led scientists to classify E_1 and E_2 as epigenetic carcinogens that function mainly by stimulating abnormal cell proliferation via estrogen receptor (ER)-mediated processes [5–10]. The stimulated cell proliferation would generate more opportunities for mutations leading to carcinogenesis [7, 9, 11]. These ER-mediated events, however, do not play a critical role in cancer initiation, because the hypothetical mutations obtained during cell proliferation are random.

The discovery that specific oxidative metabolites of estrogens, the electrophilic catechol estrogen quinones [12, 13], react with DNA led to and supports the hypothesis that estrogens are genotoxic agents and can become endogenous chemical carcinogens by generating mutations leading to the initiation of cancer [14, 15]. This paradigm supports the concept that specific, critical mutations generate abnormal cell proliferation leading to cancer, rather than ER-mediated abnormal cell proliferation giving rise to random mutations. The specificity of the critical mutations arises from the preliminary intercalating physical complex between the estrogen and DNA before formation of a covalent bond between them. This has been demonstrated by studying the mechanism of cancer initiation of the human carcinogen diethylstilbestrol (DES) [16].

In summary, the complex problem of cancer has become simpler, because many of the most prevalent cancers have a common etiology: the estrogens. The catechol estrogen quinone metabolites react with DNA to generate the critical mutations that can lead to cancer initiation.

6.3 Metabolism of Estrogens

Metabolic formation of estrogens derives from aromatization of androstenedione and testosterone, catalyzed by cytochrome P450 (CYP)19 (aromatase) (Fig. 6.1). The estrogens E_1 and E_2 (Fig. 6.1) are interconverted by 17β-hydroxysteroid dehydrogenase. When an excess of estrogen is produced, it is stored as E_1-sulfate.

Estrogens are metabolized via two major pathways: formation of 16α-hydroxy$E_1(E_2)$ (not shown in Fig. 6.1) and formation of the catechol estrogens 2-$OHE_1(E_2)$ and 4-$OHE_1(E_2)$ (Fig. 6.1) [17]. CYP1A1 hydroxylates E_1 and E_2 preferentially at the 2-position, whereas CYP1B1 hydroxylates E_1 and E_2 almost exclusively at the 4-position [18–20]. The two catechol estrogens are inactivated by conjugation to glucuronides and sulfates, especially in the liver (not shown in Fig. 6.1). In extrahepatic tissues, the most common path of conjugation of catechol estrogens is *O*-methylation, catalyzed by catechol-*O*-methyltransferase (COMT) [21, 22]. More competitive oxidation of the catechol estrogens to semiquinones (SQ) and then to $E_1(E_2)$-2,3-quinone (Q) and $E_1(E_2)$-3,4-Q, catalyzed by CYP or peroxidase (Fig. 6.1), occurs when COMT activity is low. Oxidation of semiquinones to quinones can also be accomplished by molecular oxygen.

Completion of a redox cycle occurs by reduction of catechol estrogen quinones to semiquinones via CYP reductase (Fig. 6.1). In this process, the molecular oxygen is reduced to superoxide anion radical and then converted to hydrogen peroxide by superoxide dismutase. In the presence of Fe^{2+} ion, the hydrogen peroxide is converted to hydroxyl radical. Reaction of the hydroxyl radical with lipids produces lipid hydroperoxides (not shown in Fig. 6.1) [23]. The lipid hydroperoxides act as unregulated cofactors for the oxidation of catechol estrogens by cytochrome P450. Thus, redox cycling can become a major pathway to the formation of catechol estrogen quinones, which are the ultimate carcinogenic metabolites of estrogens.

Following the formation of catechol estrogen quinones (Fig. 6.1), they can be inactivated by glutathione (GSH). Another inactivation pathway for the quinones is reduction to their respective catechols by quinone reductase [24, 25], a protective enzyme that can be induced by a variety of compounds [26].

If the catechol estrogen quinones are not eliminated by protective processes, these quinones can react with DNA to form almost exclusively depurinating adducts. In fact, a mechanism of metabolic activation that produces extremely weak ultimate carcinogens occurs for a series of compounds, including benzene, the parent compound of aromatic chemistry, and the natural estrogens E_1 and E_2. In this mechanism, the compounds are enzymatically oxidized to yield

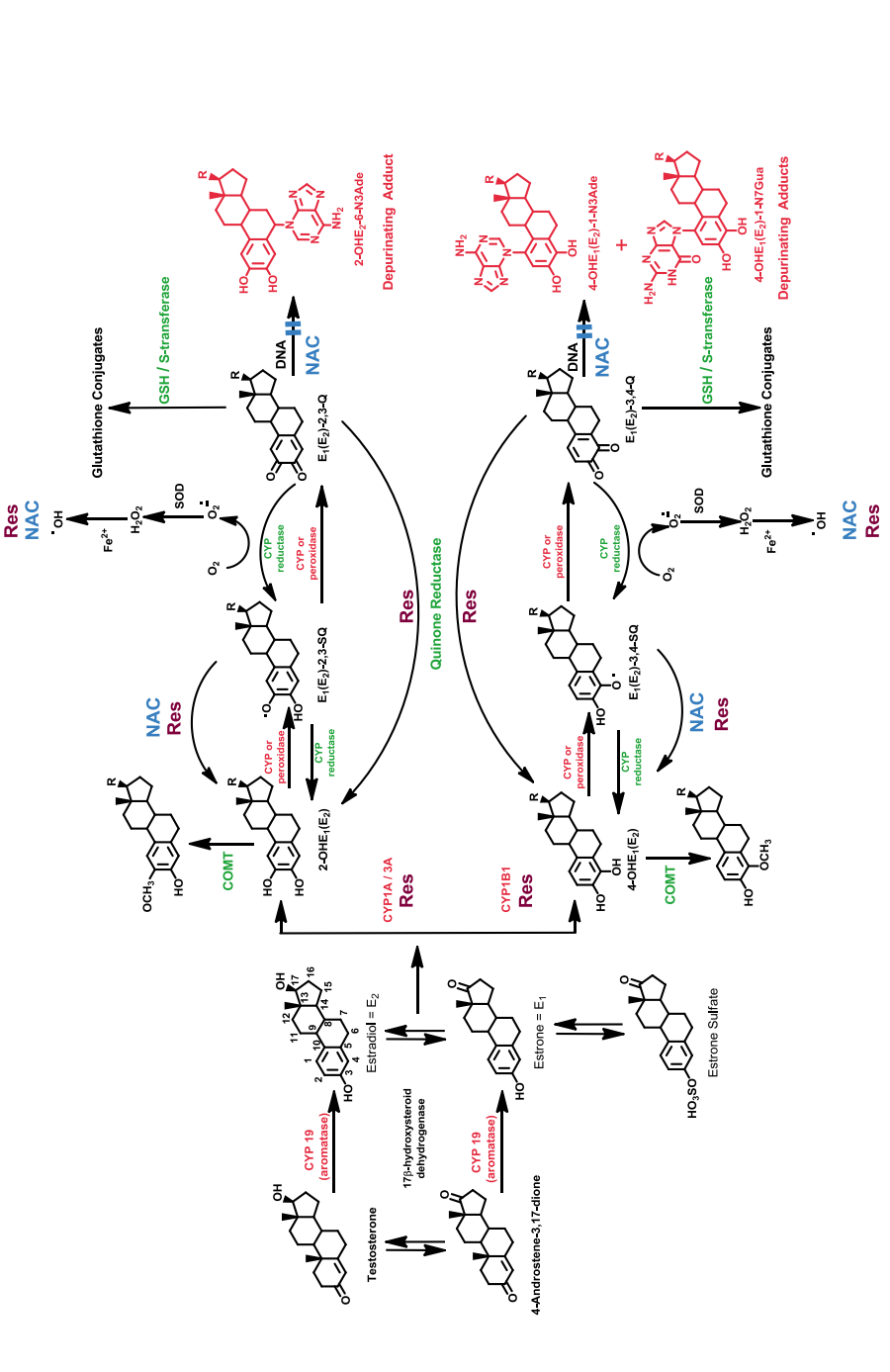

Fig. 6.1 Formation, metabolism and DNA adducts of estrogens. Activating enzymes and depurinating DNA adducts are in *red* and protective enzymes are in *green*. *N*-Acetylcysteine (NAC, shown in *blue*) and resveratrol (Res, shown in *burgundy*) indicate various steps where NAC and Res could ameliorate unbalanced estrogen metabolism and reduce formation of depurinating estrogen–DNA adducts

phenols. A second hydroxylation yields catechols, followed by a third oxidation to afford the electrophilic ultimate carcinogenic *ortho*-quinone metabolites. The metabolites can react with DNA by Michael addition to form predominantly depurinating adducts at the N-3 of adenine (Ade) and N-7 of guanine (Gua). These adducts detach from DNA, leaving behind DNA with apurinic sites. Erroneous repair of the apurinic sites can give rise to mutations that, in turn, could initiate cancer. This unifying mechanism of metabolic activation occurs with benzene [27, 28], naphthalene [29, 30], the natural estrogens E_1 and E_2 [12, 31–35], and the synthetic estrogens DES [16, 36] and hexestrol [34, 37, 38].

6.4 Depurinating Estrogen-DNA Adducts: Generators of Mutations and Cancer Initiation

Carcinogens react with DNA to form two types of adducts: stable adducts and depurinating adducts. Cancer researchers have always investigated only stable adducts, which remain in DNA unless removed by repair. These adducts are routinely detected and quantified by the ^{32}P-postlabeling technique, but their structure has not always been identified. Stable adducts are formed when electrophilic carcinogenic compounds react with the exocyclic amino group of Ade or Gua [32]. If formation of adducts takes place at the N-3 or N-7 of Ade, or the N-7 of Gua, the most nucleophilic sites in Ade and Gua [39], destabilization of the glycosyl bond and subsequent depurination of the adduct from DNA occurs [12, 32, 33]. The critical relevance of these depurinating adducts is still not recognized by cancer researchers 20 years after their discovery [40].

Evidence that depurinating DNA adducts play the predominant role in cancer initiation was first obtained from a correlation between the levels of depurinating polycyclic aromatic hydrocarbon (PAH)-DNA adducts and oncogenic Harvey (H)-*ras* mutations in mouse skin papillomas [40–42]. The very potent carcinogens 7,12-dimethylbenz[*a*]anthracene [43] and dibenzo[*a,l*]pyrene [44, 45] form predominantly depurinating Ade adducts and induce A to T transversions in codon 61 of the H-*ras* oncogene. Instead, benzo[*a*]pyrene yields approximately twice as many Gua depurinating adducts as Ade depurinating adducts in mouse skin [46], and twice as many codon 13 G to T transversions as codon 61 A to T transversions [40, 47].

A similar correlation between the sites of formation of depurinating DNA adducts and H-*ras* mutations was observed in mouse skin and rat mammary gland treated with E_2-3,4-Q [48, 49]. When $E_1(E_2)$-3,4-Q react with DNA, they form predominantly (>99 %) the depurinating adducts 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua by 1,4-Michael addition (Fig. 6.2) [12, 32, 33, 35], whereas $E_1(E_2)$-2,3-Q form a much lower amount of 2-$OHE_1(E_2)$-6-N3Ade by 1,6-Michael addition (Fig. 6.3) [35]. This product is obtained after tautomerization of the quinone to the $E_1(E_2)$-2,3-quinone methide [50].

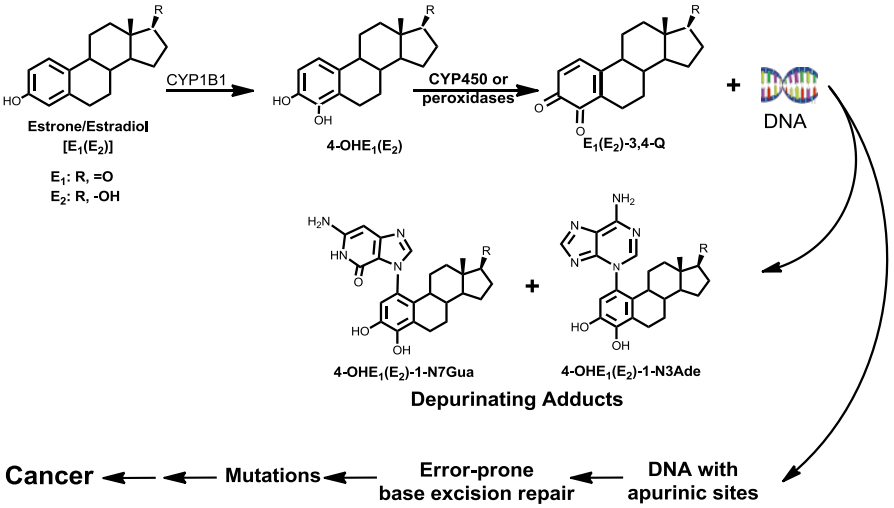

Fig. 6.2 The predominant metabolic pathway in cancer initiation by estrogens. The $E_1(E_2)$-3,4-Q react with DNA to form 97 % depurinating 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua adducts

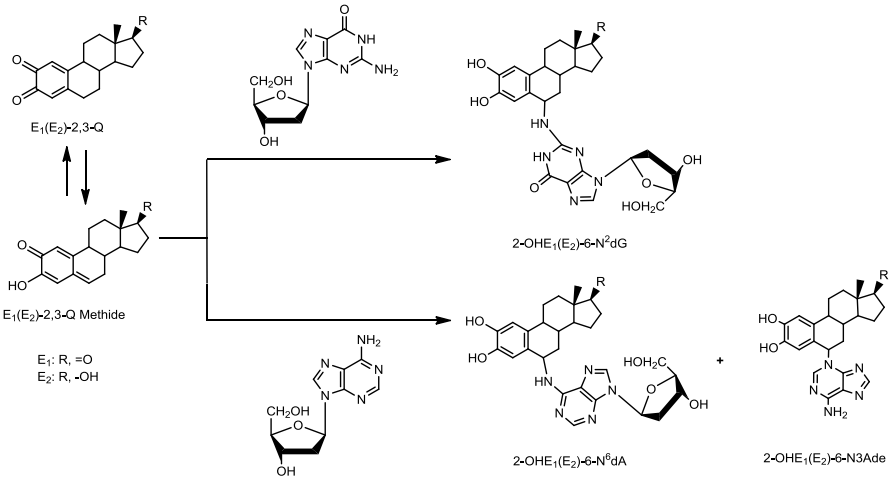

Fig. 6.3 Reaction of $E_1(E_2)$-2,3-Q with dG or dA in DNA to form stable adducts (minor) and the depurinating 2-$OHE_1(E_2)$-6-N3Ade adducts (major)

As mentioned above, the $E_1(E_2)$-3,4-Q react with DNA to form depurinating adducts much more effectively than the $E_1(E_2)$-2,3-Q do (Fig. 6.4a). In fact, when E_2-3,4-Q and E_2-2,3-Q are reacted together with DNA, to achieve comparable levels of adducts the mixture needs to contain 95 % E_2-2,3-Q and only 5 % E_2-3,4-Q [35]. Similar results are obtained from mixtures of 4-OHE_2 and 2-OHE_2 oxidized by tyrosinase in the presence of DNA (Fig. 6.4b). These results demonstrate the lesser effectiveness of E_2-2,3-Q in

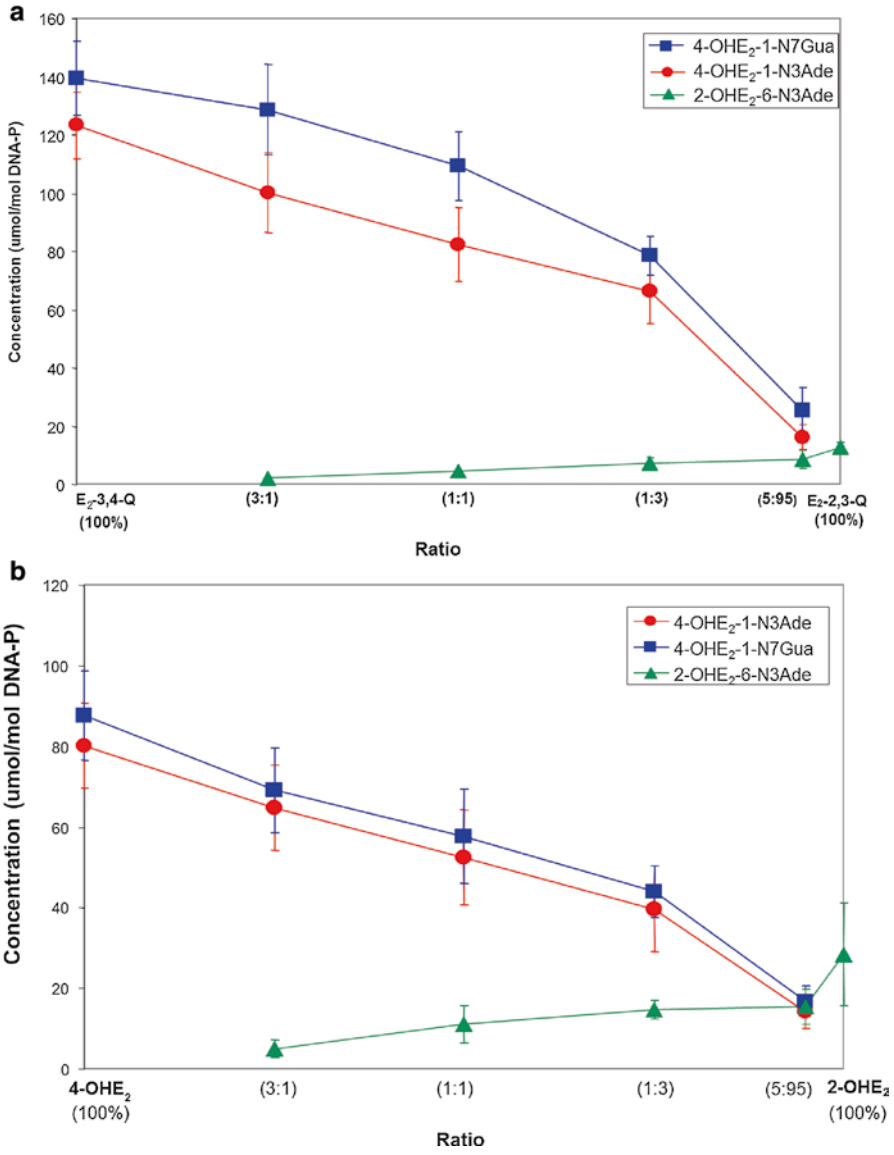

Fig. 6.4 Depurinating adducts formed by mixtures of (**a**) E_2-3,4-Q and E_2-2,3-Q at different ratios after 10 h of reaction with DNA. The level of stable adducts formed in the mixtures ranged from 0.1 to 1 % of total adducts, and (**b**) 4-OHE_2 and 2-OHE_2 in the presence of tyrosinase at different ratios after 10 h of reaction with DNA. The level of stable adducts formed in the mixtures ranged from 0.1 to 0.7 % of total adducts [35]

reacting with DNA to form depurinating adducts compared to E_2-3,4-Q. The $E_1(E_2)$-2,3-Q, instead, form 10 to 50 times higher levels of stable DNA adducts than $E_1(E_2)$-3,4-Q [31, 32]. The level of stable adducts formed by $E_1(E_2)$-2,3-Q is still much lower than the levels of the depurinating adducts, 2-$OHE_1(E_2)$-6-N3Ade [31, 35].

The levels of depurinating DNA adducts formed by the catechol estrogen quinones [35] are in agreement with the greater carcinogenic activity of 4-$OHE_1(E_2)$ compared with the borderline carcinogenic activity of 2-$OHE_1(E_2)$ [51–53]. The critical role of depurinating DNA adducts and the apurinic sites they generate has also been observed in the mutagenic activity of E_2-3,4-Q in mouse skin [48] and in rat mammary gland [49].

6.5 Error-Prone Repair as a Mechanism of Estrogen Mutagenesis

For many years estrogens were thought to be non-mutagenic, epigenetic carcinogens [54], but better, more appropriate test systems have shown that the $E_1(E_2)$-3,4-Q are mutagenic [48, 49, 55]. The mutations induced by estrogens arise from error-prone base excision repair of apurinic sites left in DNA by the depurinating estrogen-DNA adducts, rather than errors that arise during DNA replication. This is shown by the rapid appearance of mutations, in 6–12 h after treatment of mouse skin [48] or rat mammary gland [49] with E_2-3,4-Q, a period when DNA replication is repressed and excision repair is induced [56–58].

The mutagenicity of E_2-3,4-Q was demonstrated first in mouse skin [48], a model for PAH carcinogenesis. In this type of study, the dorsal skin of female SENCAR mice was treated with E_2-3,4-Q, the mice were sacrificed after several hours, and both the levels of estrogen-DNA adducts and the H-*ras* mutations were analyzed in the skin (Table 6.1). More than 99 % of the adducts formed were depurinating adducts, while only a tiny amount of stable adducts were apparent. H-*ras* mutations were detected after only 6 h and they began to diminish by 3 days [48]. These results indicate that the mutations do not arise by misreplication of the adducted DNA because induction of mutations by misreplication requires two rounds of DNA synthesis, which would take 3 days. Instead, the mutations quickly arise by error-prone base excision repair of the apurinic sites left in the DNA by depurination of the estrogen-DNA adducts.

Similar results were observed in female ACI rat mammary glands treated with E_2-3,4-Q by intramammillary injection (Table 6.1) [49]. Once again, predominant amounts of the 4-OHE_2-1-N3Ade and 4-OHE_2-1-N7Gua adducts and tiny amounts of stable adducts were detected. By 6 h after treatment, H-*ras* mutations were easily detected. These results confirm that the estrogen-induced mutations arise by error-prone repair of the DNA.

Further studies of estrogen mutagenicity were conducted in the female Big Blue (BB) rat and the BB rat2 embryonic cell line; both the rat and the cell line contain copies of the transgenic lambda-LIZ vector, and mutations in the *lac*I

Table 6.1 Mutagenicity of E_2-3,4-Quinone

	Depurinating adducts		Stable adducts	H-*ras* mutations	
	μmol/mol DNA-P		μmol/mol DNA-P	A→ G	Other
Tissue	4-OHE_2-1-N3Ade	4-OHE_2-1-N7Gua		Total clones	Total clones
SENCAR mouse skin[a]	12.5	12.1	0.004		
6 h				5/29	2/29
12 h				4/30	2/30
1 day				7/50	4/50
3 days				3/40	1/40
ACI rat mammary gland[b]	81	90	0.017		
6 h				16/29	3/29
12 h				14/34	6/34

[a]48

[b]49

and/or *cII* gene can be analyzed. Treatment of the BB rat2 cells with 4-OHE_2 or E_2-3,4-Q generated low levels of mutations, but treatment with 2-OHE_2 did not generate any detectable mutations [55]. In addition, when the female BB rats were implanted with 4-OHE_2, mutations in the *cII* gene were detected [59]. These results demonstrate that 4-OHE_2, as well as E_2-3,4-Q, induce mutations, but 2-OHE_2 does not. In addition, the estrogen-induced mutations quickly arise via error-prone repair of apurinic sites generated by depurination of estrogen-DNA adducts.

6.6 Estrogen-Initiated Transformation of Cells and Carcinogenicity in Animal Models

Treatment of cultured breast epithelial cells from women or mice has provided evidence that initiation of cancer occurs by formation of depurinating estrogen-DNA adducts. Most of these studies have been conducted in the MCF-10F cell line, an immortalized non-transformed ER-α-negative human cell line. When these cells are treated with E_2 or 4-OHE_2, the depurinating 4-OHE_2-1-N3Ade and 4-OHE_2-1-N7Gua adducts are formed [60–62]. When the MCF-10F cells are treated with E_2 or 4-OHE_2 at doses of 0.007–3.5 nM, the cells are transformed, as detected by formation of colonies in soft agar [63, 64]. Transformation occurs even when the cells are treated with both the estrogen and the antiestrogen tamoxifen or ICI-182,780 [65]. In contrast, these changes are induced by 2-OHE_2 to a much smaller extent [63, 64]. All of these results indicate that transformation of the cells is induced by the genotoxic effects of E_2-3,4-Q.

When the most invasive estrogen-transformed MCF-10F cells were implanted into severely compromised immunodeficient (SCID) mice, tumors developed [66]. In summary, treatment of human breast epithelial cells lacking ER-α with E_2 or 4-OHE_2 produces depurinating estrogen-DNA adducts that can lead to transformation of the cells, as happens in the initiation of cancer.

In a similar type of study, treatment of the immortalized non-transformed E6 mouse mammary cell line with 4-OHE_2 or E_2-3,4-Q leads to the formation of depurinating estrogen-DNA adducts and transformation of the cells to grow in soft agar [67]. These results demonstrate that estrogen genotoxicity induces transformation of breast cells in both human and animal models. Cultured human endometrial cells also form depurinating estrogen-DNA adducts [68].

The induction of cancer by estrogens has been demonstrated in several animal models. Implantation of male Syrian golden hamsters with E_1, E_2, DES or hexestrol induced kidney tumors in the animals [69]. Subsequently, 4-$OHE_1(E_2)$ were found to induce kidney tumors in the hamsters, but 2-$OHE_1(E_2)$ did not [51, 52]. In a different model, female CD-1 mice, 2-$OHE_1(E_2)$ were shown to have borderline activity in inducing uterine tumors, but the 4-$OHE_1(E_2)$ demonstrated much higher activity in this model [53]. The much smaller ability of the $E_1(E_2)$-2,3-Q to react with DNA and form adducts, compared to the greater ability of the $E_1(E_2)$-3,4-Q, is consistent with the lack or very low level of carcinogenicity of the 2-$OHE_1(E_2)$ compared to the 4-$OHE_1(E_2)$.

The carcinogenicity studies reported above do not provide any information on the mechanism of estrogen carcinogenicity, i.e., whether the mechanism involves estrogen genotoxicity or ER-α-mediated events. This question was answered, however, by studies with a strain of mice in which ER-α was knocked out (ERKO). The ERKO/*wnt*-1 mouse was developed by Bocchinfuso et al. [70, 71]. Mammary tumors develop in 100 % of female *wnt*-1 transgenic mice, and it was expected that knocking out ER-α would prevent the development of these tumors. Nonetheless, 100 % of the female ERKO/*wnt*-1 mice developed mammary tumors, although at a slower rate than the parent *wnt*-1 mice [71]. Both 4-$OHE_1(E_2)$ and the GSH conjugates of $E_1(E_2)$-3,4-Q were detected in mammary tissue harvested from female ERKO/*wnt*-1 mice [72]. In contrast, no methoxy estrogens were detected in this tissue, suggesting that the mice have poor ability to protect the 4-$OHE_1(E_2)$ from oxidation to the reactive $E_1(E_2)$-3,4-Q.

To demonstrate that the mammary tumors are induced by estrogens, formation of mammary tumors was followed in ovariectomized female ERKO/*wnt*-1 mice implanted with one of several doses of E_2. Mammary tumors developed in a dose-dependent manner [73, 74], even when mice were simultaneously implanted with E_2 and the antiestrogen ICI-182,780 [75]. In summary, these results in ERKO/*wnt*-1 mice provide stronger evidence that estrogens initiate cancer through their genotoxicity.

Therefore, the estrogens are carcinogenic in specific animal models and they are capable of inducing transformation of human breast epithelial cells. These results support the genotoxic mechanism leading to estrogen carcinogenesis.

6.7 Analysis of Depurinating Estrogen-DNA Adducts, the Generators and Biomarkers of Cancer Initiation, in Human Subjects

Development of biomarkers for cancer risk has been a major goal in cancer research for decades. Analysis of depurinating estrogen-DNA adducts, catechol estrogen metabolites and catechol estrogen conjugates provides biomarkers of risk that are related to the critical step in the initiation of a number of prevalent human cancers.

6.7.1 Breast Cancer

Three case-control studies have been conducted in women diagnosed with breast cancer or at normal or high risk of breast cancer [76–78]. The high-risk women were identified by using the Gail model score to estimate a 5-year risk greater than 1.66 % [79]. Calculation of the Gail model score is based on age, age at menarche, age at first birth, prior breast biopsies or atypical hyperplasia, and number of first degree relatives with breast cancer.

In the first two studies, each subject provided a spot urine sample (~50 mL), and an aliquot was partially purified by solid-phase extraction and analyzed for 38 estrogen metabolites, conjugates and depurinating DNA adducts [76, 77]. The estrogen analytes were identified and quantified by using ultraperformance liquid chromatography/tandem mass spectrometry, and the ratio of the adducts, 4-$OHE_1(E_2)$-1-N3Ade, 4-$OHE_1(E_2)$-1-N7Gua and 2-$OHE_1(E_2)$-6-N3Ade, to their respective metabolites and conjugates was calculated for each subject (Fig. 6.5a, b).

$$\text{ratio} = \left(\frac{\text{4-OHE}_1(\text{E}_2)\text{-1-N3Ade} + \text{4-OHE}_1(\text{E}_2)\text{-1-N7Gua}}{\text{4-catechol estrogens} + \text{4-catechol estrogen conjugates}} + \frac{\text{2-OHE}_1(\text{E}_2)\text{-1-N3Ade}}{\text{2-catechol estrogens} + \text{2-catechol estrogen conjugates}} \right) \times 1000$$

In the first study (Fig. 6.5a) of 46 normal-risk women, 12 high-risk women and 17 women diagnosed with breast cancer, the ratios in the high-risk ($p < 0.001$) and breast cancer ($p < 0.001$) were significantly higher than the ratios in the normal-risk women [76]. Similar differences were observed in the second study (Fig. 6.5b) between 40 normal-risk women, 40 high-risk women and 40 women with breast cancer (both $p < 0.001$) [77].

In the third study, serum was collected from each of the 74 normal-risk women, 80 high-risk women and 79 women diagnosed with breast cancer [78]. Once again, the ratio of adducts to metabolites and conjugates was significantly lower in the women at normal risk, compared to the high-risk and breast cancer groups (both $p < 0.001$) (Fig. 6.5c). The same significant differences were observed between the three groups

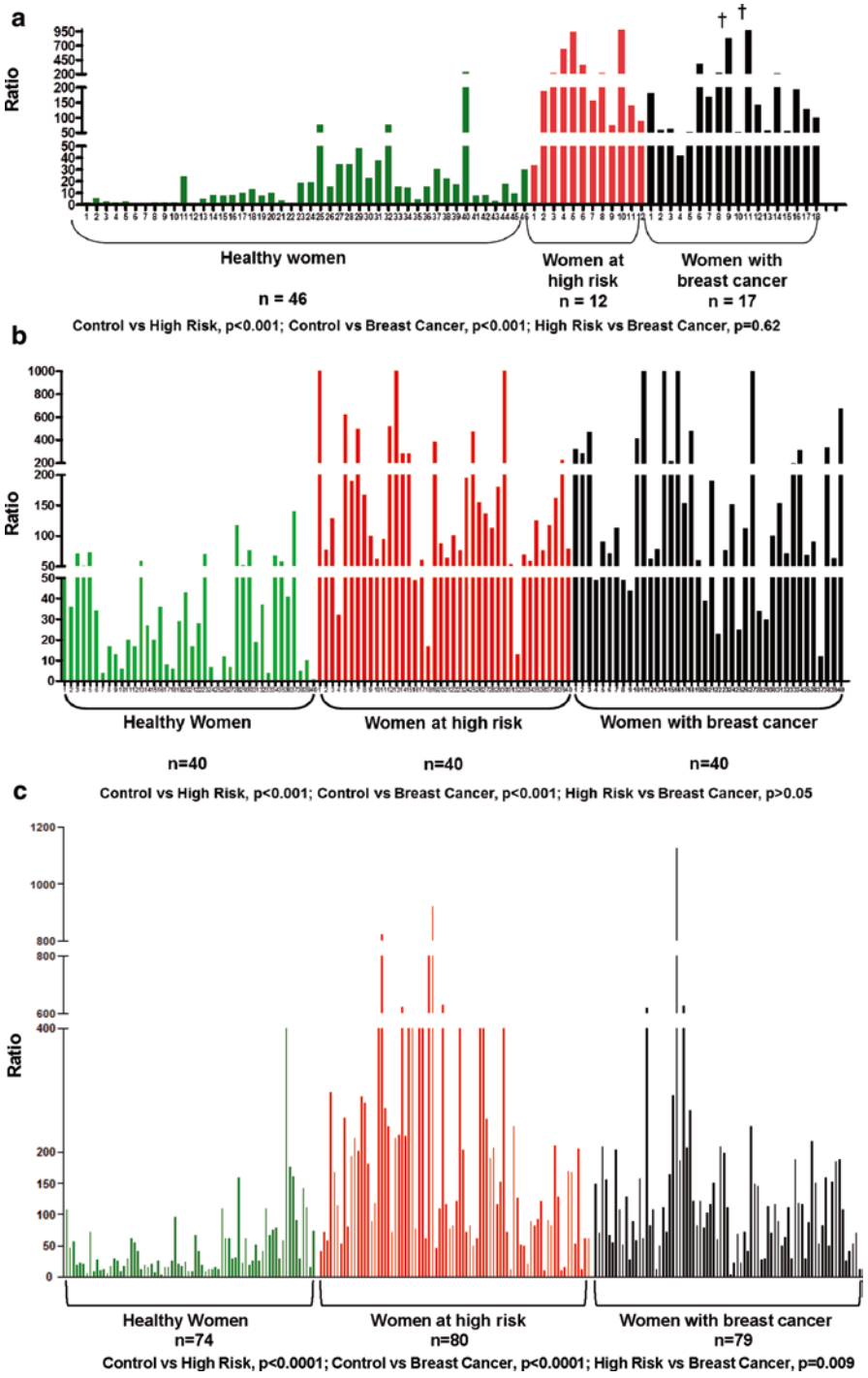

Fig. 6.5 Ratios of depurinating estrogen-DNA adducts to estrogen metabolites and conjugates in (**a**) urine of healthy women, high-risk women and women with breast cancer—first study [76]; (**b**) urine of healthy women, high-risk women and women with breast cancer—second study [77] and (**c**) serum of healthy women, high-risk women and women with breast cancer [78]

when the subjects were separated into premenopausal and peri- plus post-menopausal women, demonstrating that menopausal status had no effect on the findings [78].

In all three studies, the high ratios typically arose from high levels of adducts and low levels of metabolites and conjugates, although in some samples the levels of adducts were average, but the levels of metabolites and conjugates were very low [76–78]. These results indicate that in either case most of the estrogen present had been metabolized into catechol estrogen quinones, which could react with DNA to form adducts.

It was also observed in all three studies that the 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua adducts played the predominant role (>97 %), and the 2-$OHE_1(E_2)$-6-N3Ade adducts played a very minor role. This finding derives from the poor ability of $E_1(E_2)$-2,3-Q to react with DNA compared to $E_1(E_2)$-3,4-Q [35], and it correlates with the borderline carcinogenic activity of 2-$OHE_1(E_2)$ in animal models [51–53].

Overall, the high ratio of depurinating estrogen-DNA adducts to metabolites and conjugates serves as a biomarker of women at high risk for breast cancer. Similar results have been observed with other types of cancer.

6.7.2 Ovarian Cancer

A similar study was conducted using urine samples from 34 women diagnosed with ovarian cancer and 33 healthy control women [80]. Once again, the women diagnosed with epithelial ovarian cancer had significantly higher ratios of estrogen-DNA adducts to estrogen metabolites and conjugates ($p < 0.0001$) (Fig. 6.6), indicating that estrogen metabolism is unbalanced in women with ovarian cancer.

As part of the study of women with and without ovarian cancer, saliva samples were collected, DNA was purified and single nucleotide polymorphisms (SNPs) in CYP1B1 (V432L) and COMT (V158M) were analyzed. The DNA adduct ratio was higher in women with one or two high-activity alleles of CYP1B1, with a dose-response relationship. In women with two copies of the low-activity COMT allele, the high-activity CYP1B1 allele was associated with a significantly increased DNA adduct ratio. The combination of two high-activity CYP1B1 alleles and two low-activity COMT alleles raised the odds ratio of having ovarian cancer almost 6-fold, compared to women with the normal-activity alleles of these two enzymes [80]. These results indicate a strong association between unbalanced estrogen metabolism and initiation of ovarian cancer.

6.7.3 Thyroid Cancer

Finally, a study was conducted in women diagnosed with thyroid cancer. Well-differentiated thyroid cancer is observed most frequently in premenopausal women [81], and women with thyroid cancer seem to be at greater risk for also developing breast cancer [82]. Estrogen-DNA adducts, metabolites and conjugates were

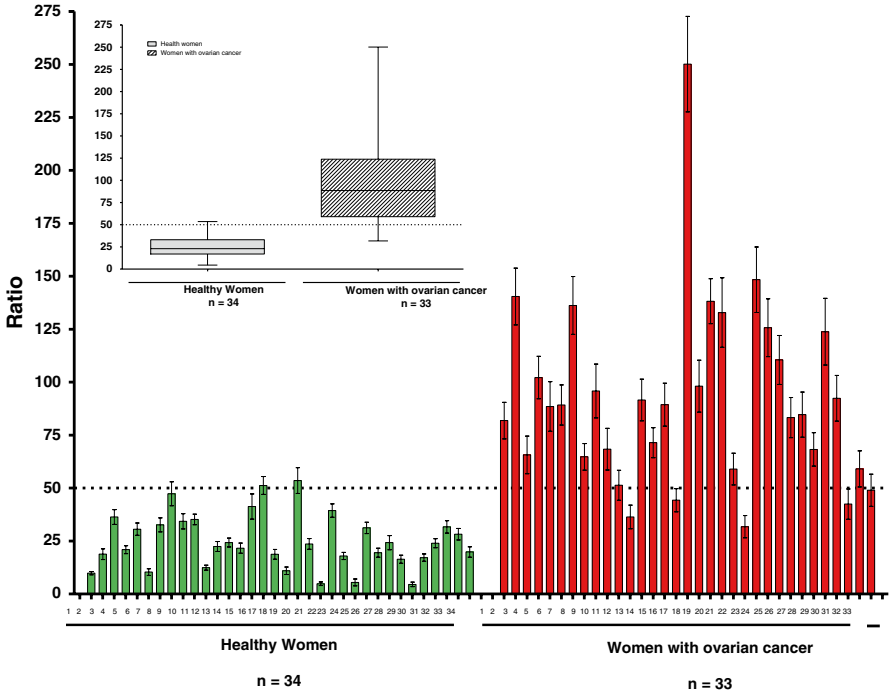

Fig. 6.6 Ratios of depurinating estrogen-DNA adducts to estrogen metabolites and conjugates in urine samples from healthy control women and women diagnosed with ovarian cancer. The ratios were significantly higher in cases ($p < 0.0001$) [80]

analyzed in urine samples from 40 women diagnosed with thyroid cancer and 40 healthy controls [83]. Once again, the women diagnosed with thyroid cancer had much higher ratios of estrogen-DNA adducts to estrogen metabolites and conjugates ($p < 0.0001$) (Fig. 6.7), suggesting that the ratio could be used as a biomarker for risk of developing thyroid cancer.

6.7.4 Prostate Cancer

Unbalanced estrogen metabolism leading to the formation of estrogen-DNA adducts occurs in men as well as women. A case-control study of men with and without prostate cancer was conducted by analyzing 38 estrogen metabolites, conjugates and depurinating DNA adducts in urine samples from 14 men diagnosed with prostate cancer (age 50 or older) and 125 healthy control men (age 45–83). The ratio of depurinating estrogen-DNA adducts to estrogen metabolites and conjugates was significantly higher in the men diagnosed with prostate cancer compared to the healthy control men ($p < 0.001$) (Fig. 6.8) [84]. These results suggest that the DNA adduct ratio could be a biomarker for risk of developing prostate cancer.

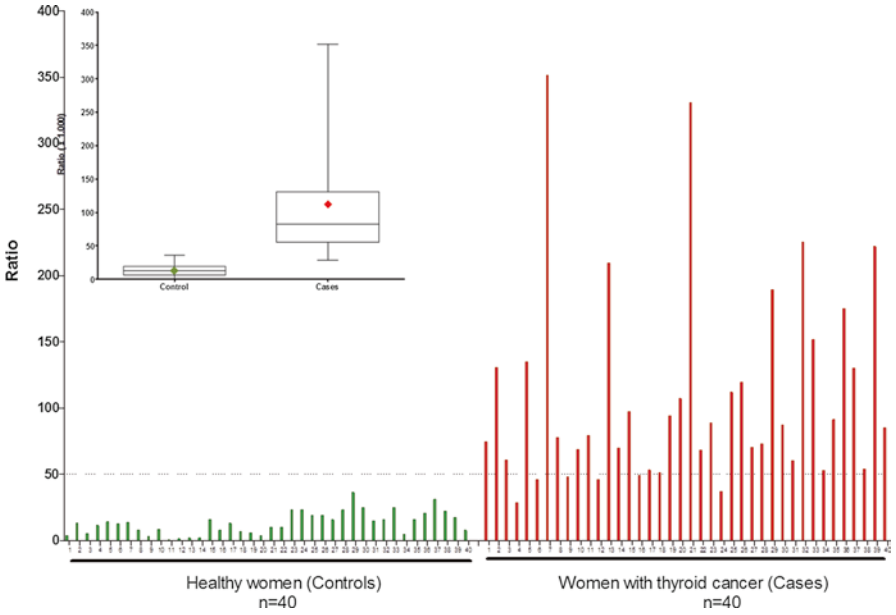

Fig. 6.7 Ratios of urinary depurinating estrogen-DNA adducts to estrogen metabolites and conjugates for women diagnosed with thyroid cancer (cases) or not diagnosed with cancer (controls). The dotted line representing a ratio of 50 is the cross-over point for sensitivity and specificity of the ratio. *Inset*: Ratios presented as median values and ranges (min to max). The *diamonds* represent the mean values ($p < 0.0001$) [83]

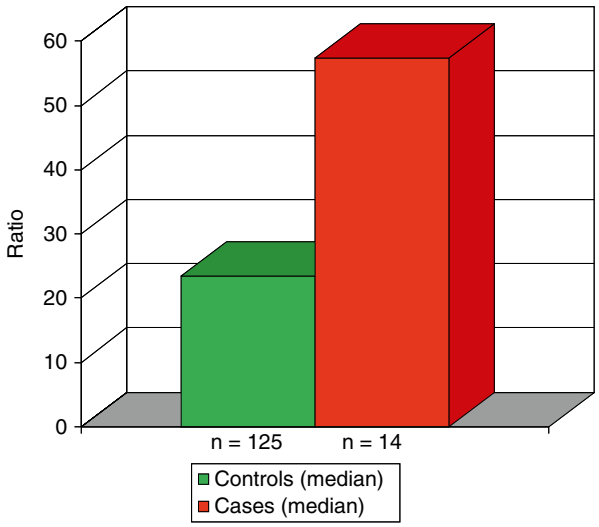

Fig. 6.8 Average levels of the ratios of estrogen-DNA adducts to estrogen metabolites and conjugates in urine samples from men with and without prostate cancer, $p < 0.001$ [84]

6.7.5 Non-Hodgkin Lymphoma

A similar study was conducted by using urine samples from 15 men diagnosed with non-Hodgkin lymphoma and 30 healthy control men [85]. Once again, men diagnosed with non-Hodgkin lymphoma had significantly higher ratios of estrogen-DNA adducts to estrogen metabolites and conjugates, compared to the healthy control men ($p < 0.0007$) (Fig. 6.9).

6.7.6 Related Studies

Estrogen quinone-derived hemoglobin adducts have been detected in blood samples from women with breast cancer [86], and depurinating estrogen-DNA adducts have been observed in breast tissue from women with breast cancer [87]. The ratio of depurinating estrogen-DNA adducts to their estrogen metabolites and conjugates, however, provides a biomarker of risk that is related to the initiating step of breast and other prevalent types of human cancer.

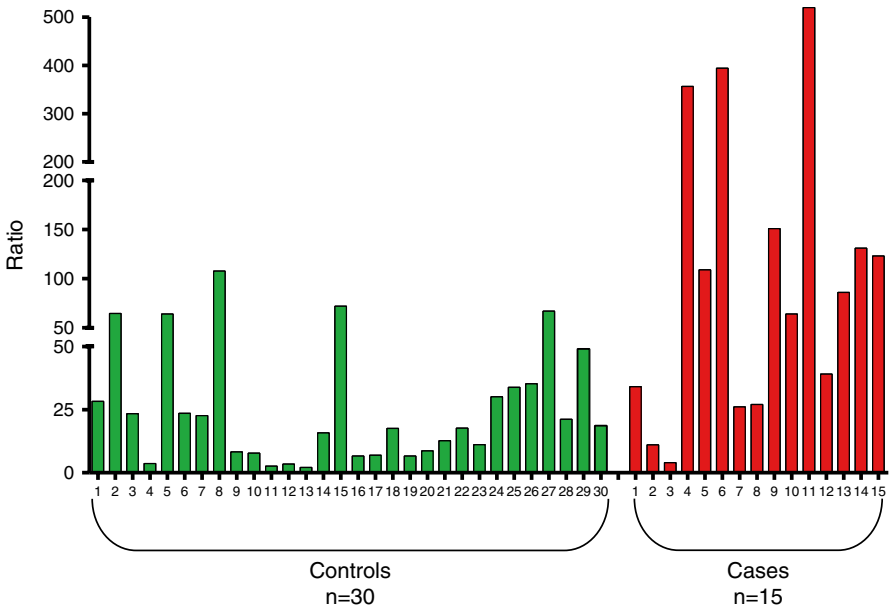

Fig. 6.9 Individual ratios of depurinating estrogen–DNA adducts to estrogen metabolites and conjugates in urine of healthy control men and men with non-Hodgkin lymphoma (NHL). Healthy controls vs. NHL, $p < 0.007$ [85]

6.7.7 *Summation*

In summary, unbalanced estrogen metabolism leading to higher ratios of estrogen-DNA adducts to estrogen metabolites and conjugates is observed in women at high risk for breast cancer or diagnosed with breast, ovarian or thyroid cancer, as well as men diagnosed with prostate cancer or non-Hodgkin lymphoma. We think that other prevalent types of cancer, which have not yet been investigated for estrogen-DNA adduct formation, are also initiated by estrogens.

Comparison of the sensitivity and specificity curves for the ratio levels in the groups of women studied shows a cut-point of 77 for breast cancer [78], 43 for ovarian cancer [80], and 30 for thyroid cancer [83]. This suggests a DNA adduct ratio in the range of 30–77 is a borderline value for indicating high risk for developing these cancers. For example, based on these initial results in women with breast, ovarian or thyroid cancer, a ratio above 77 would indicate high risk, while a ratio below 30 would indicate low risk. With the current data, DNA adduct ratios between 30 and 77 can only be considered indeterminate. With larger groups of subjects and continued collection of data, this diagnostic value for the DNA adduct ratio can be refined.

6.8 Formation of Dopamine-DNA Adducts: Potential Role in the Etiology of Parkinson's Disease

The neurotransmitter dopamine (DA) is formed in the cell bodies of the dopaminergic neurons of the *substantia nigra*. Degeneration of the nigrostriatal dopaminergic neurons and decreased production of DA results in Parkinson's disease (PD).

One of the major metabolic pathways of DA is the oxidation of DA to its quinone, which at neutral pH regularly undergoes intramolecular cyclization by 1,4-Michael addition to form leukochrome, followed by oxidation to aminochrome. Polymerization of the aminochrome leads to neuromelanin (Fig. 6.10). At lower pH (pH 5–6), however, the amino group of DA becomes partially protonated, slowing down the intramolecular cyclization of DA and leading to a competitive intermolecular 1,4-Michael addition with nucleophiles, including those of DNA (Figs. 6.10 and 6.11). Under these conditions, reaction of DA quinone with DNA leads to formation of the depurinating DA adducts, DA-6-N3Ade and DA-6-N7Gua (Figs. 6.10 and 6.11) [27, 28, 88]. The mutations generated by DNA damage may play the crucial role in initiating the series of events leading to neurodegeneration and PD (Fig. 6.10).

The reaction of DA quinone with DNA under slightly acidic conditions (pH 5–6) is analogous to the mechanism of metabolic activation of the natural estrogens E_1 and E_2 [12, 31–33, 35, 61], the synthetic estrogens DES and hexestrol [16, 34, 36–38], benzene [27, 28] and naphthalene [29, 30], in which the quinones react with DNA by 1,4-Michael addition to form analogous depurinating N3Ade and N7Gua adducts that generate the critical mutations leading to cancer initiation [15, 48, 49].

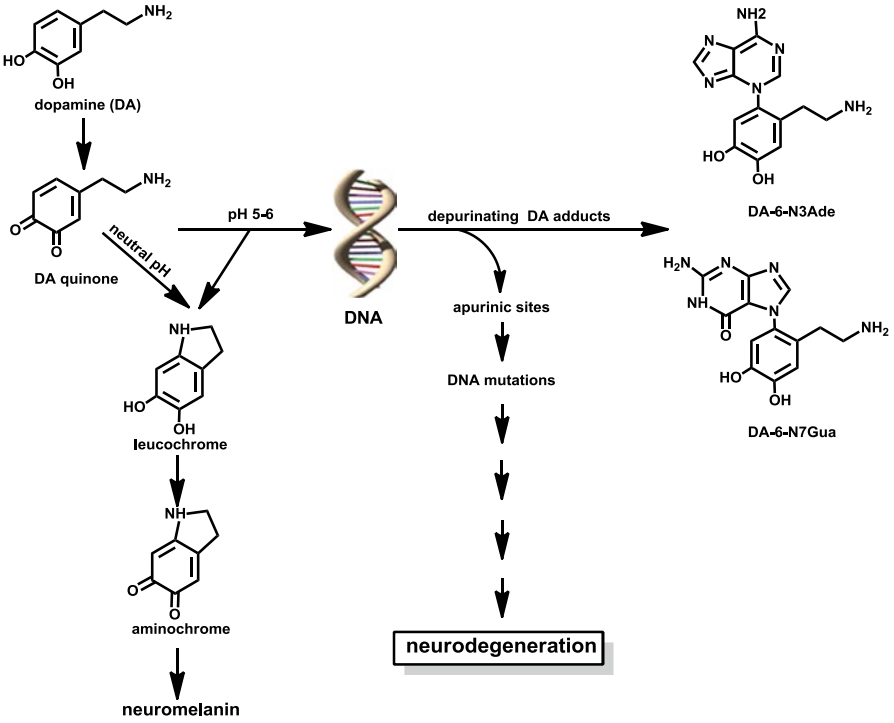

Fig. 6.10 Intramolecular 1,4-Michael addition of dopamine quinone at neutral pH to form neuromelanin and competitive intermolecular 1,4-Michael addition at pH 5–6, with formation of the depurinating adducts DA-6-N3Ade and DA-6-N7Gua

It has been reported that an excessive amount of glutamate, as occurs in excitotoxicity, can give rise to a stable pH 5.5 during glutamate and DA corelease from synaptic vesicles [89]. These data suggest that DA quinone could form DNA adducts *in vivo* under these conditions. This proposed mechanism provides a solid foundation for the specific loss of dopaminergic neurons observed in PD.

In conclusion, DA under slightly acidic conditions (pH 5–6) would be the initiator of PD by forming the depurinating N3Ade and N7Gua adducts (Fig. 6.10), similarly to the estrogens in the initiation of cancer (Fig. 6.2). The apurinic sites generated in DNA would give rise to mutations leading to neurodegeneration and development of PD (Fig. 6.10).

6.9 Prevention of Estrogen-Initiated Cancer by *N*-Acetylcysteine and Resveratrol

Metabolism of estrogens via the catechol estrogen pathway is normally in homeostasis, with a balanced set of activating and protective enzymes (Fig. 6.1). In homeostasis, oxidation of catechol estrogens to quinones, which can react with DNA, is minimized. When homeostasis is disrupted, excessive formation of catechol estrogen

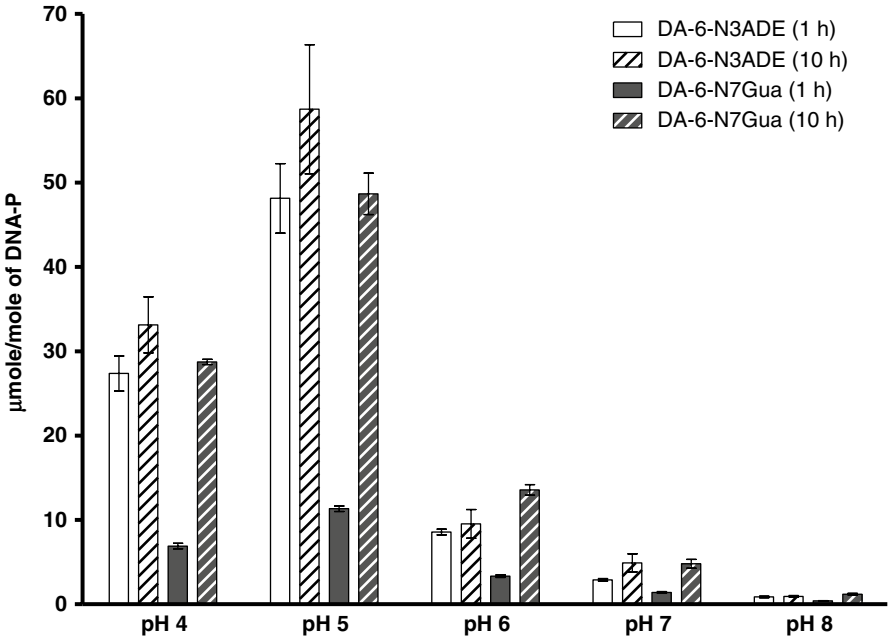

Fig. 6.11 Effect of pH on the formation of depurinating adducts after reaction of tyrosinase-activated DA with DNA [88]

quinones can occur and then reaction with DNA can lead to cancer initiation. Such disruptions can be caused by a variety of factors, including diet, environment, genetics, lifestyle and aging.

The catechol estrogen pathway includes five key enzymes. These are the activating enzymes CYP19 (aromatase) and CYP1B1 (Fig. 6.1), which promote oxidation of 4-OHE$_1$(E$_2$) to E$_1$(E$_2$)-3,4-Q. These quinones react with DNA to form estrogen-DNA adducts, generate apurinic sites and initiate cancer. The protective enzymes, COMT, quinone reductase and glutathione-*S*-transferase (GST), reduce the levels of E$_1$(E$_2$)-3,4-Q, thereby inhibiting formation of adducts and decreasing the risk of initiating cancer (Fig. 6.1) [14, 90]. GSH itself can reduce the E$_1$(E$_2$)-3,4-Q, but GST catalyzes this reaction more efficiently.

Non-tumor breast tissue from women diagnosed with breast cancer tends to have high levels of the activating enzymes CYP19 and CYP1B1 and low levels of the protective enzymes COMT and quinone reductase [91]. In contrast, breast tissue from women not diagnosed with breast cancer tends to have high levels of the protective enzymes COMT and quinone reductase and low levels of the activating enzymes CYP19 and CYP1B1 [91].

A major factor contributing to the risk of developing estrogen-initiated cancers is the levels of these activating and protective enzymes. Maintaining homeostasis or mitigating disrupted homeostasis can be accomplished by using specific dietary supplements, such as *N*-acetylcysteine (NAC) and resveratrol (Res) (Fig. 6.1). Each of these compounds can affect the biosynthesis of catechol estrogen quinones and their reaction with DNA by multiple similar and different protective mechanisms.

6.9.1 N-Acetylcysteine

The ability of NAC (Fig. 6.12) to prevent formation of estrogen-DNA adducts derives from its nucleophilicity, antioxidant properties, and reaction with the quinones themselves. Hydrolysis of NAC by acylase in the liver and gut yields cysteine (Cys), a precursor to intracellular GSH, which guarantees replenishment of this critical cellular scavenger. Low levels of GSH have been implicated in cancer etiology and progression [92], but GSH is not effective as a dietary supplement because it cannot cross cell membranes. Cys also cannot be used as a human dietary supplement because of its toxicity. In contrast, NAC not only has very low toxicity in humans, but it also has the very important property of crossing the blood-brain barrier [93].

Since 1998, NAC has been known to react efficiently with catechol estrogen quinones (Fig. 6.1) [94, 95] to prevent their reaction with DNA. When E_2-3,4-Q or peroxidase-activated 4-OHE_2 was reacted with DNA *in vitro*, NAC inhibited formation of the 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua adducts in a dose-dependent manner [96]. In inhibiting the reaction of E_2-3,4-Q with DNA, NAC reacted with the quinone itself, diminishing adduct formation approximately 70 %. In the reaction of peroxidase-activated 4-OHE_2, NAC not only reacted with the quinone, but also reduced the E_2-3,4-SQ back to 4-OHE_2, inhibiting the formation of adducts by about 84 % [96]. The ability of NAC to reduce estrogen semiquinones to catechols (Fig. 6.1) was previously demonstrated by Samuni et al. [97]. In cells, the ability of NAC to react with $E_1(E_2)$-3,4-Q is enhanced by the similar ability of GSH to react with them; in addition, NAC supports biosynthesis of GSH, which in turn can generate NAC by the mercapturic acid biosynthesis pathway [98].

The ability of NAC to inhibit formation of the 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua adducts can be studied in mammalian cell lines. The MCF-10F cell line is particularly useful because it is an immortalized but not transformed line of human breast epithelial cells and the cells lack ER-α [66]. Formation of these depurinating adducts is inhibited when the cells are incubated with both E_2-3,4-Q and NAC (Fig. 6.13a) [99]. When MCF-10F cells are treated with both 4-OHE_2 and NAC, even greater inhibition is observed (Fig. 6.13b), because NAC reduces any E_2-3,4-SQ back to 4-OHE_2, as well as reacting with E_2-3,4-Q [97, 99].

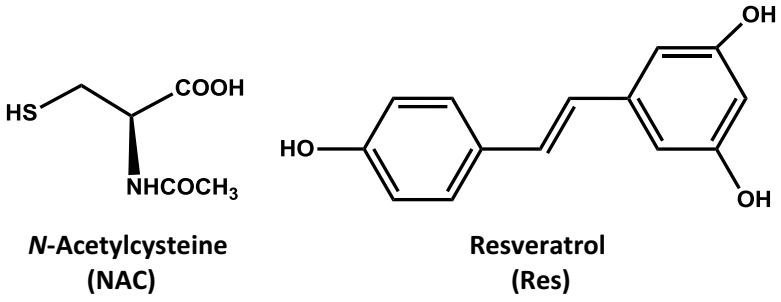

Fig. 6.12 Structures of *N*-acetylcysteine and resveratrol

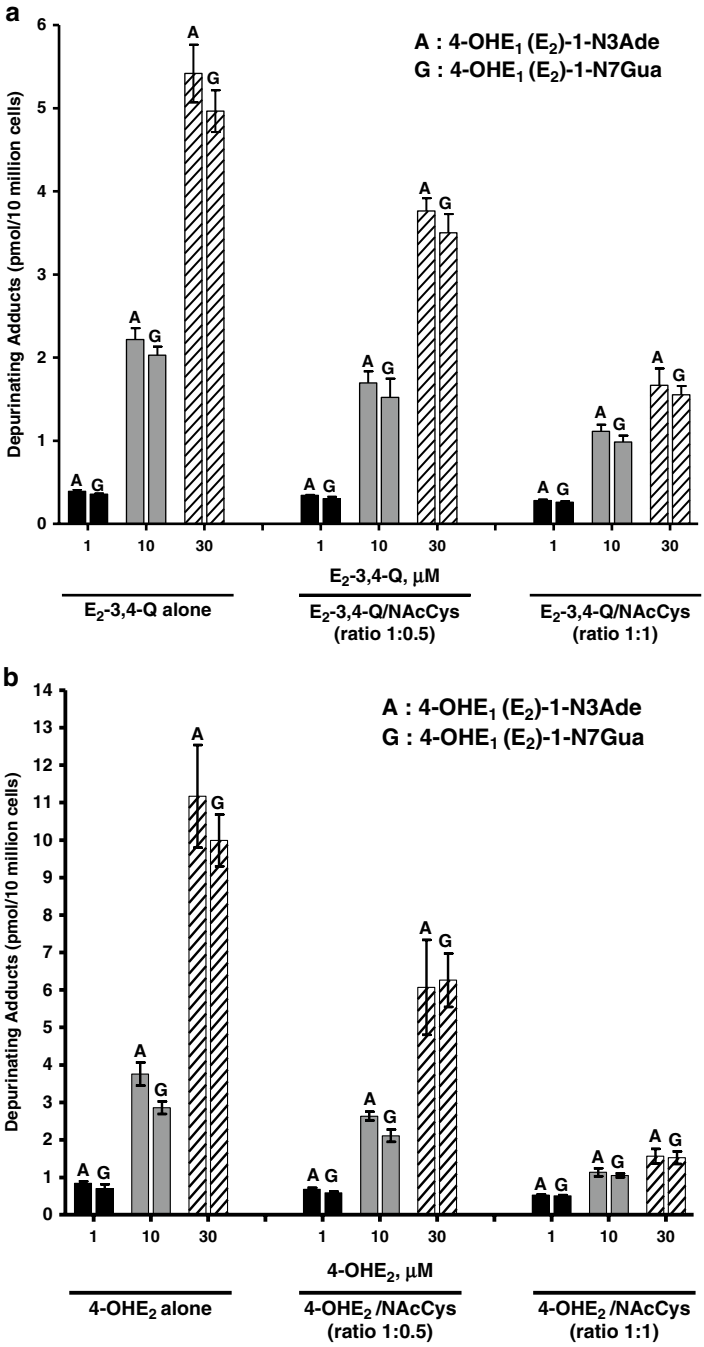

Fig. 6.13 Effects of NAC on the formation of (**a**) estrogen-DNA adducts in MCF-10F cells treated with E_2-3,4-Q and (**b**) estrogen-DNA adducts in MCF-10F cells treated with 4-OHE_2 [99]

In the immortalized mouse mammary cell line E6, similar results were observed in cells treated with 4-OHE_2 or E_2-3,4-Q and NAC [67]. E6 cells form similar amounts of the 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua adducts as the MCF-10F cells, as well as having similar levels of inhibition by NAC. With simultaneous treatment of E6 cells with 4-OHE_2 or E_2-3,4-Q and NAC, transformation of E6 cells is also inhibited [67]. Once again, greater inhibition is observed in cells treated with 4-OHE_2 plus NAC because it not only reduces any E_2-3,4-SQ formed back to 4-OHE_2, but also reacts with the E_2-3,4-Q that is formed [97, 99].

The above results demonstrate that NAC acts as an antioxidant by reducing the estrogen semiquinones back to catechol estrogens, it replenishes GSH in the cell, and acts as an antimutagen and anticarcinogen by reacting with $E_1(E_2)$-3,4-Q to prevent formation of the estrogen-DNA adducts.

6.9.2 Resveratrol

Grapes, peanuts, wine and various plants are good sources of Res. It exerts multiple effects in cells and has been shown to be chemopreventive in a variety of systems [100, 101], to modulate CYP1A1 and CYP1B1 (Fig. 6.1) [102–105], to have antimutagenic and anticarcinogenic properties [101, 106], to have antioxidant and anti-inflammatory properties [107–109], to reduce estrogen semiquinones to catechol estrogens [110, 111] and to induce quinone reductase [110–113]. Easy extraction of hydrogen from the 4′-OH bond occurs, with formation of a 4′-oxyradical that has great resonance stabilization energy (Fig. 6.12) [114]. All of the biological effects of Res have been demonstrated despite its low bioavailability, as determined following single doses to human subjects [115, 116].

Res does not inhibit the reaction of E_2-3,4-Q with DNA to form the 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua adducts by reacting with $E_1(E_2)$-3,4-Q [96]. When 4-OHE_2 is activated by lactoperoxidase in the presence of DNA and Res, however, formation of the adducts was almost completely inhibited because the Res reduced any E_2-3,4-SQ back to 4-OHE_2 (Fig. 6.1) [96].

In cells, the major inhibitory effect of Res on estrogen-DNA adduct formation is achieved by its ability to induce quinone reductase (Fig. 6.1), as seen in MCF-10F cells [110, 111]. Pretreatment of MCF-10F cells with Res for 48 h before treatment with 4-OHE_2 for 24 h significantly reduced the amounts of 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua adducts formed; if fresh Res was also included during the incubation with 4-OHE_2, even further reduction of adduct levels was observed, demonstrating the multiple modes of inhibition by Res [111]. Treatment of female ACI rats with Res reduced E_2-initiated mammary tumors by inducing quinone reductase and other protective pathways [117].

These inhibitory effects of Res could also be seen in MCF-10F cells pre-treated with dioxin to induce CYP1B1 and then treated with E_2 with or without Res. In the absence of Res, the cells formed depurinating estrogen-DNA adducts (Fig. 6.14a) [110], but no adducts could be detected in the presence of Res (Fig. 6.14a).

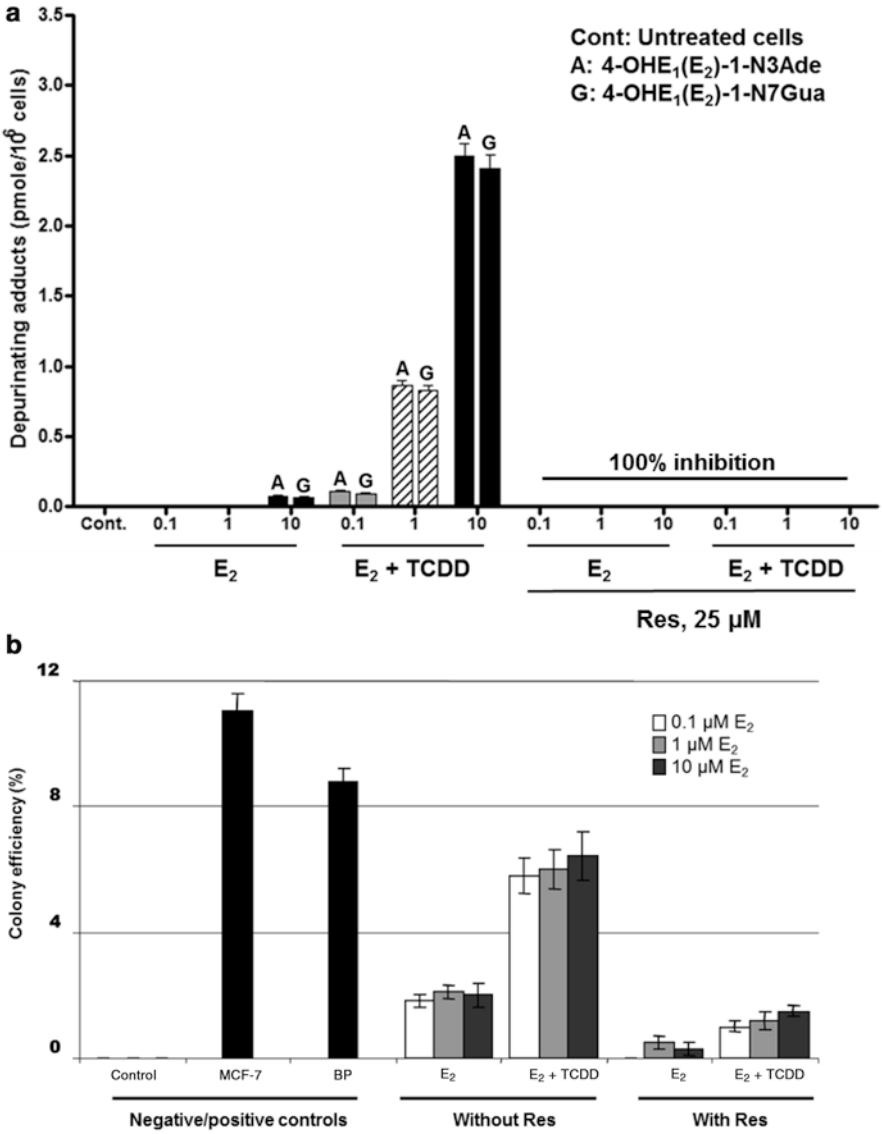

Fig. 6.14 Effects of Res on (**a**) levels of depurinating DNA adducts in MCF-10F cells pretreated with TCDD with and without Res and treated with increasing concentrations of E_2 for 24 h. The levels of DNA adducts in Res-pretreated cells are significantly different from those in the cells not pretreated with Res, $p<0.05$ as determined by ANOVA, and (**b**) antitransformation effects of Res on E_2-induced transformation of MCF-10F cells. MCF-10F cells were pretreated with TCDD with and without Res, then treated with E_2. The results are expressed as colony efficiency (%): the number of colonies formed per number of cells plated × 100; $p<0.05$. A negative control was conducted with MCF-10F cells cultured without any treatment. Two positive controls were included. One was cultured MCF-7 cells, which are a transformed cell line. In the other, MCF-10F cells were transformed with benzo[*a*]pyrene (BP) [110]

In addition, Res was able to greatly inhibit transformation of dioxin-pre-treated MCF-10F cells treated with E_2 (Fig. 6.14b) [110].

The above results demonstrate that Res inhibits formation of the depurinating 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua adducts by reducing the catechol estrogen semiquinones back to catechol estrogens, modulating CYP1A1 and CYP1B1 activity, and inducing quinone reductase activity. These three characteristics of Res lead to lower effective levels of $E_1(E_2)$-3,4-Q that can react with DNA to form the depurinating adducts and generate the initiation of cancer.

6.9.3 N-Acetylcysteine plus Resveratrol

Having investigated the ability of NAC and Res to inhibit formation of estrogen-DNA adducts and malignant transformation of mammary cells, it became important to discover their combined effects. MCF-10F cells were incubated with 4-OHE_2 plus NAC and Res. The molar ratio of Res to NAC in this experiment was 0.6 and the concentration of the combined NAC plus Res was varied from 0 to above the concentration of 4-OHE_2, which was held constant (Fig. 6.15) [118]. NAC and Res combined together were more inhibiting than each compound alone, and at higher concentrations of the two compounds, formation of estrogen-DNA adducts was completely inhibited.

6.9.4 N-Acetylcysteine plus Resveratrol Administered to Women

A group of 21 women (ages 30–70) who had never been diagnosed with cancer participated in a study of NAC plus Res [119]. They took the two dietary supplements daily for 3 months and provided a spot urine sample before starting the supplements and after the 3 month period. The urine samples were analyzed for estrogen metabolites, conjugates and depurinating DNA adducts by ultraperformance liquid chromatography/tandem mass spectrometry and the ratio of adducts to metabolites and conjugates was calculated for each sample (Fig. 6.16). Of the 21 participants, 16 women experienced a decrease in the ratio of adducts to metabolites and conjugates, one had an increase and four remained the same. Therefore, these results indicate that NAC plus Res can reduce estrogen-DNA adduct levels in people.

The two compounds, NAC and Res, work together to inhibit formation of estrogen-DNA adducts both chemically and biologically (Fig. 6.1). NAC acts chemically by reducing catechol estrogen semiquinones back to catechol estrogens and by directly reacting with catechol estrogen quinones, thereby preventing them from reacting with DNA. In addition, NAC can generate Cys and GSH, as well as additional NAC. Res inhibits adduct formation chemically by reducing catechol estrogen semiquinones back to catechol estrogens, as well as biologically by inducing

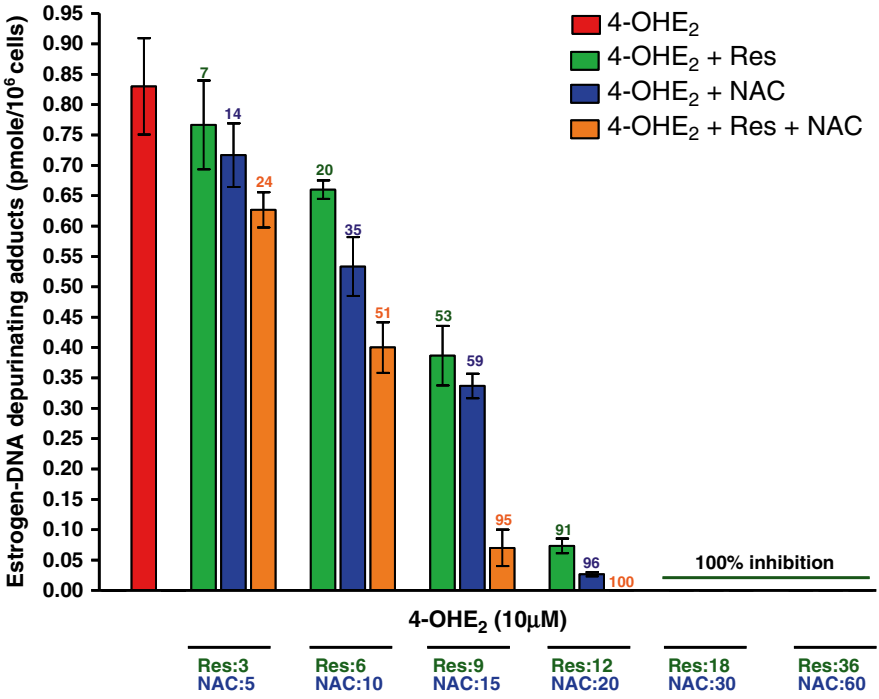

Fig. 6.15 Effects of NAC, Res, or NAC + Res on the formation of depurinating estrogen-DNA adducts in MCF-10F cells treated with 4-OHE_2. The number above each *bar* indicates the percent inhibition compared to treatment with 4-OHE_2 alone [118]

quinone reductase and modulating CYP1A1 and CYP1B1. All of these inhibitory processes of NAC and Res combine in complementary ways to minimize the amount of catechol estrogen quinones available to react with DNA to form adducts. Lower levels of estrogen-DNA adducts reduce the risk of initiating cancer.

6.10 Conclusions

The complexity of cancer has become simpler because we have found a common origin for many prevalent types of cancer. In fact, compelling evidence has led to a new paradigm of cancer initiation by estrogens. Studies on estrogen metabolism [17–25], formation of DNA adducts [12, 32, 33, 35, 48, 49], mutagenicity [48, 49, 55], cell transformation [63–67] and carcinogenicity [51–53, 69–71, 73–75] have led to and support the hypothesis that reaction of specific endogenous estrogen metabolites, predominantly $E_1(E_2)$-3,4-Q, with DNA can generate the critical mutations that initiate breast, prostate, ovarian and other prevalent types of human cancer [14, 15, 59, 90].

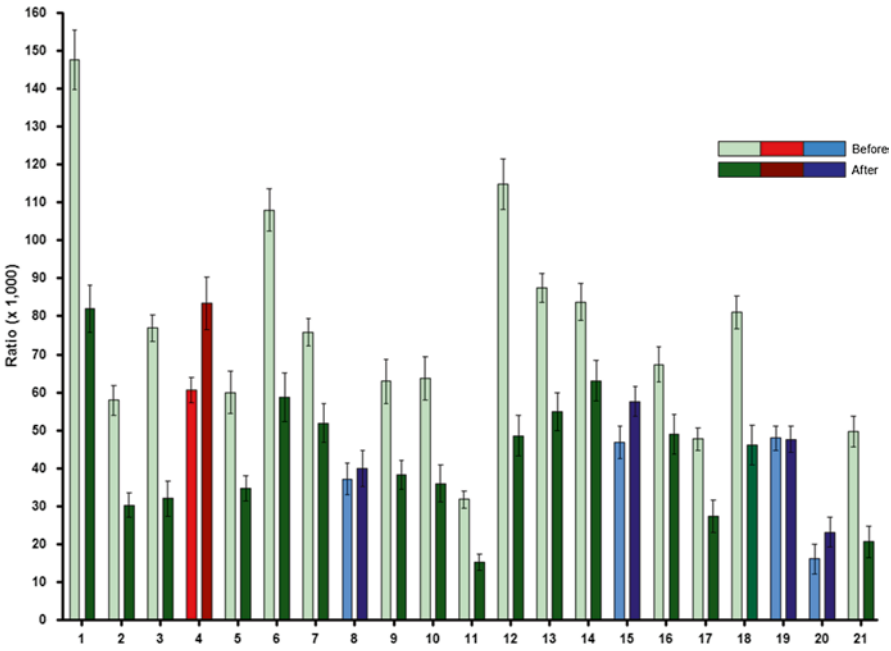

Fig. 6.16 Assessment of estrogen-DNA adduct ratios before women began taking NAC plus Res and after having taken the supplements daily for 3 months. *Green bars* represent women whose adduct ratios decreased; *blue bars* represent women whose adduct ratios remained the same; and the *red bar* represents a woman whose adduct ratio increased over the course of the study [119]

Metabolism of estrogens is characterized by a homeostatic set of activating and protective pathways. Homeostasis minimizes formation of the catechol estrogen quinones, the ultimate carcinogenic metabolites of estrogens, and their reaction with DNA. When homeostasis is disrupted, excessive oxidation of catechol estrogens to semiquinones and quinones occurs. The quinones can react with DNA to form predominantly the depurinating adducts 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua. These adducts generate apurinic sites leading to the mutations that can initiate breast, prostate and other prevalent types of cancer.

Substantial evidence for the genotoxicity of the endogenous estrogens has been obtained in studies conducted *in vitro*, in cell culture and in laboratory animals. The role of estrogen-DNA adducts in the initiation of cancer has been evaluated in the etiology of five types of human cancer: breast (Fig. 6.5), ovarian (Fig. 6.6) and thyroid (Fig. 6.7) in women, and prostate (Fig. 6.8) and non-Hodgkin lymphoma (Figure. 6.9) in men, by analyzing estrogen metabolites, conjugates and depurinating DNA adducts in urine or serum and showing that people diagnosed with these cancers have higher levels of adducts [78–80, 82, 85–87]. These cancers have a common origin, which is formation of depurinating estrogen-DNA adducts, as the first step in generating the cancer-initiating mutations. We hypothesize that other prevalent types of cancer, which include brain, colon, endometrial, kidney, leukemia, melanoma,

lung of non-smokers, myeloma and pancreas, are also initiated by catechol estrogen-3,4-quinones, which by reaction with DNA lead to the specific mutations that initiate cancer. An analogous mechanism of activation could occur with dopamine quinone under slightly acidic conditions to give rise to the initiation of PD (Fig. 6.10). In fact, DA quinone at pH 5-6 produces the DA-6-N3Ade and DA-6-N7Gua adducts, which are analogous to the adducts formed by $E_1(E_2)$-3,4-Q [88].

The above findings suggest that depurinating estrogen-DNA adducts can serve as biomarkers for increased risk of developing cancer. In addition, knowledge of the mechanism by which estrogens initiate cancer suggests that prevention can be achieved by blocking formation of estrogen-DNA adducts. This can occur by inhibiting formation of catechol estrogen quinones or their reaction with DNA.

Based on the etiology of estrogen-initiated cancers, prevention can be achieved by using selected compounds that can reduce formation of $E_1(E_2)$-3,4-Q and/or their reaction with DNA. Both NAC and Res reduce formation of estrogen-DNA adducts and malignant transformation of cultured breast epithelial cells. In combination, they are even more effective in reducing adduct formation in human breast cells [99, 110, 111, 118]. In addition, they have been shown to reduce adduct formation in women [119].

If the initiation of cancer is blocked, promotion, progression and development of the disease would be prevented. This approach to cancer prevention does not require knowledge of the genes involved or the series of events that follow cancer initiation. In conclusion, formation of depurinating estrogen-DNA adducts plays the critical role in the initiation of the most prevalent types of human cancer.

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Chapter 7
Chromatin Remodeling as the New Target for Breast Cancer Prevention

Julia Santucci-Pereira, Yanrong Su, and Jose Russo

Abstract Increased breast cancer incidence and mortality have been associated with nulliparity since 1700s. Pregnancy exerts a protective effect in women who delivered their first child before late 20s, when compared to women that never had a full term pregnancy. In addition, multiple pregnancies significantly decrease the risk of developing breast cancer after 50 years of age. This chapter addresses the mechanisms that determine the long lasting preventive effect of pregnancy against breast cancer, how to mimic this protective effect using pregnancy-hormones or smaller targeting molecules, and the participation of chromatin remodeling in breast cancer prevention.

Keywords Breast cancer prevention • Pregnancy • Human chorionic gonadotropin (hCG) • Chromatin remodeling • Genomic signature • Long non-coding RNAs (lncRNAs) • Breast development • Differentiation • Histone methylation • Gene expression • MCF10F

7.1 Introduction

Breast cancer is a heterogeneous and complex disease in which epidemiological, clinical and pathological studies have uncovered novel aspects regarding its complexity [1–9]. Among these, the knowledge that age at diagnosis and ethnicity are associated with a specific tumor type and tumor behavior, and that they are in turn differently influenced by a woman's age at the first pregnancy [10, 11]. The global incidence of breast cancer changes over time in relation to geography, race and

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lifestyle, suggesting that breast cancer risk is influenced by a multiplicity of still undefined factors. However, a common denominator for the risk of developing breast cancer is the reproductive history [6–8, 10]. Increased breast cancer incidence and mortality were associated with nulliparity as early as the 1700s, as reported by Bernardino Ramazzini, who attributed the phenomenon to the childlessness of nuns in Italian convents [10]. MacMahon et al. [6] reported that pregnancy exerted a protective effect in women whose first child was born from the early teen years to the middle twenties relative to the risk for nulliparous women. Numerous studies have confirmed these results and have additionally reported that multiple pregnancies significantly decrease the risk of developing breast cancer after 50 years of age [7–9], whereas postponement of the delivery increases a woman's breast cancer risk, which reaches the same levels observed in nulliparous women when it occurs between 30 and 34 years of age, increasing even further after 35 years [7, 8]. An understanding of the mechanisms that determine whether a pregnancy would prevent breast cancer or would increase its risk [12–15] provides the key for the question of how a physiological process like pregnancy produce breast cancer protection. *Therefore, the answer to this question should provide firsthand information of the mechanism of prevention and its knowledge brings us to the following question: how to mimic pregnancy without the pregnancy consequences and use this knowledge for preventing breast cancer?*

This chapter is divided in four parts: the first part analyzes our knowledge on the mechanisms associated with breast cancer prevention in parous women (Sect. 7.2); the second part describes how the use of pregnancy hormones, like human Chorionic Gonadotropin (hCG), can mimic pregnancy and protect the breast against cancer (Sect. 7.3); third section discusses the development of small specific molecules that can produce the same molecular mechanisms induced by either pregnancy or hCG (Sect. 7.4), and lastly, this chapter describes how chromatin remodeling is a central mechanism in breast cancer prevention (Sect. 7.5).

7.2 The Physiological Process of Pregnancy as a Clue to Understand Prevention of Breast Cancer

Pregnancy itself is a complex process that only succeeds when a woman's ovaries are fully functional and secrete estrogen and progesterone, hormones that are essential for the maintenance of pregnancy. The ovaries work under the control of the hypothalamic-pituitary-gonadal (HPG) axis [16, 17]. The HPG axis synchronizes ovarian secretions with pituitary and placental hormones, including hCG, which stimulate breast development in preparation for milk production [17, 18]. Primiparous women younger than 25 years old that have elevated serum levels of hCG during the first trimester of pregnancy have a 33 % decrease in risk of breast cancer diagnosis after the age of 50, whereas estrogen concentrations have been positively associated with risk of breast cancer before age 40, supporting the role of this or other pregnancy hormones in the development of breast cancer [10, 19–23].

7.2.1 *Experimental Basis on the Role of Pregnancy in Breast Cancer Prevention*

In experiments performed in rats, pregnancy, which is the gold standard for induction of mammary gland differentiation, needs to be completed for preventing mammary cancer. It has been demonstrated in rats that when their first pregnancy was interrupted 12 days after conception and these rats receive the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) 21 days later [22], the tumor incidence and number of tumors per animal in pregnancy-interrupted rats were similar to age-matched virgin rats, whereas rats that completed their pregnancy had a significantly reduced tumorigenic response. Completion of the first pregnancy results in full differentiation of the mammary gland, which culminates in milk secretion and persists during the length of the lactational period [17, 20]. At post-weaning, the lobular structures regress and the remaining cells exhibit a marked reduction in proliferative rate, lengthening of the G_1 phase of the cell cycle, greater capabilities to repair DNA damage, and lower binding affinity between DMBA and DNA [19]. These structural, functional and molecular changes persist in the mammary gland, resulting in a significant reduction in mammary cancer incidence, evident in various strains of rats and mice [19, 23], in spite of histopathological differences in tumor type between these species. Blakely at al. [24] have confirmed that in four genetically distinct inbred strains of rats (Lewis, Wistar-Furth, Fischer 344, and Copenhagen) and in mice, pregnancy and lactation induce similar structural and genomic changes in mammary glands [24]. Gene expression analysis identified a genomic signature that sufficed for distinguishing nulliparous from parous animals and explain the almost total refractoriness of the parous rat mammary gland to develop carcinomas after carcinogen administration [23–25]. Structural and gene expression changes induced by a full term pregnancy (FTP) are also identified in the human breast [26–31]. Studies indicate that when the development of the mammary gland has been completed by an early pregnancy, steroid hormone or hCG treatment, stem cells 1, which are susceptible to noxious effects of carcinogens, become stem cells 2. Stem cells 1 have a euchromatin-rich nucleus (EUN), while the stem cells 2, which have completed full differentiation under hormonal influences, have a more compact nucleus (heterochromatin—HTN), and are resistant to carcinogens [26, 27]. Although more differentiated, the HTN cells still retain the capacity to regenerate the complete lobular system required by subsequent pregnancies. This concept has been further demonstrated in transgenic WAP-driven Cre and Rosa 26-fl-stop-fl-LacZ mice [32]. This study showed that parity-induced mammary epithelial cells (PI-MEC) originated from differentiated cells during pregnancy, survived post-lactational involution and increased their percentage with successive pregnancies [32]. PI-MEC, like the HTN cells, show capacity for self-renewal and contribute to mammary outgrowth in transplantation studies. PI-MEC can function as alveolar progenitors in subsequent pregnancies, and they can be related to differences in response to hormonal stimulation and carcinogenic agents observed between nulliparous and parous females [32–35].

The findings that the first full term pregnancy in rodents, occurring during the high risk susceptibility window (HRSW) but before exposure to a carcinogen, prevents cancer initiation [33, 36, 37] is equivalent to the well demonstrated protective effect of an early FTP in women [6, 8, 38]. On the other hand, a first FTP initiated approximately 2 weeks after carcinogen exposure results in a high incidence of mammary cancer. This phenomenon could explain the increased cancer risk observed in parous women which had their first FTP after age 30 [7, 8], supporting the assumption that during that lengthened HRSW the breast had been exposed to carcinogenic stimuli before pregnancy. These data emphasize the importance of discriminating whether the first pregnancy would produce protection by inducing complete differentiation of the breast activating the same mechanisms that hormonal treatments do, or would increase breast cancer risk as a consequence of genotoxic or epigenetic exposures during the HRSW.

7.2.2 The Human Breast in Pregnancy

The development of the breast is a continuous process initiated by the fourth week of intrauterine life that progresses under the influence of maternal, placental and environmental factors until birth, and then by hormones, diet and environmental exposures after weaning. During these periods, the maturation of the hypothalamic gonadal (HPG) axis [16, 17, 39] and endogenous hormone secretions play essential roles on the development of the breast at puberty, which is driven by the initiation of ovulation and the establishment of regular menstrual cycles [40]. The architecture of the breast of normally cycling women has been widely described as composed of three main lobular structures. These lobules are classified based on their degree of development into lobules type 1 (Lob 1), lobules type 2 (Lob 2) and lobules type 3 (Lob 3) [19, 41, 42]. The breast of women that never conceived a child remains composed of Lob 1, with moderate formation of Lob 2 after successive menstrual cycles; Lob 3 become present only occasionally during the early reproductive years. After menopause, the breast regresses, resulting in increased number of Lob 1 in response to the decline of Lob 2 and Lob 3 with aging. It has been shown that the breast parenchyma of postmenopausal nulliparous women contains predominantly euchromatin nucleus (EUN) cells [27], which did not achieve the most differentiated stage due to the absence of pregnancy. These EUN cells retain their susceptibility to be transformed, therefore, a carcinogenic insult or an inappropriate hormonal stimulus, such as hormone replacement therapy [43], could transform the EUN cells into a cancer stem cell. On the other hand, postmenopausal parous woman breast contains predominantly heterochromatin nucleus (HTN) cells [27], which are less susceptible to carcinogenic insults (further discussed later in this chapter).

The development of the breast from birth to puberty follows a general pattern common for all normally cycling women, with the formation of Lob 1, Lob 2 and Lob 3 [41, 42]. The progression of lobular development under the cyclic influence

of ovarian hormones is rapidly accelerated during the first pregnancy, which to be successful, requires the timely fertilization of an ovocyte followed by its uterine implantation. The embryo drives a process that establishes a collaboration of the newly formed placenta with the maternal environment [44]. The placenta alone elaborates a myriad of proteins, glycoproteins, steroid hormones, growth factors, tumor suppressor factors and cytokines that control the local environment of the fetus and regulate the metabolic activities of both the mother and the fetus [45]. In addition to estrogen and progesterone, newly secreted hormones, such as human growth hormone (hGH), hCG, human placental lactogen (hPL), inhibin stimulate breast development and differentiation [46, 47]. Elevated serum levels of Metastin (KISS1) have been detected during pregnancy [48], but the role of this hormone in breast development has not been identified as yet. LH, progesterone and hCG are the main hormones driving the initial phase of growth, which is followed by the secretion of the pituitary hormone prolactin that stimulates milk secretion and contributes to the development of the fully differentiated Lob 4 during the last trimester of pregnancy and lactation. After weaning, Lob 4 regresses to Lob 3, which persists in the breast as long as women continue cycling. At peri-menopause the number of Lob 3 progressively decreases due to their involution to Lob 2 and Lob 1 [19].

7.2.3 Cellular and Molecular Basis of the Protective Effect of Early Pregnancy in the Postmenopausal Women

The morphological, physiological and genomic changes resulting from pregnancy and hormonally-induced differentiation of the breast and their influence on breast cancer risk have been addressed above and in the literature [27–30, 49–51]. The observations that during the post-menopausal years, the breast of both parous and nulliparous women contains preponderantly Lob 1 and the fact that nulliparous women are at higher risk of developing breast cancer than parous women indicate that Lob 1 in these two groups of women differ biologically and exhibit different susceptibility to carcinogenesis [51]. This concept has been further clarified by the demonstration that there are changes in cell types and increases in chromatin condensation as novel markers for cell differentiation in the adult breast [27]. These findings confirm the universality of the histone 3 dimethylation in lysine 9 and trimethylation in lysine 27 during differentiation, since a similar phenomenon has been described to occur during embryonic stem cell differentiation [52].

The observed chromatin changes in parous epithelial cells are accompanied by the expression of genes related to increasing cell adhesion, such as NRXN1, DSC3, COL27A1, PNN, COL4A6, LAMC2, COL7A1, COL16A1,and LAMA3, and differentiation, that include MGP, KRT5, GATA3 and LAMA3 [27, 28].

In contrast to the findings of other authors [53] that down-regulation of the expression of ER-α following recent (0–2 years since last pregnancy) and distant (5–10 years since pregnancy) pregnancies in premenopausal women, the study in

postmenopausal breast did not reveal differences in the expression levels of ER-α in the epithelial cells of ducts and Lob 1 between parous and nulliparous postmenopausal breast. Nevertheless, numerous genes that are regulated downstream by the ER-α were found to be up regulated in the parous breast, supporting a parity-mediated protective effect evident in younger parous women [53] but lasting until menopause. Among the ER-α downstream regulated genes was GATA3, which encodes a protein that belongs to the GATA family of transcription factors that regulates T lymphocyte differentiation and maturation. GATA3 is crucial to mammary gland morphogenesis and differentiation of progenitor cells and a putative tumor suppressor [54]. Induction of GATA 3 expression in GATA3-negative undifferentiated carcinoma cells is sufficient to induce tumor differentiation and inhibition of tumor dissemination [55]. Therefore, the observation that genes involved in the estrogen receptor regulated pathways are up-regulated in the parous breast, in spite of the lack of transcription differences of this receptor's levels between parous and nulliparous postmenopausal breast tissues, suggests that they could be under permanent transcriptional modification as a manifestation of a higher degree of cell differentiation.

Studies of breast development under the influence of parity in women and in animal models are in agreement on the pregnancy-induced differentiation of the breast, a process that ultimately becomes manifested as a specific genomic signature in the mammary gland [25, 28–30, 49, 50, 53, 56, 57]. Although variations in gene expression among different studies and species are expected, an increase in immune activity, including overexpression of lipopolysaccharide binding protein (LBP/Lbp) has been reported in the post-pregnancy breast of premenopausal women [53] and in the mammary gland of four different strains of rats [57]. Interestingly, this response observed in both recently pregnant in distant pregnant groups was not observed in the postmenopausal group. These discrepancies might indicate that the up regulation of inflammation/immune response–related genes persists during postpartum involution, but wanes after menopause sets in (see Sect. 7.2.4).

Importantly, it has been reported a shift in cell population of the postmenopausal breast as a manifestation of the reprogramming of the organ after pregnancy [27]. It was observed an increase in heterochromatin-rich nucleus cells in parous women, while the majority of the cells in the breast of nulliparous women contains euchromatin-rich nucleus (Fig. 7.1). These observations are in agreement with what is observed in the rat mammary gland, which also contains two types of luminal epithelial cells, designated dark (DC) and intermediate (IC) cells, in addition to the myoepithelial cells [58]. The DC and IC are equivalent to the HTN (heterochromatin-rich) and EUN (euchromatin-rich) cells described in the parous breast, respectively [27]. DCs increase after pregnancy and lactational involution; whereas the ICs significantly outnumber the DC in ductal hyperplasias and ductal carcinomas [58, 59]. The analysis of nuclear ultrastructural and morphometric parameters of rodent IC have allowed us to differentiate the mammary progenitor stem cell from the cancer stem cells [51, 58, 59]. Nuclear morphometric analyses of breast and ovarian carcinomas have confirmed the predictive value of nuclear grade on the progression of

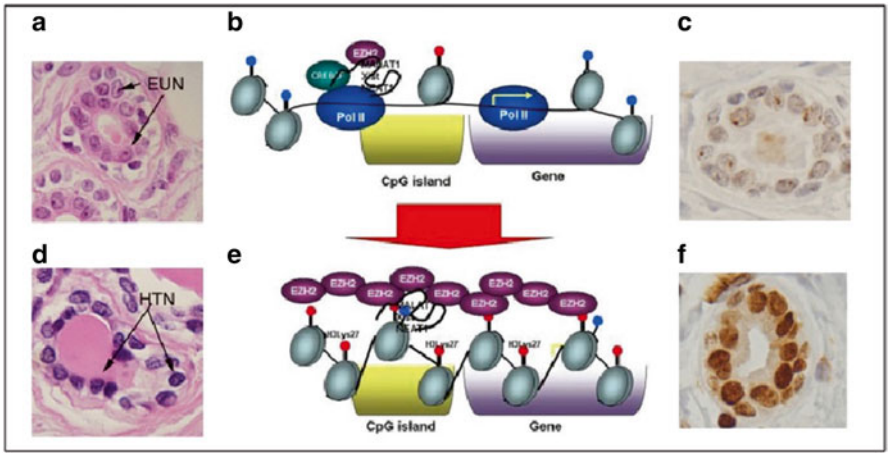

Fig. 7.1 (**a**–**c**) Ductules of nulliparous women's breast. (**a**) Epithelial cells contain euchromatin-rich nuclei (EUN) (*arrows*); (**b**) Transcriptional elongation by RNA polymerase II (Pol II) and DNA methylation-histone code link via methyl-CpG–binding proteins mediate transcriptional de-repression in EUN nuclei; (**c**) Low to negative immunohistochemistry against H3K27me3 in EUN nuclei. (**d**–**f**) Ductules of parous women's breast. (**d**) Heterochromatin-rich nuclei (HTN) (*arrows*); (**e**) Up-regulation of XIST is associated with CpG island methylation and gene silencing; (**f**). Intense nuclear immunohistochemistry reactivity with H3K27me3 antibody (Modified from: Russo et al. *Int. J Cancer, 2011* [27] and Russo, J. and Russo, I.H. "The transcriptome of the human breast" *Springer, New York 2012* [96])

premalignant lesions to invasiveness [60–62]. The findings of a significant decrease in the number of EUN with a subsequent increase in the number of HTN cells expressing specific biomarkers identified at the chromatin and transcriptional levels support the value of morphometric analyses as an adjuvant to molecular studies. The data clearly show [27] morphological indicators of chromatin remodeling in the parous breast, such as the increase in the number of epithelial cells with condensed chromatin and increased reactivity with anti-H3K9me^2 and H3K27me^3 antibodies (Fig. 7.1). Histone methylation is a major determinant for the formation of active and inactive regions of the genome and is crucial for the proper programming of the genome during development [63].

In the parous breast, the observed changes in chromatin where concomitant to up regulation of transcription factors and chromatin remodeling genes such as CHD2 (chromodomain helicase DNA binding protein 2) and the CBX3 (Chromobox homolog 3), whose products are required for controlling recruitment of protein/protein or DNA/protein interactions. CBX3 is involved in transcriptional silencing in heterochromatin-like complexes, and recognizes and binds H3 tails methylated at lysine 9, leading to epigenetic repression. Two other important genes related to the polycomb group (PcG) protein that are up regulated in the parous breast are the L3MBTL gene or l(3)mbt-like and histone-lysine N-methyltransferase or EZH2.

Members of the PcG form multimeric protein complexes that maintain the transcriptional repressive state of genes over successive cell generations. EZH2 is an enzyme that acts mainly as a gene silencer, performing this role by the addition of three methyl groups to lysine 27 of histone 3, a modification that leads to chromatin condensation [52, 64, 65] (Fig. 7.1).

Recent studies indicate that noncoding RNA (ncRNAs) molecules recruit PcG complexes to the locus of transcription or to sites located elsewhere in the genome [66, 67]. It has been postulated that the increased chromatin condensation in the parous breast could have been initiated by ncRNAs [27], which is supported by the observed up regulation of several ncRNAs in parous [27–29, 31]. Some of the ncRNAs observed up-regulated in the parous breast are X inactive specific transcript (XIST), nuclear paraspeckle assembly transcript 1 (NEAT1) and metastasis-associated lung adenocarcinoma transcript 1 (MALAT1 or NEAT2) [27–29, 31], these last two are critical components of the speckles [68].

The importance of the long non-coding RNA (lncRNAs) in transcription regulation of genes involved in different cellular processes, including differentiation, cancer initiation and progression has been emphasized [69–71]. Using next generation sequencing, Barton et al. showed that there are 42 lncRNAs differentially expressed between parous and nulliparous post-menopausal breast tissue [72–74]. Of which, 21 are up-regulated and 21 are down-regulated in the parous. In addition, eight lncRNAs showed significant correlation in expression with their nearby gene, indicating a possible role as cis-regulators [72–74]. This work provides novel information on lncRNAs differentially expressed in breast cells when comparing pregnancy vs. the lack of FTP, and places lncRNAs as potential key regulators in differentiation and protection against cancer initiation.

There is a relationship between the chromatin remodeling process and post transcriptional control maintained by the spliceosome machinery that is stored in nuclear speckles [27, 28]. Among the components of the spliceosome machinery that are up-regulated in the parous breast are the heterogeneous nuclear ribonucleoproteins HNRPA3, HNRPA2B1, HNRPD and the HNRPU [27, 28]. The functional role of these HNRPs in the postmenopausal breast could be implicated in the regulation of mRNA stability, other functions like mammary gland involution, acting as negative regulators of telomere length maintenance [75] or regulating the trafficking of mRNA molecules [76]. Other members of the spliceosome complex are the small nuclear ribonucleoproteins (snRNPs), which function as suppressors of tumor cell growth and may have major implications as cancer therapeutic targets. Among these we have found that the transcripts SF3B1, SFRS2, SFRS7, SFRS8, SFRS14, SFRS16, SNRP70, SNRPB, SNRPA1, PRF3 and PHF5A are over expressed in the parous breast [27, 28]. Other members of the splicing factor compartment that are localized in the nuclear speckles are CCNL1 and CCNL2. It has been demonstrated that CCNL2 protein is overexpressed in the nucleus of epithelial cells composing the Lob 1 of the parous breast [27]. CCNL1 and CCNL2 are transcriptional regulators that participate in the pre-mRNA splicing process and the expression of critical factors leading to cell apoptosis, possibly through the Wnt signal transduction pathway [77, 78], which we found to be methylated in the parous breast [31].

Another component of the spliceosome complex that regulates genes involved in the apoptotic process is the RNA binding motif protein 5 (RBM5). The over expression of RBM5 retards ascites associated tumor growth and enhances p53-mediated inhibition of cell growth and colony formation [79, 80] mechanisms that could also be operational in the parous breast. In studies using RNA sequencing, Santucci-Pereira et al. also found that significant differences in splicing events between parous and nulliparous women [72, 81]. The spliceosome plays a critical role in differentiating mouse embryonic stem cells, and self-renewal, pluripotency and tissue lineage specification of human embryonic stem cells [82]. Post-transcriptional modifications of RNA, including packaging into the nuclear speckles of the breast epithelial cells and recognition by RNA-binding proteins and/or microRNAs are crucial processes in differentiating breast epithelial cells. Although it is known that these regulatory mechanisms decrease the susceptibility of the cell to carcinogenesis, more studies need to be conducted for identifying the specific pathways involved in this process and furthermore this knowledge contribute to emphasize the importance of post-transcriptional regulatory mechanisms as a critical component underlying the differentiation of the breast.

7.2.4 Basis of the Dual Effect of Late Pregnancy in the Premenopausal Woman

Recently Santucci-Pereira et al. reported gene expression differences in the breast of parous vs. nulliparous healthy premenopausal women [73, 83]. The authors have used Affymetrix Human Genome U133Plus2.0 microarrays to analyze the gene expression profile of breast tissue from nulliparous and parous premenopausal volunteers between the ages of 30 and 47 years who were free of breast pathology. Because of the known short-term increase in breast cancer risk preceding the long-term protective effect of FTP, the authors also examined gene expression differences in parous vs. nulliparous women as a function of time since last FTP. They found 286 genes differentially expressed comparing all parous vs. all nulliparous, and/or, parous women whose last FTP was less than 5 years before biopsy vs. all nulliparous women. Among these, 238 genes were up-regulated, in parous compared to nulliparous breast [73, 83]. Santucci-Pereira et al. found that the up-regulated genes had three expression patterns. The genes were either (a) transiently up-regulated, (b) up-regulated following FTP but with decreased expression levels with increasing time since last FTP, or (c) constantly up-regulated, independently of time since last FTP. Interestingly, the genes transiently up-regulated were mainly involved in immune response, while the genes constantly up-regulated in the parous breast were mainly involved in developmental processes, cell differentiation and chromatin remodeling. This study shows that a FTP induces long-term expression changes in genes related to the processes of development, cell differentiation and chromatin remodeling [73, 83], as it was also found in the parous postmenopausal breast [27–29, 31]. The transiently activated genes

related to immune response may play a role in the short-term increase of breast cancer risk following FTP [73, 83]. Genes activated during the first 5 years after pregnancy related to the immune response may contribute to the increased risk experimented by certain women immediately after pregnancy, but at the same time, pregnancy induces a long lasting genomic signature around the chromatin remodeling that explains its preventive effect [27–29, 31, 73, 83].

7.3 Mimicking Pregnancy Using Human Chorionic Gonadotropin

The preventive effect of an early full term pregnancy has been seen in various rodent models including out-bred Sprague-Dawley (SD) rats, inbred Lewis and Wistar-Furth rats, and inbred mice (for review see [84]). Most of these studies have revealed that the early FTP results in a greater than 75 % inhibition of the incidence of breast carcinomas [84, 85]. Pregnancy is associated with increased levels of several pregnancy-related hormones including estrogen (E) and progesterone (P), which play leading roles in orchestrating proper development and function of breast tissue [86]. In fact, pregnancy can be mimicked by the administration of E plus P, which are thought to be responsible for the diminished risk for breast cancer among women following a FTP [86, 87]. It has been shown that a short-term administration of E plus P mimics the protective effect of parity in rats and that a treatment period as short as one-third of the gestation time is sufficient to induce protection against mammary carcinogenesis [85]. A study by Rajkumar et al. [88] has demonstrated that in nulliparous rats, a long-term protection against mammary carcinogenesis could be achieved by short-term treatments with a various combinations of both natural and synthetic E and P. In addition to E and P, another key pregnancy-related hormone that is highly secreted during pregnancy is human chorionic gonadotropin (hCG). HCG is a glycoprotein produced by the developing embryo soon after conception, and later by the syncytiotrophoblast. The protective effect against mammary carcinogenesis conferred by hCG is due to its capability of inducing complete mammary gland differentiation, similar to the one induced by pregnancy and lactation [25, 89–94]. The greater differentiation of the mammary gland is manifested as permanent structural changes, consisting of the disappearance of terminal end buds (TEBs) and diminution of the number of terminal ducts (TDs) due to their differentiation into lobules. These events are fully reached through exogenous administration of hCG alone [25, 90–94]. More importantly, it has been shown in rats that a short-term hCG treatment induced the molecular, cellular, pathophysiological and morphological changes that similarly occur during pregnancy [93–97]. These studies have revealed that hCG induced specific changes in genomic signature similar to those occurring during pregnancy, which were associated with inhibition of not only initiation and progression of chemically-induced mammary carcinomas, but also, development of early lesions, such as intraductal proliferations and carcinoma *in situ* [25, 96, 98]. HCG was found to inhibit mammary tumorigenesis through both induction of differentiation and inhibition of the proliferation of human

breast epithelial cells *in vitro* [99]. Consistent with these observations made in studies with animal models, a nested case-control study with a female population-based cohort has revealed that women with hCG levels in the top tertile consistently had lower risks of breast cancer than women with hCG levels in the lowest tertile [100]. All together, these observations strongly support the original notion that the elevated levels of hCG, a key placental hormone during pregnancy, confer potent protection on the long-term risk of breast cancer. These observations prompted to continually pursue the potential applications of hCG in prevention of human breast cancer. In this chapter, studies that have helped to establish the powerful role of hCG in breast cancer prevention are documented. The studies are presented in Sects. 7.3.1 and 7.3.2.

7.3.1 The Differential Effect of Urinary hCG (u-hCG) vs. Recombinant hCG (r-hCG)

Up to now, the main known physiological function of hCG in the female is the maintenance of the corpus luteum of pregnancy through its interaction with the ovarian lutropin-choriogonadotropin-receptor (LH-CG-R), which has been recently described in the human breast. This interaction activates a cascade of effects that results in increase in serum levels of estrogen and progesterone. Administration of hCG to virgin rats elicits a similar response [25, 32–35, 39]. In addition, it has been shown that hCG has a direct effect on the rat mammary epithelium and on human breast epithelial cells (HBEC) in culture [101]. HCG is currently used in the treatment of infertility and hypogonadotropic hypogonadism in males. It has also been used for the treatment of obesity [102], and recently has been successfully used in Phase I/II trials in the United States for the treatment of Kaposi's Sarcoma lesions in acquired immunodeficiency syndrome (AIDS) patients [103–105]. A drawback in these studies is the fact that the most common source of all commercially available hCG preparations is the urine of pregnant women, which carries numerous bioactive ovarian and placental hormone and peptide metabolites. The assessment of the specific effects of hCG, therefore, requires the use of a pure form of the hormone, such as a recombinant preparation.

Pregnancy can be considered to be the most physiological mechanism for protecting the mammary gland from malignant transformation, a conclusion supported by epidemiological data [37, 38]. The fact that hCG appears to mimic the effect of pregnancy makes the use of this hormone for cancer prevention an appealing idea that needs further exploration. In order to identify the ultimate mechanisms responsible of the pregnancy/hCG mediated protection, it was compared the mammary cancer preventive and therapeutic effects of hCG obtained from the urine of pregnant women (u-hCG) with those of the pure hormone, recombinant hCG (r-hCG) [96]. The placebo or hormonal treatments were initiated when the rats were 45 days old and were administered consecutively for 21 days. Twenty one days after the last injection, when the animals were 87 days old,

they received a single intra gastric dose of DMBA. In animals treated with placebo, 44 palpable masses were found in 22 of 49 animals treated (44.9 %). The tumors were predominantly located in the mammary glands located in the thoracic region, mammary gland pairs 2 (MG2) and 3 (MG3). Second in frequency were those tumors located in the neck and ear regions (Table 7.1). After histopathological evaluation and diagnosis of the masses dissected, it was found that 26 of them were invasive mammary adenocarcinomas, which were found in 18 animals (37 %), representing an average of 0.53 adenocarcinomas per animal (Table 7.1). These tumors were predominantly papillary adenocarcinomas, either type I (4 tumors), type II (5 tumors), types II and I combined (10 tumors), and papillary carcinomas types I and II combined with cribriform pattern (7 tumors). In addition the mammary glands of the same animals contained a total of 27 *in situ* carcinomas, or 0.55 *in situ* carcinomas per animal (Table 7.1). None of these tumors exhibited necrosis or regressive changes. In four of the animals, the enlarged masses represented lymph node or salivary gland hyperplasia (3 enlarged lymph nodes, 5 enlarged salivary glands), or swollen portions of muscle, fibroadipose tissue, or mammary gland, but they were free of neoplasms [96].

Treatment of the animals with r-hCG significantly reduced the number of palpable tumors in the mammary gland, neck, and ear regions. Five tumors were palpated in 4 out of 49 animals (8.1 %). When the tumors were histopathologically classified, it was found that three of them were papillary adenocarcinomas, either type I, combined types I and II, or cribriform adenocarcinoma, reducing the number of animals with mammary neoplasms to three (6 %) (Table 7.1, Fig. 7.2). Foci of carcinoma *in situ* were found within invasive adenocarcinomas, but this type of lesion was not found in animals free of invasive carcinomas or bearing only benign lesions. One of the tumors detected by palpation was a benign epidermal inclusion cyst and the other an enlarged lymph node. A total of 50 animals treated with u-hCG were evaluated at the end of the experiment. Seven tumor masses were palpable in six of the animals (12 %). Four of them were invasive papillary adenocarcinomas type I and II, and one was a papillary adenocarcinoma type I and II combined with cribriform carcinoma. These lesions and two *in situ* carcinomas were found in 4 out of the 50 animals (8 %). The other two palpable masses were an enlarged lymph node and a benign keratoacanthoma, respectively (Table 7.1). This experiment clearly demonstrated that either u-hCG or r-hCG has a preventive effect in mammary carcinogenesis [96].

7.3.2 Time-Dependent Preventive Effects of Human Chorionic Gonadotropin (hCG) on Rat Mammary Carcinogenesis

The degree of protection by hCG against the development of breast cancer is dependent on the duration of hCG action at specific stages of breast development. In addition, the duration of hCG-treatment prior to carcinogen inoculation affects the degree of

Table 7.1 Effect of r-hCG and u-hCG on the progression of DMBA-induced mammary carcinomas

Regimen treatment	No. animals	Animals with tumors/%	No. tumors/ tumors per An.	Animals with carcinomas/%	No. carcinomas/ carcinoma per An.	No. *CIS*/*CIS* per An.	No. benign lesions[a]/ benign lesions/An.
4-Placebo	49	48/98	273/5.6	44/90	210/4.29	87/1.70	63/1.3
5-r-hCG	49	38/78	111/2.2	38/78	81/1.65	41/0.84	30/0.61
6-u-hCG	46	30/65	88/1.9	27/59	72/1.57	31/0.67	16/0.34

Statistical analysis

Regimen	Animals with tumors/%	No. tumors/tumors per An.	Animals with carcinomas/%	No. CIS/CIS per An.
4 vs. 5	$P<0.01$	$P<0.01$	$P<0.01$	$P<0.01$
4 vs. 6	$P<0.01$	$P<0.01$	$P<0.01$	$P<0.01$

[a]Benign lesions include both tumoral and non-tumoral lesions of mammary and non-mammary origin, i.e., fibromas, fibroadenomas, epidermal inclusion cyst, keratoacanthomas, lymph node and salivary gland hyperplasia, mammary cysts and duct ectasia, etc.

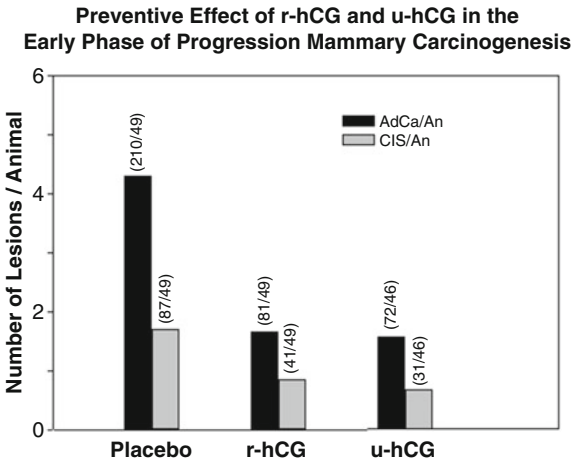

Fig. 7.2 Histogram showing in the ordinate the number of adenocarcinomas (AdCa) and carcinoma *in situ* (CIS) per animal. The abscissa shows the treatments received: Placebo, r-hCG, and u-hCG. The numbers in *parentheses* represent the total number of lesions in the total number of animals studied (From: Russo, J. and Russo, I.H. "The transcriptome of the human breast" *Springer, New York 2012* [96])

its protective effects [96, 97]. It has been demonstrated that even a short exposure to hCG, as short as 5 days, confers a preventive effect against development of chemically-induced mammary cancer in rats [97]. Additionally, the preventive effects of hCG increases significantly with the increasing duration of its treatment. The potent inhibition of DMBA-induced mammary carcinomas in female rats by hCG treatment is reflected by significant reduction in tumor rate, the tumor load per rat, tumor size and incidence and latency [96, 97]. Animals were divided into a control group, which received the vehicle of hCG, and three hCG-treated groups, which received 100 IU hCG for 5, 10 or 15 days. The hCG treatment was initiated when animals were 50 days old. Five rats from each group were sacrificed 3 weeks after the last hCG injection for evaluation of the direct effect of hCG on mammary gland differentiation. The differentiation level was assessed through the counting of terminal end buds (TEB) in whole mounts preparations. The number of TEBs in all hCG-treated groups was significantly lower, around five TEBs per animal, compared to the control group which presented an average of 21 TEBs per mammary gland. The rest of the rats, at age of 86 days, received a single dose of the chemical carcinogen DMBA. Four months after the DMBA administration, the animals were euthanized and the tumorigenic response was evaluated [96, 97]. DMBA induced mammary tumors in 91 % of the rats in the control group. Pretreatment of the animals with hCG for 5, 10 and 15 days progressively decreased the incidence of DMBA-induced mammary tumors with a statistically substantial percentage ($X^2 = 98.2$, $df = 3$, $p < 0.0001$). Among the hCG-treated groups, tumor frequencies were 69.2 % in 5-day, 53.8 % in 10-day, and 15 % in 15-day groups. Furthermore, there was also

statistically significant decrease in tumor incidence among the hCG-treated groups. Tumor frequency in hCG-5 days was statistically significant higher than in hCG-10 days ($X^2=6.42$; $p=0.011$) and in hCG-15 days ($X^2=13.6$; $p<0.0001$). Tumor frequency in the 15-day hCG-treated group was remarkably lower than that in the 10-day hCG-treated group ($X^2=7.53$; $p=0.006$) (Table 7.2). The total number of thoracic tumors is statistically significantly higher in the control groups than that the hCG-treated groups. While a total of 31 thoracic tumors were found in the control group ($n=11$), only 12, 13 and 5 thoracic tumors were found in the 5-day ($n=14$), 10-day ($n=14$) and 15-day ($n=13$) hCG-treated groups, respectively. The similar trend was also observed for the abdominal tumors. A total of 19 abdominal tumors were found in the control group ($n=11$) whereas only 1 abdominal tumor was found in the 15-day hCG-treated group ($n=13$) [96, 97].

The tumor load per rat in the control group was 4.5 ± 1.4 (mean ± SEM), while this number dropped to 1.2 ± 0.3, 1.4 ± 0.5 and 0.4 ± 0.2 in the 5-day, 10-day and 15-day hCG-treated groups, respectively ($p<0.03$) (Fig. 7.3). There is also a statistically significant difference between 5-day and 15-days of hCG-treated groups ($p<0.04$). The lowest number of tumor per rat was found in the 15-day hCG-treated group; however, there was no statistically significant difference in terms of tumor quantity among pretreatment groups. These data indicate that the 5-days of hCG treatment is as effective as the 10 days and 15 days treatment in reducing tumor multiplicity. The histopathological examination on the tumor samples (Table 7.3) revealed that the percentages of adenocarcinomas in all the groups ranged from 94 to 100 % of the tumors [96, 97].

Although the preventive effects of hCG on human breast cancer and chemically-induced mammary tumor in animals have been well recognized [90, 91, 101], the data described above provided additional lines of evidence that strongly support our original notion that hCG plays a pivotal role in gaining the preventive effects of an early pregnancy against breast cancer. More importantly, this study has clearly demonstrated that the duration of hCG-treatment significantly affects the degree of protection of rats against the DMBA-induced development of breast cancer. Furthermore, this study indicates that while the animals that received hCG treatment still developed breast cancer, they delay the development of tumors (Fig. 7.3), further demonstrating the protective effects of hCG against mammary tumors. The key finding is that the preventive effect of hCG is evident as early as 5 days of hCG treatment and is significantly enhanced as the duration of the treatment is increased,

Table 7.2 Number of tumors

Group	Control	hCG—5 days	hCG—10 days	hCG—15 days
Animal per group	11	14	14	13
Animal with tumors	10	10	8	2
Total number of tumors	50	17	19	5
Thoracic tumors	62 %	71 %	68 %	80 %
Abdominal tumors	38 %	29 %	32 %	20 %

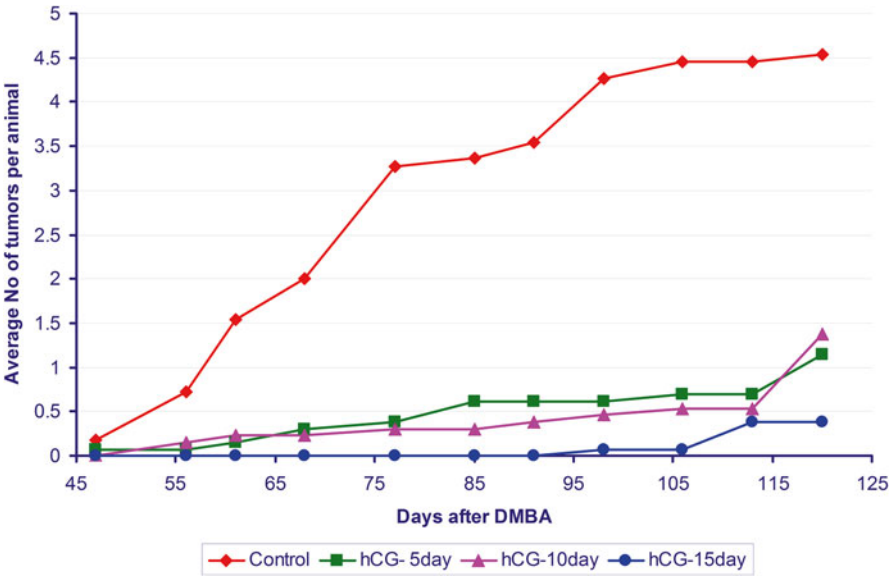

Fig. 7.3 Tumor multiplicity and latency. The number of tumors per animal rapidly increases in the animals that did not received hCG (From: Russo, J. and Russo, I.H. "The transcriptome of the human breast" *Springer, New York 2012* [96])

Table 7.3 Tumor histopathology

Group	Control	hCG—5 days	hCG—10 days	hCG—15 days
Total number of tumors	50	16	19	5
Adenocarcinomas (n)	49	15	19	5
Adenocarcinomas (%)	98	94	100	100
AdCa average per rat	4.5	1.2	1.4	0.4

and reaching the maximum level after 15 days of hCG treatment. This study indicates that in order to obtain the maximum preventive effect, an optimum application period is necessary along with the optimum dose. In previous studies [106], the preventive effect of hCG in a 21-day period with different doses (1, 5, 10, 100 IU r-hCG in 50-day-old SD rats) was tested. The percentages of adenocarcinoma frequency and tumor burdens showed a noticeable decrease as the dose increased [106]. Adenocarcinoma frequency was found to be 42 % in 1 IU, 14 % in 5 IU and 11 % in 10 IU. Strikingly, no malignant tumors were encountered in 100 IU dose application. Consequently, it was determined the optimum effective dose as 100 IU and it was observed similar results in full term pregnant rats before carcinogen gavage without the hCG treatment. In previous study, it was compared rats that

received a 3-week hCG treatment (100 IU per day) before carcinogen gavage with rats that underwent 3 weeks of pregnancy [94, 96]. When the carcinogen gavage was applied on the 92nd day, adenocarcinoma frequency was found to be 48 % in the control group, 5.6 % in the pregnancy group and 6.1 % in the hCG-treated group. When the carcinogen gavage was shifted for a later time (134th day), carcinoma frequency decreased in the control group (18.5 %), and a significant decrease was observed in pregnancy and hCG pretreatment groups (9.2 % and 7.4 %, respectively). Together, these observations indicate that hCG confers preventive effects against chemically-induced mammary tumors in a time- and dose-dependent manner. The fact that hCG at even smaller doses and for a much short duration of its treatment confers protective effects on breast tissue reflects the effectiveness of this hormone in breast cancer prevention.

Several mechanisms have been implicated in the hCG-induced protection against mammary tumors and it has been extensively discussed in previous publication [96]. In this chapter we discuss some of the major mechanisms by which hCG inhibits the initiation and the progression of chemically induced mammary carcinomas, such as inducing differentiation of the mammary gland during pregnancy. As mentioned previously in this chapter, the breast tissue of normally cycling women contains three types of lobules: the undifferentiated Lob 1, the more developed Lob 2 and Lob 3. The breast attains its maximum development, Lob 4, during pregnancy and lactation. After menopause the breast regresses in both nulliparous and parous women containing only Lob 1. It has been proposed [26, 107] that Lob 1 in the breast of nulliparous women and parous women with breast cancer never went through the process of differentiation, retaining a high concentration of epithelial cells that are targets for carcinogens. Breast cancer initiates in Lob 1, the most undifferentiated structures frequently found in the breast of young nulliparous women. It has been shown that a determining factor in the susceptibility of the human breast to cancer is the mammary gland architecture [89, 93, 107]. As early full pregnancy, treatment of rats with hCG stimulates mammary gland differentiation [36, 91, 102, 108]. In particular, it has been demonstrated that pregnancy or hCG treatment for 21 days induces differentiation of mammary gland shifting of EUN cells, which is susceptible to carcinogenesis, to HTN cells, which are refractory to carcinogenesis (Fig. 7.1) [27, 36, 91, 102, 108]. Consistent with these observations, the number of TEBs after hCG treatment decreases significantly compared to untreated rats.

Pregnancy imprints in the breast permanent changes that reduces the susceptibility of this organ to cancer. Previous genetic signature analysis has revealed that full term pregnancy induces changes in genomic signature that are related to the control of growth and differentiation in the human breast and that hCG induced the similar genomic signature [24, 25, 107]. The genomic signature induced by either pregnancy or hCG included activators or repressors of transcription genes, apoptosis, growth factors, cell division control, DNA repair, tumor suppressor, and cell-surface antigen genes [25, 30, 96]. Thus, secretion of hCG during pregnancy induces changes in genetic signature and thus, differentiation of the mammary gland, thereby making the breast tissue less susceptible to carcinogenesis.

Consistent with the role of hCG in induction of mammary gland differentiation, it has been reported that hCG induces the expression of inhibin [46, 106, 109, 110], a gonadal glycoprotein which is a member of the TGF superfamily of growth and differentiation factors. Inhibin regulates the production of follicle-stimulating hormone and is present in cells of the cyto-trophoblast layer of human placenta at term and in primary cultures of human trophoblasts [111]. Inhibin-b subunit, considered as a tumor suppressor, contributes to the process of initiation, promotion, or progression of endocrine-related cancers [112]. High levels of inhibin-a are present in the maternal serum throughout human pregnancy, derived from a placental source [113]. HCG stimulates the secretion of inhibin from these cultured placental cells [111] and human breast MCF-7 cells [46]. The induction of inhibin by hCG was associated with inhibition of cell proliferation [46]. In rats, hCG treatment induced the expression of inhibin in the cytoplasm of alveolar cells but not in ductal cells [109]. The induction of inhibin by hCG was evident by 10-day of hCG treatment and reached maximal by day 15. Thereafter, the hCG-mediated induction of inhibin was detected in the stroma, which exhibited maximal expression by day 20. Once hCG treatment was terminated, the mammary gland regressed to its pre-treatment condition, appearing similar both in morphology and inhibin content to that of control animals. The expression of inhibin in the mammary gland after hCG administration at the time of maximal lobulo-alveolar development, and its diffusion towards the stroma during regression, suggest a critical role of inhibin as a modulator of mammary growth and differentiation. The time-course of induction of inhibin expression by hCG treatment appears to be parallel to that of hCG-induced inhibition of mammary gland tumor, indicating its role in these events. Furthermore, inhibin has been shown to regulate mammary epithelial cell differentiation through mesenchymal-epithelial interactions [114]. In these animals, inhibin-a and inhibin-b were found to be elevated in the non-tumoral mammary glands in association with lobule formation and in the tumors. Their mRNAs were also elevated in the mammary tissue, associated with increased levels of c-myc and c-jun induced by the hCG treatment. DMBA alone did not modify the expression of these genes. These findings indicate that inhibin production and gene activation are associated with both mammary gland differentiation and tumor regression [114].

Another mechanism by which hCG prevents the initiation and the progression of chemically induced mammary carcinomas is through its activation of programmed cell death [101, 115] and inhibition of cell proliferation [116]. Guo et al. analyzed gene expression profiles of human breast cancer cells MCF-7 cells treated with hCG for 24, 48, and 96 h and identified 48 genes affected by this hormone [117]. A cluster of genes was found to be over-expressed during the first 24 h and level off thereafter whereas other genes were maximally expressed at 96 h of treatment. These genes are involved in the regulation of cell proliferation, apoptosis, cell trafficking, and DNA [117]. It has been shown that naturally derived hCG induced apoptosis in human breast xenografts from a mean of 5 % in control to a mean of 28 % in hCG-treated tumors [115]. The hCG activates apoptosis likely via up-regulation of tumor suppressor factors and the modulation of apoptotic gene expression. Several tumor suppressors including p53 [56], OKL38 [118] and VHL [119] have been shown to be

up-regulated by hCG. The tumor suppressor p53 is extensively involved in activation of apoptosis induced by a various oxidative stresses. The expression of p53 protein was increased in hCG-pretreated mice and rats and the p53-regulated gene p21Cip1 was also increased concomitantly with p53 [99, 120]. A role of p53 in hCG-induced protection has been indicated by the study using BALB/c p53 null mammary epithelium [121]. In the mammary epithelium, the absence of p53 gene expression abrogated the protective effect of prior pregnancy. The tumor incidence curves were superimposable in p53 null mammary epithelium treated with DMBA or pregnancy plus DMBA. These results demonstrate that p53 plays a pivotal role in hCG-induced protection. The role of p53 in hCG-induced protection has been described previously [101]. Another tumor suppressor, OKL38, is a pregnancy-induced growth inhibitory gene [118]. It was enhanced by hCG in the rat mammary gland and ovary. The overexpression of this gene in Buffalo rat liver cells resulted in growth inhibition and cell death. Interestingly, Yao et al. reported that expression of OKL38 was enhanced by activation of p53 following DNA damage, and that OKL38 induced apoptosis through localization to mitochondria and induction of cytochrome *c* release [122]. OKL38 has been shown to be an oxidative stress response gene stimulated by oxidized phospholipids, indicating a potential role in protection against oxidative stress [123]. The VHL gene is a tumor suppressor gene encoding an E3 ubiquitin ligase that results in specific target proteins being marked for degradation. It has been shown that hCG up-regulated the transcript level of VHL, associated with increased expression of p53 in human granulose lutein cells [119]. In addition, several apoptotic genes including TRPM2, ICE, and TGF-b have been shown to be up-regulated by hCG in associated with up-regulation of p53/p21Cip1in MCF-10 F cells and MCF-7 [99]. It is likely that p53/OKL38/VHL pathway may be involved in mediation of hCG-induced apoptosis in mammary gland. Through up-regulation of these tumor suppressors, as well as other apoptotic-related genes, hCG induces programmed cell death.

Several studies have reported that hCG inhibited cell proliferation. The transcription factors, NF-kB, AP-1 and estrogen receptors (ERs) are involved in up-regulation of a large number of growth-related genes in breast cells [46, 116]. HCG treatment decreased proliferation and invasion of breast cancer MCF-7 cells by inhibiting NF-kB, and AP-1. Estrogens are potent stimuli of cell proliferation in breast epithelial cells and their proliferative effects are mediated mainly via ERa in more than 80 % of all breast cancers. Pregnancy and breast-feeding pregnancy and lactation reduce estrogen levels in breast cells. Treatment of MCF-7 cells with highly purified hCG resulted in a dose- and time-dependent significant decrease in steady-state ER mRNA and protein levels as compared to controls, with the maximal decrease occurring after 4 h of culture with 10 ng/mL hCG. Furthermore, it has been observed that hCG, through up-regulation of inhibin, down-regulated the expression of the ERa by methylation of the CpG islands within the promoter region of this gene [46]. It is likely that hCG inhibits estrogen-mediated breast cell proliferation by reducing the E_2/ERa-mediated signal pathways during pregnancy.

All these data leads us to conclude that the hormonally-induced differentiation offers enormous promise for the primary prevention of breast cancer and that the ability of hCG to replicate the naturally protective effects of pregnancy against breast

cancer hold a significant public health value. More importantly, hCG enhanced the radio-sensitivity of the MCF-7 breast cancer cell line, resulting in an 8–10 % reduction in MCF-7 cell survival at a dose of 2Gy, a typical dose used in conventional cancer therapy [124]. In this regard, hCG alone and in combination with other therapies represent a new approach effective also for breast cancer treatment.

7.4 Development of Small Specific Molecules That Can Produce the Same Molecular Mechanisms Induced by Either Pregnancy or HCG

The well proven role of hCG in inducing molecular changes resulting in a lifetime reduction in breast cancer incidence is hampered by the need of administering the hormone either subcutaneously (sc) or intramuscularly (im) due to its large size and its inactivation when given orally. Therefore, it is important to investigate the possibilities of activating the hCG receptor by the use of small peptides from the beta chain or using chimaeras from the alpha and beta chains of this hormone to confer the protective effects against breast cancer. Preliminary data show that one of the peptides of the beta chain exerts a differentiating effect on the breast epithelium similar to that induced by both pregnancy and the placental hormone hCG.

It has been demonstrated that the human breast epithelial cells (HBEC) MCF-10F reproduce the normal processes of ductulogenesis and branching, mimicking the architectural pattern of the normal breast *in vivo* when seeded in a 3D collagen matrix. The cells grow along hollow branches forming ductules lined by a monolayer of epithelial cells. These normal-appearing ductules become disarrayed when the cells are treated with chemical carcinogens [125] or with E_2 [126–128], forming instead spherical structures with a multilayered epithelium that exhibits marked atypia, similar to that observed in atypical hyperplasia and *in situ* carcinomas reported in primary breast lesions. Treatment of E_2-transformed MCF-10F cells with 2.5mcg/mL r-hCG resulted in a significant decrease in the number of solid masses in comparison with the controls. The hormonal treatment also increased the number of secondary and tertiary branching in the ductular structures, a phenomenon that characterizes the differentiating properties of r-hCG [129]. Selected oligopeptides of the hCG beta subunit were synthesized and evaluated to test their abilities to mimic the complete hormone. A 15 aa peptide with a sequence "N"-SYAVALSCQCALCRR-"C" that encompasses aa 81–95, designated peptide 81–95, was tested in the *in vitro* system described above. Its addition to the culture medium increased the branching pattern of MCF-10 F cells by increasing the number of secondary and tertiary ducts (Fig. 7.4A). It also abrogated the formation of solid masses of 17 beta-estradiol transformed cells (E_2 cells) in collagen (Fig. 7.4B), and inhibited invasiveness more efficiently than r-hCG in MCF-10 F cells, in their derived E_2 cells and tumor derived cells (E_2T4), and in the breast cancer cell lines MCF-7 and MDA-MB-435 (Fig. 7.4C).

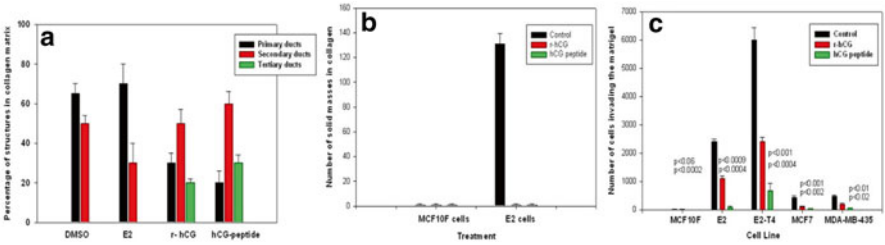

Fig. 7.4 (**a**) Effect of 17b-estradiol (E_2), r-hCG and hCG peptide on formation of primary, secondary, and tertiary ducts in MCF10F cells grown in a collagen matrix. DMSO served as control. Ordinate, percentage of structures. (**b**) Effect of r-hCG and hCG peptide on solid masses formation. Only DMSO-treated (control) E2-cells formed solid masses. C. Effect of r-hCG and hCG peptide on the invasive index of MCF10F, E_2-transformed, E_2T4, MCF-7 and MDA-MB-435 cells. Ordinate, number of cells traversing a matrigel membrane

Given the evident limitations of currently existing strategies for breast cancer prevention [6, 7, 14, 26, 126, 130–148], it is of significant importance to develop new approaches capitalizing on the preventive effect of hCG, on hormonally induced differentiation, on the ability to change the genomic signatures into one that reduces the risk for breast cancer, and on the novel findings that specific oligopeptides can be tailored to target pathways for optimal induction of breast differentiation and cancer prevention.

The hCG receptor is a member of the subfamily of glycoprotein hormone receptors within the superfamily of G protein–coupled receptors (GPCR). The hormone-binding domain has been localized to exons 1–7 in the extracellular (EC) domain/region of the receptor, which contains several leucine rich repeats. High-affinity binding of hCG and LHR causes secondary hormone or receptor contacts to be established with regions of the EC loop/transmembrane module that initiate signal transduction. CG/LH-R coupling functions are exerted primarily through cAMP/protein kinase A-mediated events in the gonads [149, 150]. To verify the presence and functionality of the receptor in normal and transformed MCF10F cells we used the monoclonal antibody (mAb) 20C3 raised against the human LHR-transfected Chinese hamster ovary (CHO-LHR) cells, which was kindly provided by Drs. A. Funaro and F. Malavasi, from the Dept. Genetics, Biology and Biochemistry at the University of Torino, Italy. MCF10F and E_2-transformed cells exhibited a punctuate positive reaction along the cytoplasma membrane (Fig. 7.5), in a distribution similar to that seen in the positive control MA-10A cells. For testing the functional capacity of hCG and the 81–95 peptide, MA-10 [151] and MCF10F cells were treated with 2.5 μg r-hCG/mL or 20 μM 81–95 peptide by measuring their effect on intracellular cAMP production following the manufacturer's recommended procedures (Fig. 7.6). Both treatments induced in MA-10 and MCF10F cells a time-dependent increase in intracellular cAMP production, indicating that the expressed human LH/hCG-receptor functionally couples with endogenous adenylyl cyclase.

Preliminary studies performed in the Breast Cancer Research Laboratory at the Fox Chase Cancer Center constructed a model of hCG bound to the CG/LH-R, based

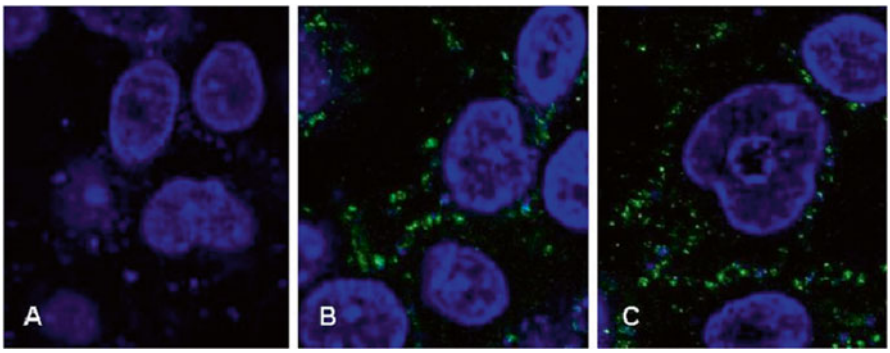

Fig. 7.5 Immunocytochemical detection of CG/LH-receptor in MCF10F cells. (**a**) MCF10F negative control; (**b**) MCF10F cells, and (**c**) E_2 transformed cells. Cells incubated with 20C3 mAb exhibited a punctated reaction along the cyto.plasma membrane stained with goat-antimouse 488 AlexaFluor; blue stained nuclei (DAPI)

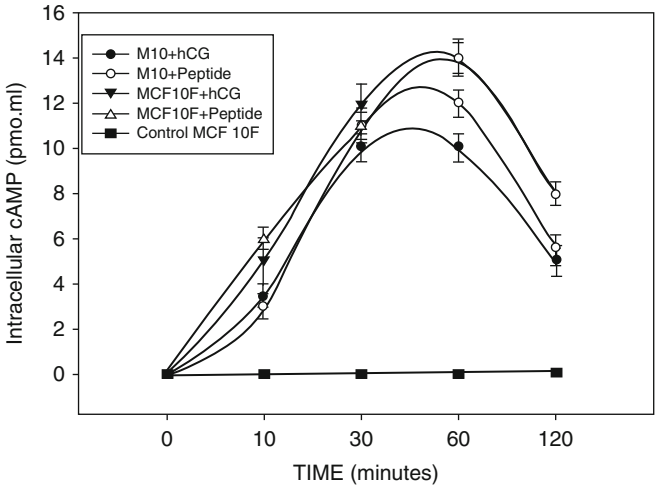

Fig. 7.6 Intracellular cAMP concentration in pmol/mL (ordinate) measured in acetylated MA-10 and MCF10F cells using a cAMP enzyme immunoassay kit (EIA CA201, Sigma-Aldrich, St. Louis, MO)

on the PDB structure 1XWD [152]. The sequences of hCG and CG/LH-R were aligned to their respective templates using the program MolIDE, and side chain conformations of the protein and peptide were predicted with the program SCWRL [152–154], allowing all side chains to move in both hormone and receptor. The structural details of this model reveal several aspects of the functional specificity conferred by the beta chain of the hormone. There is an exceptionally high charge density within the interface between receptor and hormone, and correspondingly, it is expect that a significant portion of the specificity of a particular hormone for its cognate receptor will be conferred by a constellation of complementary charge interactions. One such interaction that could contribute to specificity of hCG for its cognate recep-

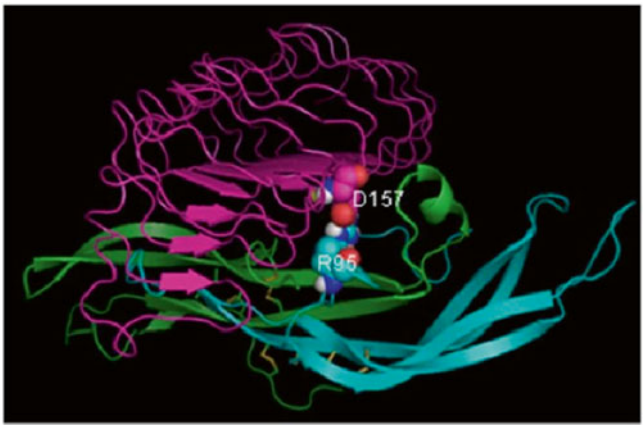

Fig. 7.7 Specificity determining hCG residue R95/115 making contact with CG/LH-R residue D157

tor involves R95 in hCG (R115 in full-length sequence) is predicted by this model to make intimate contact with the receptor at position D157 (Fig. 7.7). This is only one example of several interactions predicted from this model which could be tested for exploring the determinants of hormone specificity and also to target the CG/LH-R for response that could mimic the hormone activation.

7.5 Chromatin Remodeling, the Essential Event for Breast Cancer Prevention

7.5.1 Chromatin Remodeling in the Human Breast of Parous Women

The reduction in breast cancer risk conferred by the first pregnancy has been attributed to the induction of complete differentiation of the virginal breast, which in nulliparous women remains undifferentiated and susceptible to carcinogenic insults [98, 155]. In the breast of parous women, the epithelial cells have a condensed chromatin and increased reactivity with anti-H3K9me^2 and H3K27me^3 antibodies [27]. Histone methylation is a major determinant for the formation of active and inactive regions of the genome and is crucial for the proper programming of the genome during development [63], observations that led us to conclude that chromatin remodeling is the driving force of the observed differences between the parous and nulliparous breast (Fig. 7.1). The differentiated breast of parous women is characterized by up-regulation of different genes, including chromatin remodeling genes, such as *CHD2, L3MBTL* and *CBX3*; this latter one recognizes and binds H3 tails methylated at lysine 9, leading to transcriptional silencing in heterochromatin-like complexes and epigenetic repression. *L3MBTL,* up-regulated in the parous breast, is an important gene

related to the polycomb group (PcG) protein and forms multimeric protein complexes that maintain the transcriptional repressive state of genes over successive cell generations. This gene is also up-regulated in women that received hCG. The *PcG* acts mainly as a gene silencer through the addition of three methyl groups to lysine 27 of histone 3, a modification that leads to chromatin condensation (Fig. 7.1) [52, 64, 65]. It is possible to postulate that the increased chromatin condensation observed in the parous breast is the surrogate end point of the preventive effect of pregnancy. This postulate is based on the study of human breast tissue biopsies following stringent criteria of transcriptome analysis in nulliparous and parous women [27–29]. In addition, when we studied the methylation pattern in the breast of nulliparous and parous women, we found differences mainly at Wnt/β-catenin pathway [31], which has important role in development and also in chromatin remodeling [156].

Studies of the human breast have clearly indicated that there are morphological and immunohistochemical evidences of chromatin remodeling in the parous breast, such as the increase in the number of epithelial cells with condensed chromatin and increased reactivity with anti-H3K9me2 and H3K27me3 antibodies in combination with up-regulation of genes controlling these processes [27] (Fig. 7.1). The chromatin remodeling process is demonstrated not only by the shifting of the EUN to the HTN cells, but also confirmed by the increase in methylation of histones H3K9me2 and H3K27me3. This is an indication that methylation of other genes could also be involved in the process. Using the DNA from five nulliparous and five parous breast core biopsies and applying the MBD-cap sequencing methodology [157], 583 genes showing different levels of methylation between the parous and nulliparous breasts [31, 158]. From the 583 genes, 455 were hypermethylated in the parous while 128 were hypermethylated in the nulliparous breast, confirming the reprogramming of the chromatin to a more silenced or resting stage [31, 158]. Using Integrative Genomics Viewer (IGV) software [159, 160], the distinct areas, throughout the entire gene, where the methylation levels differed between the sample groups were identified. The identification of these areas, known as differentially methylated regions (DMRs) is important because it may indicate if a methylation is more likely to affect gene expression [161]. Using IGV, DMRs of 53 genes were identified. Analysis and research into the functions of these genes showed that several of them interact to each other in either the Wnt signaling pathway or in its controlling PI3K/AKT/mTOR pathways [31, 158]. Interestingly, the analyses of gene expression differences between parous and nulliparous [28, 29] also identified components of this pathway, such as CSNK1A1 and SOX family. The overall methylation and gene expression profiles indicates that the beta-catenin, a downstream protein of the Wnt signaling pathway, is being down-regulated in the parous women [31, 158].

7.5.2 r-hCG Induces In Vitro Chromatin Remodeling

In order to determine if hCG is targeting chromatin remodeling, we have used MCF-10F cells treated with 50 IU/mL of r-hCG for 2 weeks. The treated and untreated cells were grown in collagen matrix after the treatment and used to

quantify the level of methylation of the histone 3 (Fig. 7.8). Immunohistochemistry (IHC) was performed using antibody against the H3 trimethylated at lysine 4 ($H3K4me^3$) and at lysine 27 ($H3K27me^3$). Evaluation of IHC reactions was performed by a count of ~800 cells per case and results were expressed as the percentage of positive nuclei over the total number of cells counted and statistically analyzed by t-test. Cells were evaluated according the intensity of brown staining as strongly positive (+++); moderately positive (±), or negative (−). More than 80 % of the MCF-10 F control cells (Fig. 7.8. a,b) were strongly positive with the $H3K4me^3$ antibody, consistent with the presence of an active euchromatin, whereas 60 % of the r-hCG treated cells (Fig. 7.8 c,d) were moderately positive or negative. The opposite reactivity was observed in the cells immunoreacted with the $H3K27me^3$ antibody, showed in Fig. 7.8 (g,h), whereas greater than 80 % of control cells were either weakly positive or negative (Fig. 7.8 e,f).

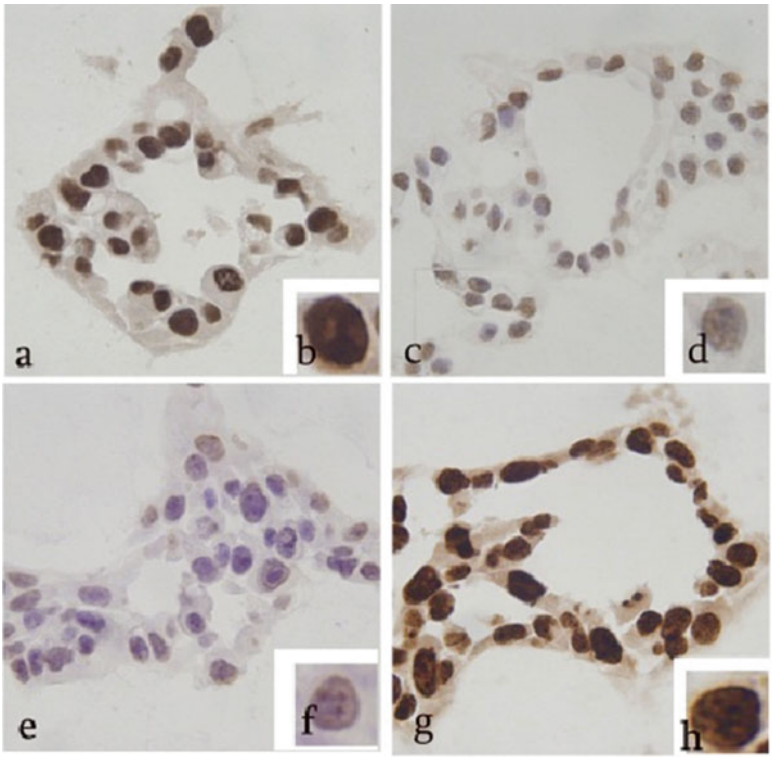

Fig. 7.8 MCF-10F cells growing in collagen after a 2-week treatment with 50 IU r-hCG. IHC reactivity with antibody against the H3K4me3 (a-d) or H3K27me3 (**e**–**h**). Untreated controls (**a**, **b**) were strongly positive with the $H3K4me^3$ antibody, consistent with the presence of an active euchromatin, whereas r-hCG treated cells (**c**, **d**) were predominantly negative. MCF-10F cells immunoreacted with H3K27me3 antibody showed strong nuclear reactivity in r-hCG treated cells (**g**, **h**), whereas the untreated control cells were negative (**e**, **f**) (From: Russo, J. and Russo, I.H. "The transcriptome of the human breast" *Springer, New York 2012* [96])

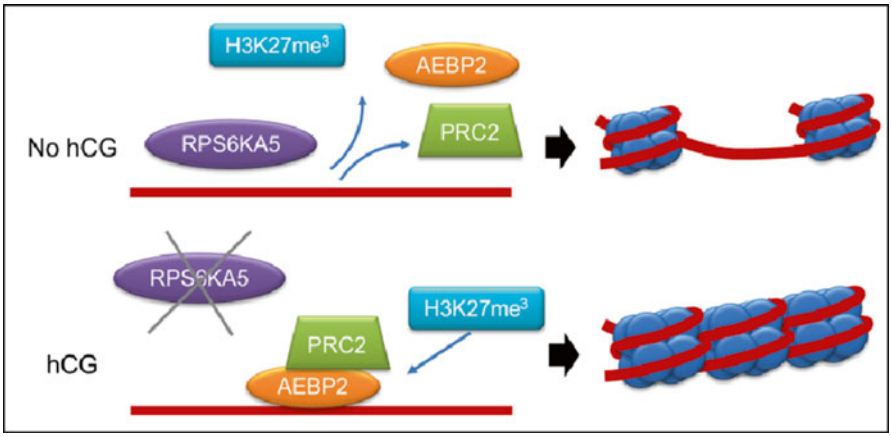

Fig. 7.9 Hypothesis of the interaction of the genes altered by hCG. After hCG treatment, AEBP2 expression is increased and RPS6KA5 expression is decreased. This allows the recruitment of PRC2, resulting in trimethylation of H3K27 and condensation of the chromatin

In addition to these evidences, when we performed gene expression microarray analysis comparing MCF-10F cells treated with 50 IU r-hCG for 2 weeks against untreated cells, we also observed genes related to chromatin remodeling being altered. Some of these genes were RPS6KA5, AEBP2, and CHD2. This last one, found up-regulated by r-hCG, was also up-regulated in parous women [28, 29]. RPS6KA5, also known as MSK1, has been described to antagonize polycomb silencing through the displacement of Polycomb Repression Complex (PRCs) and the removal of H3K27me^3 [162], this gene was down-regulated by r-hCG, supporting more activity of H3K27me^3. Yet, we found up-regulation of AEBP2, which is a DNA-binding protein that recruits Polycomb Repression Complex 2 (PRC2), resulting in the trimethylation of H3K27 [163]. The interaction of these genes could induce the condensation of the chromatin in the hCG-treated MCF-10 F cells (Fig. 7.9).

7.6 Future Perspectives in the Use of R-HCG and Short Peptides in Cancer Prevention

Pregnancy exerts a protective effect in women who had completed a FTP in their early twenties. The same protective effect is observed in rodent models and it is possible to be mimicked with the use of r-hCG. Therefore the usage of this hormone, already used for other applications by women, has the potential to be used as a preventative agent against breast cancer, especially for those women with high risk for this disease, such as those with BRCA1/2 mutations. Previous studies in rats showed that even the r-hCG usage for periods shorter than the length of pregnancy was able

to induce protection against mammary tumors, meaning that the prophylactic effect of r-hCG probably can be reached with treatments shorter than 40 weeks.

One disadvantage of the use of the whole hormone is the via of administration, however this issue can be avoided if smaller, specific molecules, targeting the same receptor are developed to trigger the same effects induced by hCG, similarly to the peptide mentioned in this chapter. Further studies in this area are needed for the development of more specific and efficient molecules. In addition, better understanding of the molecular effects of parity and hCG on the mammary gland may help the development of additional, novel strategies to prevent breast cancer.

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Chapter 8
Risk-Reducing (Chemopreventive) Agents in Breast Cancer Prevention

Naomi Gronich and Gad Rennert

Abstract The use of risk-reducing medications to prevent the development of malignant diseases has been promoted for more than 20 years. Successful examples have mostly involved the use of hormone antagonists and SERMs as well as anti-inflammatory drugs, and were mostly reserved for high risk patients.

In this chapter we discuss the current evidence available for the association between breast cancer and commonly used drugs suggested in the literature as carrying potential preventive activity against breast cancer in vitro, in animal models and in humans. These include vitamin D, bisphosphonates, statins and metformin, all of which are in use for a variety of non-cancer related indications.

While all of these compounds have shown a high level of anti-breast cancer activity, in one or more of the different experimental platforms, none have been shown to be preventive in randomized controlled trials (RCTs). Therefore these drugs have not been formally approved for actual use in prevention, in either average-risk or high-risk women. This might reflect the fact that it is extremely hard to use RCTs that employ medications that are in common use, because of a major bias that is introduced if one randomizes only the fraction of the population that is not already using the drug for other indications. However, a common use of these compounds by the population, if actually have a true preventive effect, would lead to reduction in incidence of breast cancer in the population at large by way of a "natural experiment". The current reduction in breast cancer incidence and mortality seen in many western countries can actually be attributed, at least in part, to an inadvertent effect of these drugs.

Keywords Breast cancer • Chemoprevention • Prevention • Risk reduction • Metformin • Vitamin D • Statins • Bisphosphonates

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8.1 Introduction

Breast cancer affects approximately 1.7 million women worldwide annually, and is the most common cancer in women. Breast cancer survival rates vary greatly worldwide, ranging from 80 % or over in North America, Sweden and Japan to around 60 % in middle-income countries and below 40 % in low-income countries [1]. It is estimated that worldwide over 500,000 women die annually due to breast cancer [1]. Effort should be put on preventing cancer. Prevention requires a multidimensional approach that combines behavioral/life-style changes, use of risk-reducing agents (chemoprevention), surgical prevention and early detection (screening) efforts.

Drugs or agents that interfere with the carcinogenic process to prevent or reduce the risk of cancer are termed chemopreventive agents [2]. Premalignant lesions, that have not yet gained many genetic alterations, are probably more easily treatable than malignant cells and are the targets for the prevention endeavor. Chemopreventive agents that are effective in the premalignant setting might not be effective in cells that have become malignant. Chemopreventive agents can interfere with tumor initiation by, for example, preventing carcinogen activation, or they might interfere in tumor promotion processes by changing the tumor microenvironment and blocking signal transduction. Inflammation is a potential target for chemoprevention through interference with tumor promotion. An example is the COX-2 inhibitor celecoxib that was shown to reduce the risk of colorectal cancer. Agents might be effective thus on specific stage of tumor development, and if given before or after that stage they might not be active [3].

New potential targets and treatments for preventive intervention are constantly being searched for. One of the most accessible approaches is using drugs that are already being used for other indications. Such medications, especially if widely used, already have an established safety profile and are relatively easy to evaluate using association studies. As such, tamoxifen and raloxifene were proven effective in preventing breast cancer in randomized controlled trials. A strong rationale derived from many independent lines of evidence, including mechanistic, animal model and clinical trials data, formed the basis for the successful tamoxifen/raloxifene cancer prevention trials. However, adverse effects concerns (increased risk of endometrial carcinoma, stroke and venous thromboembolism) affected widespread acceptance of these drugs as a primary preventive measure [3].

Understanding the overall biology of the malignant process and the mechanisms of site-specific cancer development is crucial in identifying candidate chemopreventive agents.

The establishment of the efficacy of chemopreventive agents is based on evidence collected from animal models, association studies in humans and experimental studies in humans. Mechanistic insights from in vitro studies and from animal models are of major importance. However, animal models many times do not parallel the biological mechanisms and metabolic processes in humans.

Observational data in humans, derived from case-control and cohort studies provide important evidence of association between the use of medication and the risk of disease. These enable assessment of the intervention in large population, and in "real-life" situations. However, such observational studies are prone to a variety of biases that could confound the results.

Randomized controlled clinical trials are considered the gold standard for proving drug efficacy and for risk/benefit assessment of pharmacological agents. Phase 3 trials may not be feasible when dealing with widely used drugs, as excluding current consumers of the drug will introduce a significant selection bias to the trial. Phase 3 trials are also of only limited time duration; therefore long term effects on clinical outcomes might not be identified.

Chemoprevention should first be aimed at high risk populations where the benefit to risk ratio is maximal. If found effective, and if side-effects profile is minimal—such medication can be considered for use by the population at large. It is as yet unclear how many healthy people at average risk will be ready to take medications for extended periods or for life to prevent disease. Experience with patterns of vitamins use by the general population could suggest that such an approach is feasible.

This chapter will review evidence on non-hormonal pharmacological measures considered for breast cancer prevention. These include bisphosphonates, metformin, statins and vitamin D.

8.2 Bisphosphonates

Bisphosphonates are analogues of pyrophosphate, used in the treatment of osteoporosis, Paget's disease, tumor-associated osteolysis and hypercalcemia [4]. Bisphosphonates contain two phosphonate groups attached to a central carbon. The carbon replacing the oxygen bridge from the natural pyrophosphate allows the attachment of various side chains. Some compounds exhibit short side chains such as etidronate or clodronate. The length of side chains can be increased and amino groups added at their end. Such aminobisphosphonates (N-BPs), including pamidronate and alendronate, are 100- to 1000-fold more potent inhibitors of bone resorption than clodronate or etidronate. Zoledronate, a cyclic bisphosphonate of the newest generation that contains two nitrogen atoms in an imidazole ring, is the most potent compound. Capable of chelating divalent cations such as Ca^{+2}, the bisphosphonates have strong affinity for bone, targeting especially bone surfaces undergoing remodeling. Bisphosphonates remain in the matrix until the bone is remodeled and then are released to the resorption lacunae beneath the osteoclast as the overlying mineral matrix is dissolved. The half-life in bone is very long with ongoing biological activity after a single dose of a few years.

Non-N-BPs are metabolized to methylene-containing, hydrolysis-resistant, analogues of ATP, which accumulate intracellularly and cause inhibition of essential metabolic enzymes, leading to osteoclast apoptosis. The mode of action of first

generation bisphosphonates (clodronate, etidronate, tiludronate) on bone, thus, involves direct induction of apoptosis. Second and third generation N-BPs (alendronate, pamidronate, risedronate, zoledronate) inhibit isoprenoid lipids synthesis in the mevalonate pathway, a biosynthetic pathway responsible for the synthesis of cholesterol and several isoprenoid lipids including farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Fig. 8.1). FPP and GGPP are essential intermediates required for the post-translational prenylation (lipid modification) of important regulatory and signaling GTPases, notably Ras, Rho and Rac. The incorporation of lipid molecules within GTPases is important for their targeted localization and anchorage on the inner side of the cell membrane and for consequent signal activation. The mevalonate intermediates geranylgeraniol and farnesol were shown to be able to inhibit the zoledronic acid induced suppression of

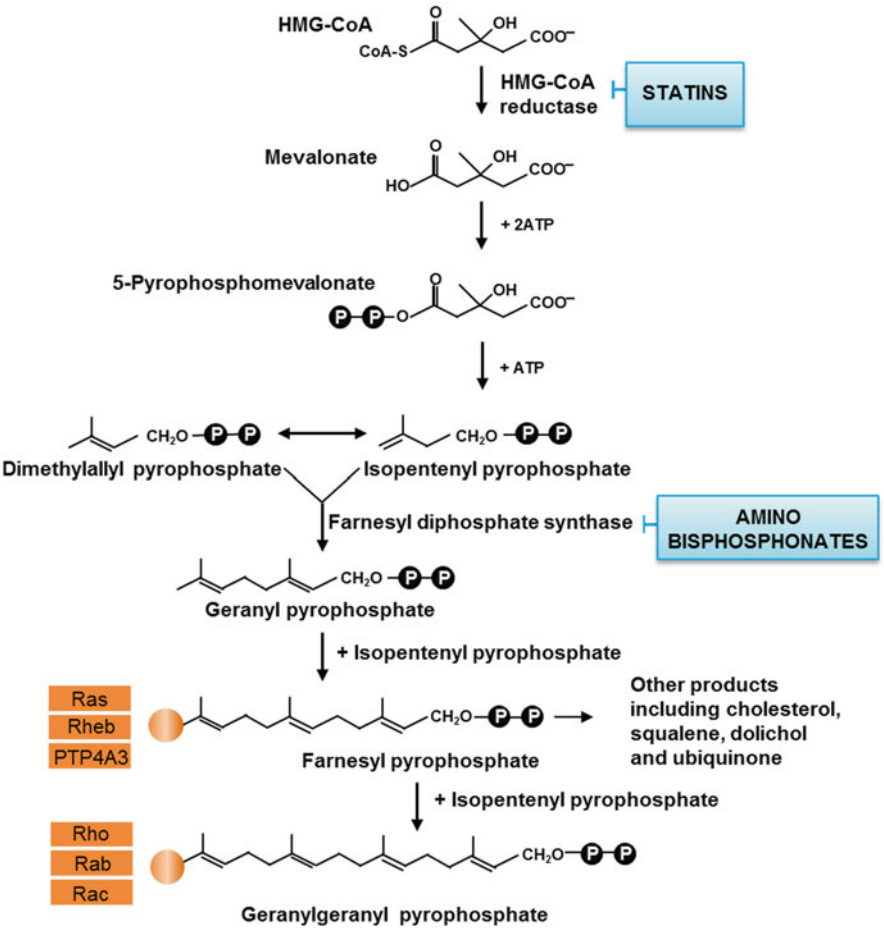

Fig. 8.1 Mevalonate pathway—sites of action of aminobisphosphonates and statins

protein prenylation, activation of the caspase pathway and apoptosis in human breast cancer cell lines [5].

Also of relevance to the development of a malignant process, it has been shown that non-N-BPs and N-BPs differ in their action on inflammatory process. While non-N-BPs can be metabolized into macrophages and may inhibit the inflammatory response of macrophages, N-BPs sensitize macrophages to an inflammatory stimulus, inducing an acute phase response, in particular, related to modifications of the cytokine network [6].

In vivo preclinical evidence showed that bisphosphonates reduce skeletal tumor burden and inhibit the formation of bone metastases in clinically relevant dosing regimen [7]. Bisphosphonates may render the bone a less favorable microenvironment for tumor cell colonization. Circulating tumor cells are attracted to bone surfaces within the bone marrow and bind to the osteoblastic niche by displacing hemopoietic stem cells. Here, tumor cells can remain quiescent for years. When later become active these cells can establish bone metastases or leave the bone microenvironment and potentially initiate metastases at other organ sites. In a study on a nude mice model, sequential treatment with doxorubicin followed by zoledronic acid elicited substantial antitumor effects in subcutaneous breast tumors in the absence of bone disease [8]. Zoledronic acid affected breast cancer metastasis to visceral organs as well as to the bone [9]. Moreover, in vitro, N-BPs have direct anti-tumor effect, with inhibition of tumor cell adhesion, migration, invasion and proliferation, and induction of tumor cell apoptosis. In human breast cancer cells, ex vivo, zoledronic acid showed an antitumor effect similar to the effect of chemotherapeutic regimen [10]. Dose-dependent suppression of cell proliferation and induction of apoptosis by zoledronic acid were shown in highly tumorigenic breast cancer cell lines by a survivin- and caspase-dependent manner [11, 12]. Reduced breast cancer cells viability due to apoptotic death was demonstrated also with pamidronate, and less potently with first generation clodronate [13, 14]. Zoledronic acid combined with letrozole induced levels of apoptosis in breast cancer cells in vitro that were significantly greater compared with treatment with each drug alone. Interestingly, this synergistic relationship was drug-sequence dependent, occurring only when cells were first treated with letrozole, followed by zoledronic acid. The converse sequence, or administering the drugs simultaneously, induced levels of apoptosis no greater than each drug alone [15].

The concentrations of zoledronic acid required to induce tumor cell apoptosis in vitro are 10–100 μM, much higher than the approximately 1 μM peak plasma concentration of zoledronic acid in the clinical setting. Zoledronic acid is rapidly eliminated from plasma by renal excretion and rapid uptake and accumulation in the bone. Osteoclasts, on the other hand, could be exposed to a concentration of 1000 μM of bisphosphonates released from mineralized bone matrix into the resorption space beneath the osteoclast [5].

Combining statins and bisphosphonates, both of which target posttranslational prenylation of proteins by targeting the mevalonate pathway, was shown to potentiate cytostatic/cytotoxic effects against breast cancer cells in vitro, and retardation of tumor growth and prolongation of mouse survival in vivo, while neither pamidronate

nor lovastatin alone affected tumor growth in these mice. N-BPs exert their effects by inhibiting protein farnesylation while statins additionally target protein geranylgeranylation, and this might explain the potentiation of effect [16].

8.2.1 *Mechanisms suggested for the anti-breast cancer effects of bisphophonates*

- Zoledronic acid caused 88 % reduction in tumor growth in bone, compared to vehicle-treated controls, in mice that lacked functional osteoclasts, strongly indicative of osteoclast-independent effects on tumor cells [17].
- Cell-cycle inhibition and induction of both intrinsic- and extrinsic-apoptotic pathways [18].
- Reduction of the stimulatory effects of growth factors (IGF-I,IGF-II, FGF-2) on cell proliferation and inhibition of their protective effects on apoptotic cell death [19].
- N-BPs directly bind to the kinase domain of HER1/2 to cause a global reduction in downstream signaling. By doing so, the drugs kill breast cancer cells that are driven by activating mutations or overexpression of HER1 [20].
- Endoplasmic reticulum stress, activating PERK-eIF2α-CHOP pathway to induce REDD1 expression and inhibit the mTOR pathway [21].
- Inhibition of angiogenesis by Zoledronic acid [22, 23]. Clodronate and pamidronate were shown to abrogate tumor angiogenesis via the HIF-1α/VEGF signaling pathways [24].
- Increased cancer surveillance via activation of γδT cells. Human Vγ9Vδ2 T cells are a subset of human T cells that exhibits anticancer activity. N-BPs are internalized by peripheral blood mononuclear cells, such as monocytes and dendritic cells, where they inhibit the mevalonate pathway, leading to the intracellular accumulation of isopentenyl pyrophosphate (IPP) which activates Vγ9Vδ2 T cells. Human Vγ9Vδ2 T-cells infiltrate and inhibit growth of these tumors. Estrogen receptor-positive tumors seem to be more sensitive to this effect. Expression of tumor cell surface receptor ICAM-1 (intercellular adhesion molecule-1) triggers the recognition by Vγ9Vδ2 T cells [25, 26].
- N-BPs decreased MMP-9 expression and the number of macrophages in tumor stroma. Inhibition of MMP-9 activity breaks the vicious loop linking tumor growth and myeloid suppressor cells expansion, thus helps to reduce immunosuppression [27].

8.2.2 *Human Studies*

Bisphosphonates were found to be associated with decreased risk of cancer including colorectal, endometrial and ovarian cancers and suggestions of the role of bisphosphonates in cancer prevention have been accumulating [28–30]. In a retrospective

cohort study within the UK General Practice Research Database studying 46,036 oral bisphosphonate users and 46,036 matched controls, there was an evidence of 20 % reduction in breast cancer risk, though slightly attenuated over time [31].

The Breast Cancer in Northern Israel Study (BCINIS) is an on-going, population-based, case-control study of incident female breast cancer with age-, clinic-, and ethnic-group matched population controls. Use of bisphosphonates, most commonly alendronate, was assessed in 4039 postmenopausal patients and controls, using pharmacy records. The use of bisphosphonates for longer than 1 year before diagnosis was associated with a significantly reduced relative risk of breast cancer (invasive and ductal carcinoma in situ) (odds ratio [OR] 0.61, 95 % CI 0.50–0.76). Breast cancer risk did not change further if bisphosphonates were used for more years. In addition, Breast tumors identified in bisphosphonates users were more often estrogen receptor (ER)-positive and less often poorly differentiated [32].

As the use of bisphosphonates in women without a cancer diagnosis is mainly for the prevention and treatment of bone loss and as women with bone loss are at lower breast cancer risk since bone density reflects cumulative estrogen exposure, observational studies of bisphosphonates could suffer from confounding by indication. Means for adjusting for differences in bone density between bisphosphonate users and non-users could control such possible confounding.

In a study that accounted for such possible bias of 154,768 postmenopausal participants of the WHI trial, 2816 oral bisphosphonate users at entry (90 % alendronate, 10 % etidronate) were compared to all other participants. Hip fracture risk was incorporated into regression analyses to adjust for bone mineral density difference between users and nonusers of bisphosphonates. After 7.8 mean years of follow-up, invasive breast cancer incidence was lower in bisphosphonate users (hazard ratio [HR] 0.68, 95 % CI 0.52–0.88) as was incidence of ER-positive invasive cancers (HR 0.70, 95 % CI 0.52–0.94). A similar but not significant trend was seen for ER-negative invasive cancers. A lower breast cancer incidence was seen in bisphosphonate users after relatively short-term use while a null association was seen with longer duration use, consistent with a direct effect of bisphosphonate on slowing or inhibiting growth of preclinical but already established breast cancers. The incidence of ductal carcinoma in situ was higher in bisphosphonate users (HR 1.58, 95 % CI 1.08–2.31). The authors suggested that bisphosphonates prevent in situ cancers from progressing to an invasive stage, thus a relative increase in in situ cancers could result [33].

In another population-based case–control study in Wisconsin from 2003 to 2006, including 2936 incident invasive breast cancer cases and 2975 population controls, younger than 70 years of age, the odds ratio for breast cancer in current bisphosphonate users compared with nonusers was 0.67 (95 % CI 0.51–0.89). Increasing duration of use was associated with a greater reduction in risk (P trend = 0.01). There was a suggestion that use of bisphosphonates was associated with a reduced risk of breast cancer only among women reporting symptoms of bone loss (postmenopausal fractures, osteoporosis, and height loss), but the differences were not statistically significant (P interaction = 0.29) [34].

Thus, three separate observational studies of the association between use of second generation N-BPs and risk of breast cancer development in postmenopausal women reached almost the exact same results.

Studies that evaluate the rate of development of contralateral breast tumors are used many times to point at possible prevention potential of drug interventions. In a nested case–control study among women diagnosed with a first primary ER-positive invasive breast cancer at ages 40–79 years, association between postdiagnostic bisphosphonate use and risk of second primary contralateral breast cancer was assessed, using multivariable-adjusted conditional logistic regression. Comparison of 351 contralateral breast cancer case subjects with 662 control subjects (i.e., breast cancer patients not diagnosed with contralateral breast cancer) who were incidence density-matched on county; race/ethnicity; and age at, year of, and stage at first breast cancer diagnosis) current use of any N-BP and use specifically of alendronate were both associated with reduced risks of contralateral breast cancer compared with never use (OR 0.41, 95 % CI 0.20–0.84 and OR 0.39, 95 % CI 0.18–0.88, respectively). The risk of contralateral breast cancer further declined with longer durations of bisphosphonate use among current users (P trend = 0.03) [35].

8.2.3 Clinical Trials

The evidence of anti-tumor activity of bisphosphonates from clinical trials of breast cancer is conflicting and inconclusive; all of the trials used zoledronic acid, while the described observational studies have reviewed mostly alendronate effect.

In the Austrian Breast and Colorectal Cancer Study Group trial-12 (ABCSG-12) zoledronic acid improved disease-free survival (DFS) in the adjuvant setting. ABSCG-12 was a randomized, controlled, multicenter trial in 1803 premenopausal women with ER-positive early-stage (stage I–II) breast cancer receiving goserelin, comparing the efficacy and safety of anastrozole or tamoxifen with or without zoledronic acid (4 mg every 6 months) for 3 years. These were patients carrying disease with a good prognosis, and less than 5 % received chemotherapy. Also, hormonal therapy resulted rapid suppression of reproductive hormones before the initiation of bisphosphonate treatment. The addition of zoledronic acid to adjuvant endocrine therapy improved DFS with reductions in contralateral breast cancer, secondary malignancies, death, and locoregional and distant recurrence [36]. At a median follow-up of 62 months, more than 2 years after treatment completion, zoledronic acid reduced risk of DFS events with an HR 0.68 (95 % CI 0.51–0.91) [37]. After 94.4-months median follow-up relative risk of disease progression was still significant HR 0.77 (95 % CI 0.60–0.99) [38].

In the ZO-FAST Study 1065 postmenopausal women with early breast cancer were randomly assigned to immediate zoledronic acid (4 mg every 6 months) or delayed ZOL (initiated only for fracture or high risk thereof). Immediate zole-

dronic acid group had a significant 41 % relative risk reduction for DFS events (P = 0.03) [39].

In the AZURE trial, an open-label, international, multicenter, randomized, controlled, parallel-group phase 3 trial, 3360 women with stage II/III breast cancer were randomly assigned to receive standard adjuvant systemic treatment alone (control group) or with intravenous zoledronic acid (4 mg every 3–4 weeks for six doses, then every 3 months for eight doses, followed by every 6 months for five doses, for a total of 5 years of treatment). Number of DFS events did not differ between groups. However, zoledronic acid reduced the development of bone metastases. Also, zoledronic acid improved extraskeletal invasive-DFS in postmenopausal women, irrespective of the ER status of the primary tumor, improving disease outcomes in these women [40, 41].

In the neoadjuvant setting, a retrospective evaluation of a subpopulation of patients (N = 205) treated with neoadjuvant chemotherapy in the AZURE study, which evaluated the effect of chemotherapy and/or hormone therapy with or without zoledronic acid in treating women with stage II/III breast cancer, has shown a significant improvement in pathological complete response of 10.9 % versus 5.8 % when therapy was complemented with zoledronic acid [42].

In the NEOZOTAC prospective randomized study comparing the efficacy of TAC (docetaxel, adriamycin and cyclophosphamide) followed by granulocyte colony- stimulating factor with or without zoledronic acid 4 mg every 3 weeks in patients with stage II/III, HER2-negative breast cancer, addition of zoledronic acid to the neoadjuvant chemotherapy did not improve pathological complete response rates. Although postmenopausal women (N = 96) had a numerical benefit from zoledronic acid treatment (pathological complete response 14.0 % for TAC + zoledronic versus 8.7 % for TAC), the difference did not reach statistical significance [43].

It seems that women with low levels of reproductive hormones resulting from either the effects of natural menopause or ovarian suppression treatment benefit more in the adjuvant and neoadjuvant settings from zoledronic acid treatment. One should note the benefit of contralateral cancer incidence reduction in the ABCSG-12 trial, to give a hint as to the role of bisphosphonates in prevention. Also, bisphosphonates effect might be masked when it is part of chemotherapeutic regimens, but might show its effect when given alone, preventively.

Nevertheless, Hue et al. have performed a post-hoc analysis of two randomized trials of the preventive effect of bisphosphonates : The Fracture Intervention Trial (FIT) that randomly assigned 6459 osteopenic/osteoporotic women aged 55–81 years to 5 mg/day alendronate or placebo for 2 years and 10 mg/day thereafter for a mean follow-up of 3.8 years, and The Health Outcomes and Reduced Incidence With Zoledronic Acid Once Yearly–Pivotal Fracture Trial (HORIZON-PFT) that randomly assigned 7765 osteoporotic women aged 65–89 years to annual intravenous zoledronic acid or placebo for a mean follow-up of 2.8 years. Rates of invasive breast cancer were low (0.8–1.8 %), and there was no significant difference between groups [44].

8.2.4 Conclusion

In conclusion, clinical data from randomized controled trials largely do not support the large amount of evidence from in vitro, in vivo and observational studies that suggests both a preventive and a prognostic effect of bisphosphonates. The reasons for such a discrepancy can be several, from differences in type of bisphosphonates used, through differences in dosage and in length of administration.

Randomized clinical trials designed to study the preventive effect of bisphosphonates in high-risk breast cancer patients might be the next reasonable step that will help elucidate the conflict.

8.3 Metformin

Metformin is an oral hypoglycemic drug in widespread clinical use for treating type 2 diabetes mellitus, stimulating fatty acid oxidation, glucose uptake and nonoxidative metabolism, and decreasing blood insulin levels, with low incidence of serious adverse effects [45]. Metformin does not cause hypoglycemia in non- diabetics and has been used safely in polycystic ovary syndrome. Metformin mechanism of action involves increased activity of the AMP-dependent protein kinase (AMPK) (Fig. 8.2), probably by inhibiting complex I of the respiratory chain and thus causing increased ADP:ATP and AMP:ATP ratios, leading to activation of AMPK. An alternative explanation of metformin induction of AMPK is alteration of carbon flow through the folate-related one-carbon metabolic pathways, similar to an anti-folate chemotherapeutic agent [46]. AMPK is a nutrient sensor that inhibits tumorigenesis by targeting tumor metabolism and inhibiting mammalian target of rapamycin (mTOR)-associated oncogenic signaling pathway. mTOR coordinates nutrient availability and energy metabolism in response to growth factors. Metformin can negatively affect growth of human tumors even in the presence of activating mutation in PIK3CA, another regulator of cell metabolism that converges on the mTOR pathway. AMPK suppresses epithelial-mesenchymal transition (EMT) that enables cancer cell invasion of basement membranes and metastasis. Exposure of cells to metformin is sufficient to reverse their mesenchymal phenotype [47]. Metformin has been the subject of much interest in the realm of cancer prevention. A concern has been raised that certain tumor cells might use AMPK activation to undergo metabolic adaptation to their benefit [48], and thus metformin anti cancerous action might be context/cancer-type specific, however, accumulated evidence has provided reassurance as to the protective effect of metformin against cancer. We will discuss metformin action in breast cancer.

Metformin suppresses breast cancer growth in vivo [49–53] and in vitro. Metformin caused concentration-dependent suppression of breast cancer cells proliferation with G1 cell cycle arrest, in both ER-positive and ER-negative cell lines, but the effect was larger in ER- positive cells. A concentration-dependent phosphorylation of AMPK was detected following metformin treatment [54]. Breast

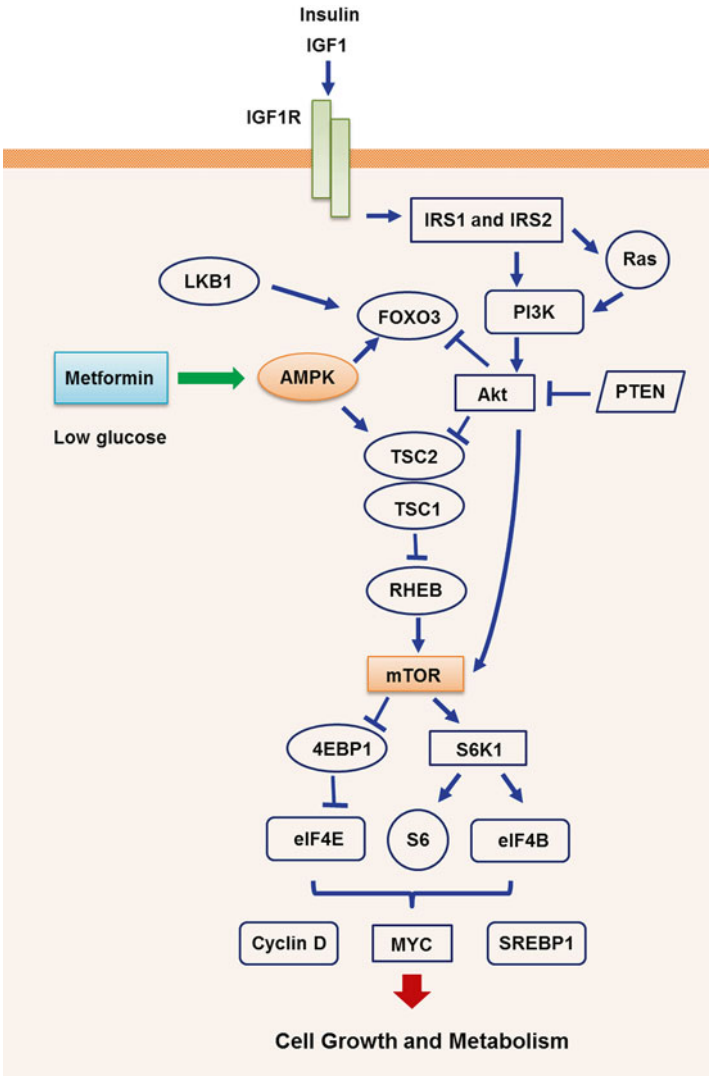

Fig. 8.2 Activation of AMPK by metformin

cancer cells exposed to metformin undergo apoptosis [55], or necrosis, and the cytotoxic effect is pronounced at clinically achievable concentrations [56]. Liu et al. have shown that metformin induces S phase arrest and apoptosis in triple negative (basal-like) breast cancer cells, whereas it inhibits cell proliferation (G1 arrest) without the induction of apoptosis in luminal A, B, and HER2 breast cancer cells [57, 58]. Interestingly, vitamin D3 combined with metformin exhibited synergistic effects on human breast carcinoma cells proliferation and apoptosis, probably through the mTOR related pathways [59].

8.3.1 Mechanisms Suggested for Metformin Anti-Breast Cancer Effect

- Activation of AMPK, that induces cell cycle arrest and cytostatic effect by switching off the synthesis of lipids, ribosomal RNA and proteins [60].
- Inhibition of mTOR pathway and its downstream effectors such as S6K1 and 4EBP1 [56].
- Reducing plasma levels of insulin and insulin-like growth factor-1 (IGF-1) [61], thus reducing signaling through Ras and PI3K pathway.
- Disrupting ErbB2/IGF-1R complexes, Src Kinase and PI3K/Akt signaling [62].
- Circumvention of the ability of cancer cells to switch to aerobic glycolysis, under glucose deprivation condition of tumor microenvironment (where there is an imbalance between poor supply and very high consumption rate) [63].
- Reduction of fatty acid synthase expression, critical for de novo fatty acid synthesis, via miRNA-193b [64].
- Inhibition of Sonic hedgehog signaling pathway that regulates expression of genes controlling cell growth, survival and differentiation, and shown to be correlated with breast cancer cells invasiveness [65].
- Inhibition of histone H2B monoubiquination in an AMPK dependent manner [66].
- Inhibition of advanced-glycation-end-products (AGEs)-induced growth and VEGF expression by suppressing RAGE gene expression through AMPK activation [67].
- Restoration of MHC-I expression on cell surface, thus preventing immune escape of cancer cells [68].
- FOXO3 nuclear localization, activation of ATM-pS1981, γ-H2AX, p53-pS15; downregulation of expression of stemness markers [49].
- Inhibition of the nuclear translocation of CRTC2, a CREB-coactivator known to increase aromatase expression [69].
- Reversal of epithelial-mesenchymal transition (EMT) that enables cancer cells invasion of basement membranes and metastasis [47].
- Inhibition of cancer stem cells survival and self-renewal [56, 65].
- Affecting metabolism and miRNA expression of chemoresistant cells to become more similar to chemosensitive cells [70]; Resensitization of multidrug resistant cells to chemotherapy [71].

8.3.2 Human Studies

Increasing age, obesity and insulin resistance are shared risk factors between diabetes mellitus and breast cancer, and diabetes mellitus slightly increases the risk of breast cancer. In a cohort study among 52,657 British women with type 2 diabetes, and 30,210 randomly selected women without diabetes, diabetes was associated with a 29 % increased overall breast cancer risk, though the association was markedly attenuated when adjusted for age, period of cohort entry, region, and body mass

index (BMI) [72]. Using Taiwan's National Health Research Institute Database diabetes patients were found to have slightly increased risk for breast cancer [73], and in a cohort study of 4216 early stage (I-II) breast cancer, diabetes and insulin use were associated with increased risk of recurrence [74]. In a case-control study in Spain of postmenopausal breast cancer cases and randomly selected controls, diabetes was not associated with the overall risk of breast cancer, but it was only linked to the risk of developing triple negative tumors (OR 2.25, 95 % CI 1.22–4.15) [75].

Metformin use is associated with reduced cancer incidence overall as compared with the use of other antihypoglycemic drugs. In particular for breast cancer, a study of 68,019 postmenopausal women followed-up over a mean of 11.8 years, from the Women's Health Initiative (WHI), found that women with diabetes receiving medications other than metformin had a slightly higher incidence of breast cancer compared with women without diabetes, and women with diabetes who were given metformin had lower ER+/PR+, and HER2- breast cancer incidence, after adjustment for breast cancer risk factors (HR 0.75, 95 % CI 0.57–0.99) [76]. Using the Danish medical registries Bosco et al. have conducted a nested case-control study among type 2 diabetic patients, and found approximately 20 % lower breast cancer incidence among metformin users compared with non-metformin users, after adjusting for diabetes complications, obesity, and predictors of breast cancer [77]. In contrast, in a retrospective cohort study within the UK Clinical Practice Research Datalink, no difference in breast cancer incidence between metformin and sulfonylurea initiators was found [78].

Biological effects of administering short courses of metformin to breast cancer patients were exhibited in prospective "window of opportunity" trials that have followed the in vitro and epidemiological studies. Women with breast cancer that did not have diabetes were given metformin after diagnostic biopsy until surgery, and biological markers were compared between pre and post metformin histological specimens. Breast cancer tumors had decreased expression of insulin receptor, following metformin treatment, and decreased phosphorylation status of protein kinase B (PKB)/Akt, ERK1/2, AMPK, and acetyl coenzyme A carboxylase [79]. However, in a second trial, up-regulation of pAMPK was demonstrated in metformin treated patients, along with downregulation of pAkt compared to control group, as well as significant fall in the proliferative index ki-67 and cleaved caspase-3 [80]. Niraula et al. have also demonstrated decreased ki-67 staining following metformin treatment [81]. Yet in another window of opportunity trial metformin did not significantly affect levels of ki-67 overall [82], but different effects of metformin treatment, according to patients' insulin resistance, particularly in luminal B tumors, were demonstrated [83].

8.3.3 Conclusion

In conclusion, in vitro data on beneficial effects of metformin are appealing, and are somewhat supported by epidemiological evidence. However the overall evidence is still relatively weak and future primary prevention clinical trials are needed before clinical recommendations can be made.

8.4 Statins

Statins, the most effective and best-tolerated agents for treating dyslipidemia, are competitive inhibitors of hydroxyl-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, which catalyzes an early, rate-limiting step in the mevalonate pathway of cholesterol biosynthesis in hepatocytes (Fig. 8.1). Serious adverse events associated with statins use such as rhabdomyolysis and liver failure are rare (44 cases per million person-years of use; and 1 case per million person-years of use, respectively), and occur more often in older age and with concomitant use of drugs that diminish statin catabolism or interfere with hepatic uptake of statin [84].

Lovastatin, simvastatin, fluvastatin and atorvastatin are lipophilic drugs, entering liver cells by diffusion, able to permeate also extrahepatic cells membrane. Pravastatin and rosuvastatin are hydrophilic and enter liver cells via the organic anion transporter protein 2 (OATP2) (Fig. 8.3).

Statins have been shown to exert broader biological effects beyond reducing cholesterol. Reduction in isoprenoids (farnesyl pyrophosphate and geranylgeranyl pyrophosphate), intermediate products in the mevalonate pathway, needed for post-translational modification of various proteins such as Ras and Rho, can explain some of statins' other documented biological activities (Fig. 8.1). Statins display anti-inflammatory and immunomodulatory effects by altering gene expression and function of cells involved in the inflammatory process. Treatment of monocytes with statins reduces CD11 expression and inhibits cellular adhesion to the endothelium. By reducing IFNγ-mediated induction of MHC-II in endothelial cells, statins reduce T-cell activation [85, 86].

Evidence from in vitro, in vivo and human studies have suggested a possible anticancer direct effect of statins, by enhancing tumor apoptosis, inhibiting angiogenesis, and impairing metastasis [87].

Lipophilic statins are able to inhibit breast cell proliferation [88, 89], in a dose dependent manner [90]. The inhibitory power was between 10 and 90 %; the potency was greater in ER-negative cancer cells [90]. Koyuturk et al. have shown that statins induce cell cycle arrest and apoptosis in both ER-positive and ER-negative cell lines [91]. The effect of statins could be reversed with mevalonate, and mimicked by geranylgeranyl transferase inhibitor but not farnesyl transferase inhibitor [92].

Mevalonate has been shown to promote the growth of human breast tumors in mice, where tumors in mevalonate treated mice were significantly larger than tumors in control mice [93]. Lovastatin prolonged tumor latency, reduced tumor formation and metastatic dissemination of a highly invasive and metastatic mammary carcinoma murine model [94]. The antineoplastic effects of simvastatin were demonstrated in chemoprevention of N-methyl-N-nitrosourea-induced mammary carcinogenesis in female rats, in which simvastatin suppressed tumor frequency by 80 % [95], or caused a shift from high grade to low grade carcinomas [96]. ER–/HER2+ mammary tumor growth was inhibited by simvastatin and fluvastatin in female mice [89].

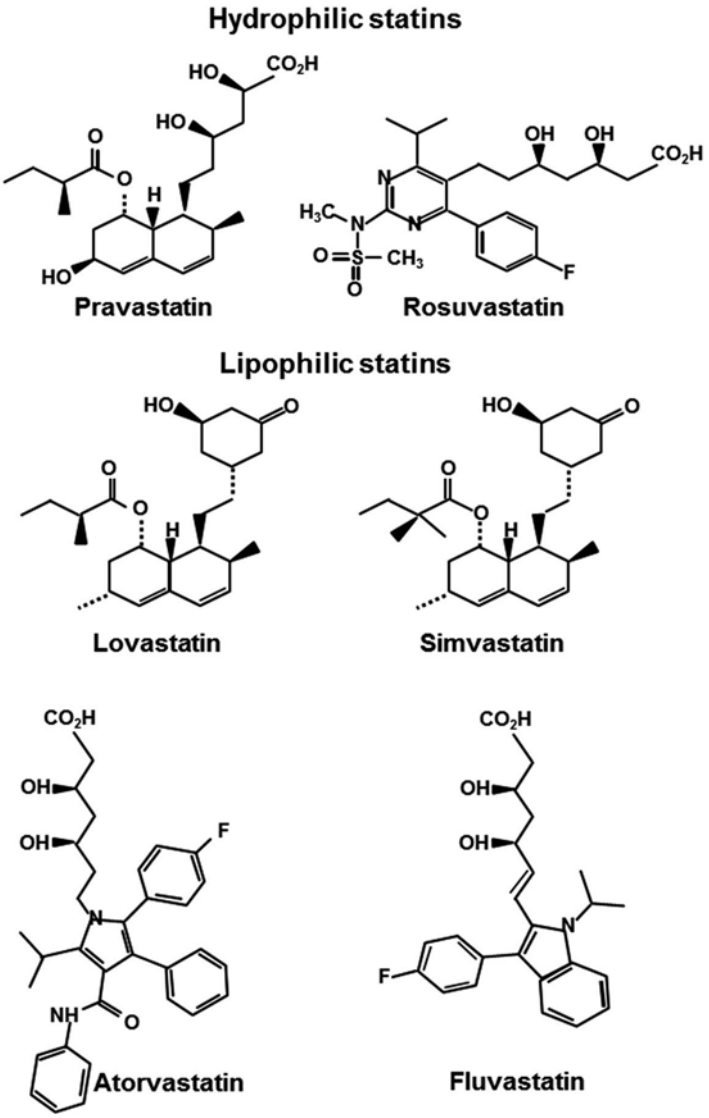

Fig. 8.3 Hydrophilic and hydrophobic statins

8.4.1 Mechanisms Suggested for the Anti-Breast Cancer Effects of Statins

- Inhibition of the proteasome, needed for proteolytic degradation of regulated proteins in the cell cycle, resulting in the accumulation of p21 and p27 and G1 arrest [97, 98].

- Modification of expression of genes related to cell proliferation (down-regulation of cyclin D1, PCNA, c-myc and up-regulation p21(Waf1), p19(INK4d), integrin beta8), and cell invasion (decrease in u-PA, MMP-9, u-PAR, PAI-1 and increase in anti-oncogenes Wnt-5a and H-cadherin) [99].
- Decline in the key p-MEK1/2 intermediate of the Ras-Raf-MEK-ERK cascade thought to drive cell proliferation; decline in activated NF-kappaB levels; decline in various MAP kinase proteins (p-ERK1/2, p-JNK, and p-p38) as well as a reduction in cyclin D1, associated with increased levels of p21[89].
- Modulation of the E2F1-pathway through the regulation of expression of prohibitin and retinoblastoma (Rb) genes, which subsequently lead to changes of E2F-downstream targets MCM7 and MSH2; Increased PTEN and decreased DJ-1 expression leading to a down-regulation of the active pAkt [100].
- Targeting the JNK signaling pathway: stimulation of phosphorylation of c-jun [91].
- Altering iron homeostasis, nitric oxide generation and antioxidant defense mechanisms [92].
- Metabolic consequences: suppression of glycolytic and Krebs cycle activity, and lipid biosynthesis [100].
- Inhibition of RhoA-dependent cell signaling [99] and reduced invasive capacity of human breast cancer cells into the endothelial cell monolayer, by inhibition of the membrane localization of RhoA and RhoC in the cells [101].
- Inhibition of angiogenesis by stimulating an anti-angiogenic gene (thrombospondin-2) [99].
- Inhibition of the effect of mutant P53 on breast cancer cells (up regulation of the mevalonate pathway by mutant p53, correlates with highly expressed sterol biosynthesis genes in human breast tumors) [102].
- Regulation of cyclin D1-CDK4-p21WAF1/CIP1 pathway in BRCA1 overexpressed cells [103].

8.4.2 Human Studies

Reduced risk of many types of cancers was noted among statin users in association studies [30, 104, 105]. A substantial lower breast cancer incidence was observed in 7528 Caucasian statin-using women, at a mean age of 77 years followed up for 6.8 years in the SOF—a prospective observational study conducted at community-based clinical centers in the United States [106]. Hydrophobic statin use was associated with reduction in breast cancer incidence within 156,351 postmenopausal women in the Women's Health Initiative (WHI), while hydrophilic statin use was not associated with reduced breast cancer incidence [107]. In a follow up study from the WHI, that used a method of censoring outcome of breast cancer after 6 years of follow up in order to resemble data from clinical trials, statins protective effect was not observed [108]. A borderline significant risk-reducing effect for long-term statin use (>5 years) was observed in a case-control study in Washington State comprising 2000 women interviewed in person [109].

In other studies, association between statin use and breast cancer incidence was not observed [110]. In a large case-control study within the Kaiser Permanente of Northern California, no association was observed between lipophilic statin use and overall breast cancer risk, nor was a reduction in risk of any of the intrinsic subtypes, luminal A, luminal B, HER2+/ER− or triple negative [111]. Incidence of breast cancer was not decreased in participants of the Cancer Prevention Study II Nutrition Cohort using cholesterol-lowering drugs (mostly statins) for 5 or more years [112], or in a retrospective cohort study of 92,788 women from Group Health Cooperative (GHC) in Washington State with a median follow-up of 3 years [113]. A higher proportion of statin users than non-users in the GHC study had a screening mammogram in the 3 years prior to end of follow-up.

In a retrospective analysis of a cohort of 1945 early stage breast cancer survivors, Kwan et al. examined the association between post-diagnosis statin initiators (mainly lovastatin) and risk of breast cancer recurrence, and found a non- significant trend towards reduced incidence of recurrence among statin users, an effect that "mimics" a preventive study in high-risk patients. Increasing duration of use was significantly associated with decreased risk of recurrence [114].

In another retrospective study that mimics a secondary prevention study, recurrence rate was evaluated in 18,769 stage I-III breast cancer patients. Simvastatin users, but not users of hydrophilic statins, experienced 10 fewer breast cancer recurrences per 1000 women after 10 years of follow up, compared with women who were not prescribed statins [115].

8.4.3 Conclusion

The in vitro/in vivo anti-cancerous activity of lipophilic statins is well established. However, clinical data pertaining to breast cancer prevention by statins is less consistent. Observational studies of risk of disease were many times negative while survival studies have mostly shown a positive association. Prospective clinical trials of statins for breast cancer prevention are lacking. Therefore, at this time a recommendation for use of statins for primary prevention of breast cancer cannot be made.

8.5 Vitamin D

Provitamin D is synthesized in the skin. Exposure of the skin to sunlight converts the provitamin to cholecaliciferol (vitamin D3). Vitamin D2 is a provitamin that presents in plants and fungi. Fortified dairy products, cereals, fish, and commercial vitamin preparations contain either D2 or D3, which practically have similar potencies in humans. Both D2 and D3 undergo first 25-hydroxylation in the liver, to form the major circulating vitamin. The biologically active forms are products of second hydroxylation 1,25(OH)2 D3 (calictriol), and 1,25(OH)2 D2 [4]. The second hydroxylation occurs in the kidney

as well as in target tissues, including the breast [116, 117]. The major action of calcitriol on effector systems is to stimulate transcellular absorption of calcium.

Vitamin D receptor (VDR) shuttles between the cytosplasm and the nucleus. Vitamin D exerts most of its cellular effects via its nuclear receptor, that heterodimerizes with the retinoid X receptor (RXR). The VDR-RXR complex binds vitamin D responsive elements (VDRE) in gene promoters and enhancers throughout the genome and regulates transcription of target genes [118]. In addition to regulating gene transcription via its specific intracellular receptor, vitamin D induces rapid, non-transcriptional responses involving activation of transmembrane signal transduction pathways, like growth factors and peptide hormones.

Vitamin D might have a role in cancer. In particular, in breast cancer, compared to normal breast tissue, up regulation of RNA for VDR, and the hydroxylases was demonstrated [119]. It is quite well established that vitamin D suppresses proliferation and induces differentiation of breast cancer cells in vitro [120, 121], participates in negative growth regulation of the mammary gland [122], and of breast cancer in vivo.

In a non-immunodeficient spontaneous breast cancer mouse model perfusion of 25(OH)D or 1,25(OH)2D delayed tumor appearance and significantly decreased lung metastasis. Both metabolites reduced Ki-67, cyclin D1, and ErbB2 levels in tumors. Perfusion with 25(OH)D caused a 50 % raise in tumor 1,25(OH)2D levels, indicating good tumor penetration and effective activation [123]. ER-negative as well as ER-positive breast tumors were suppressed in animal models [124]. Haploinsufficiency of VDR gene shortened the latency and increased the incidence of mammary tumor formation in a mouse model [125].

8.5.1 Mechanisms Suggested for the Anti-Breast Cancer Effects of Vitamin D

- Rapid inhibition of the mitogen-activated protein kinase (MAPK) cascade by inactivating Src tyrosine kinase [126].
- Regulating the ErbB2/AKT/ERK signaling pathways [127].
- Down-regulation of the Ether-àgo-go-1 potassium channel [128].
- Repression of CD44, a transmembrane glycoprotein, that has a role in tumor initiation and recurrence, by forming a complex with STAT3 and Janus kinase 2 (JAK2) [129, 130].
- Increased intracellular expression of IL1α [131].
- Induction of CYP24A1, CA2, DPP4, IL1RL1 expression [132].
- Regulation of genes including those involved in innate immunity (CD14), differentiation (Bmp6), extracellular matrix remodeling (Plau) and cell survival (Birc3). [133].
- Accumulation in G0-G1 [134], or in G1/S [135]; activating caspase-3 to undergo apoptosis [134]

- Induction of BRCA1 association with VDR, to enhance CDKN1A expression. CDKN1A encodes for the p21waf1 protein, a cell cycle regulator, critical for activation of the G1/S checkpoint. (Thus, BRCA1 heterozygote mutation carriers might exhibit an attenuated response to vitamin D growth inhibition) [136].
- Acting as a selective aromatase modulator: decreasing aromatase expression in the tumor and surrounding mammary adipose tissue, leading to a significant reduction in estrogen synthesis, while having no effect on some tissues (ovary and uterus) and a stimulatory effect on other tissues (bone marrow cells) [137].
- Maintaining the myoepithelial cell layer and the basement membrane of DCIS cells, while disappearance and breakdown of the myoepithelial cell layer and basement membrane in DCIS have been identified as major events in the development of breast cancer [138].
- Alleviation of the pro-metastatic effect of macrophages on breast cancer cells, and abrogation of the induction of epithelial-to-mesenchymal transition [139].

8.5.2 Human Studies

8.5.2.1 Human Studies Showing a Chemoprevention Effect of Vitamin D on Breast Cancer

- Within 136 women diagnosed with primary breast cancer those whose tumors contained immunocytochemically detectable VDR had a longer disease-free interval than those patients with negative tumors [140].
- In an analysis of data from the first National Health and Nutrition Examination Survey (NHANES) Epidemiologic Follow-up Study, variables that reflected vitamin D exposure were derived from an interview, dietary assessment, and dermatological examination. Sunlight exposure and dietary vitamin D consumption reduced the risk of breast cancer with RRs ranging from 0.35 to 0.75 [141]. There was no measurement of serum vitamin D levels in this analysis.
- In a case-control study nested within the Nurses' Health Study cohort, each incident case of breast cancer was matched with a control on the basis of age, menopausal status, use of postmenopausal hormones, month of blood collection, time of day of blood collection, and fasting status at the time of collection. In multivariable analyses high plasma level of 25-(OH)D were marginally significantly associated with a lower risk of breast cancer, especially in older women [142].
- In 1026 cases and 1075 controls of the Long Island Breast Cancer Study Project, a population-based case-control study, plasma 25-(OH)D concentration, measured 60 days (median) from diagnosis, was inversely associated with breast cancer risk, more pronounced among postmenopausal women [143].
- Decreased risk of breast cancer with increasing 25(OH) vitamin D3 serum concentrations (OR 0.73, 95 % CI 0.55–0.96; P trend = 0.02) was found in 636 cases compared to 1272 matched controls from the French E3N Cohort in a model that

also took into account serum calcium, PTH, and steroid hormone concentrations [144]. In subgroup analysis a more pronounced decreased breast cancer risk was observed in younger women than in older women.

- In a population-based case-control study with 289 premenopausal cases and 595 matched controls, a significant inverse association between breast cancer risk and plasma 25(OH)D concentrations was found. Compared with the lowest plasma 25(OH)D category (<30 nmol/L), the ORs (95 % CI) for the upper categories (30–45, 45–60, ≥60 nmol/L) were 0.68 (0.43–1.07), 0.59 (0.37–0.94) and 0.45 (0.29–0.70), respectively (p trend = 0.0006). The median difference between time of diagnosis and time of blood collection in cases was 189 days [145]. A similar association was reported for postmenopausal women from the same cohort [146].

Notwithstanding, association studies of vitamin D levels and cancer outcomes are prone to some inherent biases. Low serum levels of vitamin D are related to obesity, because vitamin D is sequestrated in fat tissue [147]. Obesity is associated with cancer. Lack of physical activity, is associated with higher cancer rate and with low levels of vitamin D. Individual health practices including diet and supplements are also associated with both vitamin D levels and cancer [148].

8.5.2.2 Human Studies not Showing a Chemopreventive Effect of Vitamin D on Breast Cancer

- The Women's Health Initiative (WHI) had an arm where postmenopausal women were randomized to daily supplementation of 1000 mg calcium and 400 IU vitamin D3 or placebo [149]. Personal use of calcium and vitamin D was allowed and was reported by 52 % of women in both groups which could influence the detected differences between groups in the study outcomes. Adherence to study medication was around 60 %. After a mean follow-up of 7 years there was no significant difference between groups in incidence of breast cancer. In post hoc analysis, 5 years after active intervention ended, calcium and vitamin D supplementation decreased in situ breast cancer incidence, but in a puzzling manner women with baseline vitamin D intakes > 600 IU/day, had an increased risk of invasive breast cancer [150].
- In a case-control study nested within the WHI Calcium and Vitamin D Clinical Trial the authors have shown that adjusting for health and lifestyle characteristics such as BMI and physical activity has brought the unadjusted significant association between vitamin D level and breast cancer to a non-significant value [151].
- No association was observed with a median follow up time of 11.3 years after Danish participants of three prospective cohorts that included information on education level, physical activity, smoking, alcohol intake, and BMI [152].
- A subset of female participants from the prospective Cancer Prevention Study-II (CPS-II) Nutrition Cohort who provided a blood sample was followed for 4–7 years. Serum 25(OH)D was measured in 516 incident cases and 516 controls, matched on age, race, and date of blood draw. There was no association between

25(OH)D and breast cancer (OR 1.09, 95 % CI 0.70–1.68) for the top versus bottom quintile after controlling for additional confounders [153].

- In a nested case–control study within the Malmo Diet and Cancer Study, with 764 incident breast cancer cases, and 764 controls a non-significant inverse association between breast cancer risk and 25(OH)D3 was noted [154].
- In a case-control study comprised of 1087 participants (231 cases and 856 matched controls) nested within the NSABP-P1 trial (in which women, 35 years of age or older at increased risk for breast cancer were randomized to tamoxifen or matched placebo) higher BMI was associated with a greater breast cancer risk. Serum levels of 25(OH)D were not found to be independent predictors of breast cancer risk [155].
- In a case-control study nested within two prospective cohorts, the New York University Women's Health Study and the Northern Sweden Mammary Screening Cohort blood samples were collected at enrollment and women were followed up for breast cancer ascertainment. In 678 cases and 1208 controls no association was observed between circulating levels of 25(OH)D and overall breast cancer risk, but an inverse association between 25(OH)D levels and breast cancer risk was observed among women who were younger than 45 years of age/premenopausal at enrollment [156].
- Among 1218 controls that were matched to 582 breast cancer cases from the Nurses' Health Study II (NHSII) cohort study, that were predominantly premenopausal, no association with plasma vitamin D level was observed. Only stratification by BMI yielded a significant interaction [157]. No association was observed between calculated free 25(OH)D or with vitamin D binding protein and risk of breast cancer, as well [158].
- Genome-wide association studies (GWAS) identified SNPs related to circulating 25(OH)D in or near four genes: GC, encoding vitamin D binding protein, the major transporter of circulating vitamin D compounds; CYP24A1, encoding the cytochrome p450 24-hydroxylase that initiates intracellular catabolism of 25(OH)D and 1,25-dihydroxyvitamin D; CYP2R1, encoding the 25-hydroxylase which converts vitamin D to 25(OH)D in the liver; and DHCR7, encoding the enzyme that converts 7-dehydrocholesterol to cholesterol rather than vitamin D3. These four SNPs explain 5.2 % of the variation in circulating 25(OH)D. In a large pooled study of 9456 cases and 10,816 controls from six cohorts no association was found between any of the four SNPs or their polygenic score and breast cancer risk [159].

The most important adverse effect of vitamin D supplementation is hypercalcemia. Most positive preclinical studies used high-dose, intermittent 1α,25(OH)2D3. In the WHI trial a regular clinical dose was used (400 IU/day, which equals to 10 μg). In a phase 1 dose-escalation trial of 1α,25(OH)2D3 administered orally once a week 2.8 μg/kg (body weight) was safely administered without any dose-limiting side effects. Doses of 0.48 μg/kg and above already produced mean peak calcitriol levels of 1625 pg/mL, approximately 25-fold greater than top normal levels and well within the therapeutic range suggested by in vitro experiments. Only 50 % of patients experienced self-limiting grade 1 hypercalcemia [160].

Much effort has been placed recently in developing non-toxic vitamin D analogs that would not cause hypercalcemia. The prohormone 25(OH)D3 might be less toxic [161]. Other analogues are EB-1089, KH-1060, MC-903, Gemini compounds, and 1α(OH)D5 but it is unclear whether analogues which cause less hypercalcemia are equipotent in terms of anticancer effects [118].

8.5.3 Conclusion

In conclusion, while vitamin D is a definite candidate for chemoprevention, the current conflicting evidence does not allow the recommendation of vitamin D supplementation for prevention of breast carcinoma. While biological mechanistic studies, in vivo animal models and several association studies show benefit, the few clinical studies results failed to show the same. It is possible that further understanding of gene-environment interactions in vitamin D metabolism will shed light on specific population sub-groups who might benefit from such an intervention.

8.6 A Concluding Remark

Preventing breast cancer in healthy women requires efficacious drugs with a good long-term safety and tolerability profile. Bisphosphonates, metformin, statins and vitamin D are good candidates, but for all four agents, clinical data showing an effect of breast cancer prevention are still not robust enough to set policies of use for prevention.

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Chapter 9
The Role of Diet in Breast Cancer Prevention

Niva Shapira

Abstract Breast cancer (BC), the leading cancer in women, shows increasing prevalence worldwide in parallel with western metabolic epidemics, i.e. obesity, metabolic syndrome, and diabetes. Though BC closely shares major risk factors with these diseases, the connection to its dietary prevention is not widely supported.

The present chapter aims to update the scope and evidence base underpinning the potential for nutritional contributions to BC prevention. It reviews difficulties in studying direct nutrition-BC correlations, critical periods in the life cycle, and their dietary implications for carcinogenic and pathometabolic trajectories. Evidence-based risk factors, including anthropometric measures—high birthweight, adult tallness, fatness/body mass index (BMI), and late weight gain, and reproductive events—early menarche, late childbearing without breastfeeding—are covered.

BC initiation involves diet-related pro-oxidative, inflammatory, and procarcinogenic processes, i.e. through lipid/fatty acid peroxidation, estrogen metabolism and DNA-adduct-depurination and mutation formation. The pathometabolic trajectory is affected by high estrogen, insulin, and growth factor cascades and resultant accelerated proliferation/progression. Gender-based nutrition explains women's specific risk, i.e. with high fatness, estrogen metabolism, n-6:n-3 polyunsaturated fatty acid (PUFA) ratio and n-6 conversion to pro-inflammatory/carcinogenic mediators. Recent large-scale studies have confirmed effectiveness of evidence-based recommendations for reducing BC risk, emphasizing low energy density and nutritious plant-based diets, physical activity, and body/abdominal fatness management.

Better understanding of dietary interrelationships with BC—as applied to food selection, combination, and preparation, and potential for recommended patterns, e.g. Mediterranean, DASH, plant-based, low energy density and glycemic load, with high nutrient/phytonutrient density—would increase public motivation and authoritative support for early/timely prevention, optimally merging with other dietary goals at various life stages, for lifelong BC prevention.

Keywords Breast cancer • Nutritional prevention • Omega-6 fatty acids • Antioxidants • Plant-based diet • DNA adducts • Gender-specific nutrition • Estrogen • Obesity • Metabolic syndrome

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Abbreviations

–	Negative
+	Positive
AICR	American Institute for Cancer Research
ALA	Alpha-linolenic acid
ARA	Arachidonic acid
ATP	Adenosine triphosphate
BC	Breast cancer
BCFA	Branched-chain fatty acid
BMI	Body mass index
BRCA	Breast tumor suppressor gene
cm	Centimeter
COX	Cyclo-oxygenase
CVD	Cardiovascular disease
DASH	Dietary Approaches to Stopping Hypertension
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DOHaD	Developmental Origins of Health and Disease
ED	Energy density
ϵdA	Ethenodeoxyadenosine
ϵdC	Ethenodeoxycytidine
EPA	Eicosapentaenoic acid
EPIC	European Prospective Investigation into Cancer and Nutrition
ER	Estrogen receptor
FA	Fatty acid
g	Gram
GDM	Gestational diabetes mellitus
GH	Growth hormone
GI	Glycemic index
GL	Glycemic load
GST	Glutathione-S-transferase
HC	Hydroxycholesterol
HDL	High-density lipoprotein
HER	Human epidermal growth factor receptor
HMGCR	3-Hydroxy-3-methylglutaryl-CoA reductase
HR	Hazard ratio
IGF	Insulin growth factor
IGFBP	Insulin growth factor binding protein
IGT	Impaired glucose tolerance
IL	Interleukin
kg	Kilogram
LCPUFA	Long-chain polyunsaturated fatty acid
LDL	Low-density lipoprotein

M(1)dG	A type of malondialdehyde adduct
mg	Milligram
mL	Milliliter
MetS	Metabolic syndrome
mmHG	Millimeters of mercury (blood pressure measurement)
MUFA	Monounsaturated fatty acid
n-	Omega-
ng	Nanogram
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NCEP	National Cholesterol Education Project
NQO1	NAD(P)H:quinone oxidoreductase 1
OR	Odds ratio
PCA	Principal component analysis
PR	Progesterone receptor
PUFA	Polyunsaturated fatty acid
RR	Relative risk
SCFA	Short-chain fatty acid
SERM	Selective endogenous (estrogen) receptor modulator
SFA	Saturated fatty acid
SHBG	Sex hormone binding globulin
SSB	Sugar-sweetened beverage
T2DM	Type 2 diabetes mellitus
TNF	Tumor necrosis factor
WC	Waist circumference
WCRF	World Cancer Research Fund
WHO	World Health Organization

9.1 Background

9.1.1 Breast Cancer Trends and Incidence

Breast cancer (BC) is the most common cancer among women worldwide, with prevalence increasing—particularly the postmenopausal type—in areas where the incidence had previously been low, such as Japan, China, and southern and eastern Europe [1], with half of all BC cases and 60 % of BC deaths occurring in developing countries [2]. This epidemiological pattern, which follows those of other western epidemics, and share similar risk factors—obesity, type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD)—strongly suggests it is part of the pathometabolic prevalence that is closely associated with western life style patterns, and thus may support a nutritional approach to BC prevention.

9.1.2 Genes and Environment

Among BC cases only 5–10 % were due to genetic defects, with 90–95 % attributable to environmental and lifestyle factors—tobacco, diet, infection, and obesity contributing approximately 25–30 %, 30–35 %, 15–20 % and 10–20 %, respectively—providing major opportunities for prevention [3]. Among the 5–10 % of genetically based BC cases, many were caused by inherited mutations in either the BRCA1 or BRCA2 genes [4, 5]. A marked increased penetrance of BRCA mutations over the years has been shown in a number of cohorts and is thought to reflect increased western reproductive and lifestyle risk factors, including overfatness, smoking and low physical activity [6, 7]; also associated with estrogen receptor (ER) positive (+) cancer rates increasing while ER negative (–) rates are decreasing across all ages and ethnic groups [8, 9]. Despite increasing ER+ indicated potential for medical prevention, ER– showed poor prognosis - both emphasizing their special need for development of a differential nutrition and lifestyle protective approach.

9.1.3 Difficulties in Research on the Diet and Breast Cancer Connection

The BC connection to nutrition and lifestyle is difficult to prove, as it is affected by variety of factors, changes, and critical periods throughout the life cycle. We can get clues about diet and BC relationships when we look at rates of the disease in different countries [10]. For example, Japanese women have a much lower rate of BC than American women, but when they immigrate to the US, their BC risk goes up. The same is observed upon importation of American food habits to Japan, as shown in Okinawa Japan, which traditionally had among the world's highest longevity and lowest BC rates, but is now facing increasing rates of BC due to dietary changes resulting from post-World War II American occupation. The local government recently initiated an effort to return to the original Okinawan diet in an attempt to curb the increase [11].

The present chapter aims at better understanding pathometabolic mechanisms as related to BC and their dietary implications, to increase the motivation for and effectiveness of nutritional protection against the disease.

9.2 Pathometabolic Factors in Breast Cancer

9.2.1 Evidence-Based Risk/Benefit Factors

The level of evidence supporting the correlation of a risk/protective factor to BC range from 'convincing' down to 'probable,' 'limited-suggestive,' and 'no conclusion' levels. For postmenopausal BC, the strongest evidence for protective/beneficial

contribution—'convincing'—was shown for postpartum lactation, and the second strongest—'probable'—for physical activity. Among the negative factors, 'convincing' evidence was shown for alcoholic drinks, body fatness, and adult attained body height, and 'probable' evidence was shown for abdominal fatness and adult weight gain. Interestingly, among all food factor evaluations, only total fat yielded 'limited—suggestive' evidence.

For premenopausal BC, factors that reduced the risk included postpartum lactation—'convincing,' body-fatness—'probable,' and physical activity—'limited-suggestive.' Factors that increased risk were alcoholic drinks—'convincing,' adult attained height, and greater birth weight—'probable.'

The above factors may not necessarily be the direct cause of the effect, but rather can be translational measures, i.e. attained adult height may reflect specific endocrine-metabolic conditions during puberty and adolescence—such as reduced insulin sensitivity, increase insulin and growth factors, body weight, fat, and estrogen [12, 13]—which are also known to affect the age of menarche and initiation of breast development. This corroborates similar associations for 'large birth weight' or 'large for gestational age,' known to express prenatal metabolic conditions that imprint and affect future health and disease incidence according to the Barker Hypothesis of the Developmental Origins of Health and Disease (DOHaD) [14, 15].

9.2.2 Critical Periods for Breast Cancer Risk and Prevention

The breast's glandular system remodels continuously via cell division throughout life, with developmental peaks *in utero*, at puberty, and during pregnancy. These early critical periods, prior to attaining BC protection by breast cell differentiation – through a full-term pregnancy-induced 'molecular signature' [16], are associated with endocrine/metabolic predisposition, during which environmental effects can induce modifications, leading to increased risk of future BC development, and may potentially represent the most important windows of opportunity for early and 'timely' protection and BC prevention.

Life events that protect against BC include late menarche, early pregnancy and childbearing, and early menopause—all of which have the effect of reducing the number of menstrual cycles and exposure to estrogen—while early menarche, late menopause, not bearing children, and late (over 30 years of age) first full-term pregnancy, were all found to increase BC risk [16–130].

Large birthweight, which reflects intrauterine nutritional conditions and potentially contributes to increased BC risk [1], is in accordance with the Barker Hypothesis regarding DOHaD [14, 15]. Other effects of *in utero* exposure, i.e. to high fat intake and to estrogen ([19] or to a twin-partner, particularly a male [20]), further support the effect of early exposure on future BC risk.

Later critical periods in the life cycle are also associated with increases in fat tissue mass and pathometabolic patterns. Menarche is enabled at ≈17 % body fat [21]; growth spurts and fat accumulation in adolescent girls are associated with puberty-

related impaired glucose tolerance (IGT) and insulin sensitivity [22, 23]; and overweight/obesity in pregnancy often predisposes women to IGT and gestational diabetes mellitus (GDM) [24]. All of these factors are greatly affected by diet and corresponding predisposition to BC. Overconsumption has been shown to lead to early puberty, telarche, and menarche, and to delayed menopause, while undernutrition beneficially delays puberty and advances the age of menopause [1], suggesting that puberty/adolescence could be a window of either risk—as with the western diet—or changed to be 'a window of opportunity'—if properly managed to balance endocrine-metabolic factors by diet and lifestyle [25–29]. Other similarly critical periods throughout the life cycle, i.e. pregnancy and menopause, combine endocrine-metabolic factors with major influences on BC risk, and may yield corresponding opportunities for potential prevention.

9.3 Carcinogenic Mechanisms in Breast Cancer

Estrogen's association with BC was suggested to be affected by both ER-mediated hormone stimulation of breast cell proliferation, with concomitant enhanced rate of mutations, and estradiol's genotoxic metabolites initiating DNA mutagenic processes [30] and generating oxygen free radicals and resulting mutations that accumulate over time to induce neoplastic transformation. Both mechanisms have been shown to operate in breast tissue, and to potentially be reduced by a variety of estrogen inhibitors [31]. High urine DNA adducts in at-risk or active BC cases, indicating a critical role for adduct formation in breast cancer initiation, potentially suggest use of antioxidants capable of blocking estrogen-DNA adduct formation and depurination, e.g. N-acetylcysteine and resveratrol, which have demonstrated inhibitory potential *in vitro* and *in vivo* [32]. Further, levels of M(1)dG malondialdehyde adducts in fine needle aspirations were much higher in BC cases than in controls, correlating with increasing tumor grade and pathological size [33]; and within breast cancer ductal epithelial cells, mean ethenodeoxyadenosine (ϵdA) and ethenodeoxycytidine (ϵdC) levels were much higher in benign breast disease and BC compared to healthy breast, and were 30–200 times higher than in lymphocytes [34]; both may contribute to increased depurination, mutations and genomic instability in breast cancer.

A biochemical link between estradiol catabolism, dietary n-6 polyunsaturated fatty acid (PUFA) intake, and lipid oxidation-induced DNA damage (Fig. 9.1), as supported by both *in vivo* and *in vitro* models [35], was shown by n-6 PUFA (linoleic acid [LA] 18:2) increasing the formation of miscoding etheno-DNA adducts [36] in the white blood cells of women, but not of men [36, 37]. This gender specificity could result from higher female conversion of dietary n-6 PUFA to their inflammatory eicosanoids [38] and estrogen catabolism, via redox-cycling of 4-OH-estradiol(2) (Fig. 9.2) and subsequent lipid peroxidation [35].

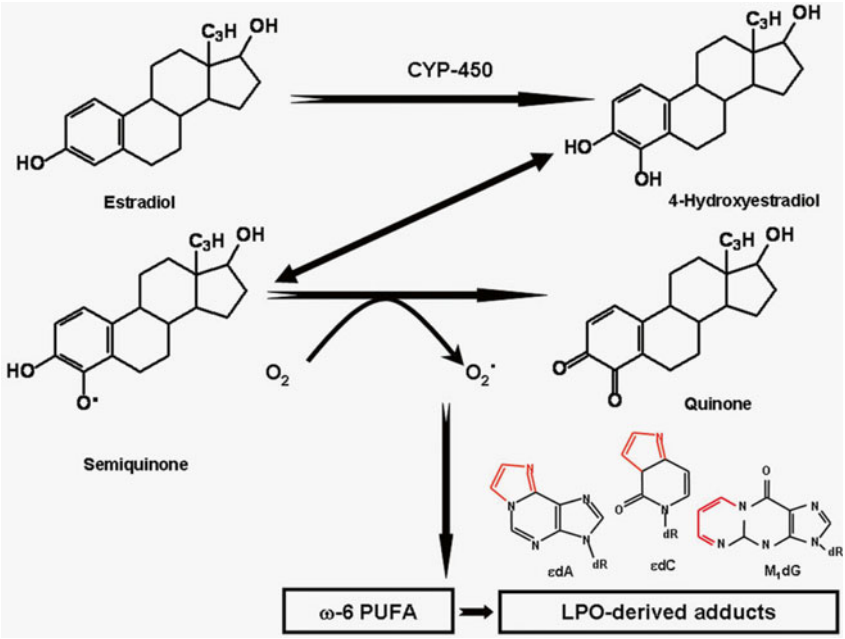

Fig. 9.1 Proposed scheme for metabolic redox-cycling of 4-hydroxyestradiol leading to reactive oxygen species and lipid peroxidation (LPO) of x-6 PUFA; the resulting LPO byproducts such as 4-hydroxy-2-nonenal and malondialdehyde generate miscoding etheno DNA adducts (edA and edC) and M1dG, respectively, that were analyzed in WBC of healthy female volunteers [35]

9.3.1 The "Israeli Gender Nutrition N-6 PUFA Paradox" Hypothesis

This hypothesis links Israeli women's higher cancer risk relative to men with female's greater n-6 PUFA conversion to proinflammatory/oxidative/carcinogenic eicosanoids as compared to males, with resultant worse international ranking for women and decreasing gender disease/life-expectancy gap when consuming very high dietary n-6 PUFA. High n-6 and n-6:n-9 fatty acid (FA) ratio, i.e. relative to traditional Arabic and Mediterranean diets/oils with much lower BC rates, corroborated by a later trend of increasing n-6 PUFA intake among Arab women, that has been associated with increasing BC risk and gradually closing of the gap in BC prevalence between Jewish and Arab women [39] (Fig. 9.3). The potential exacerbation of women's specific risk by a high n-6 PUFA diet applies to global abandonment of traditional diets/foods/oils, together with increasing global n-6 consumption and western cancer rates. This emphasizes the importance of considering gender in nutritional epidemiology and preventive strategies [40], particularly vs. BC, which is closely associated with combined estrogen and n-6 PUFA metabolism leading to

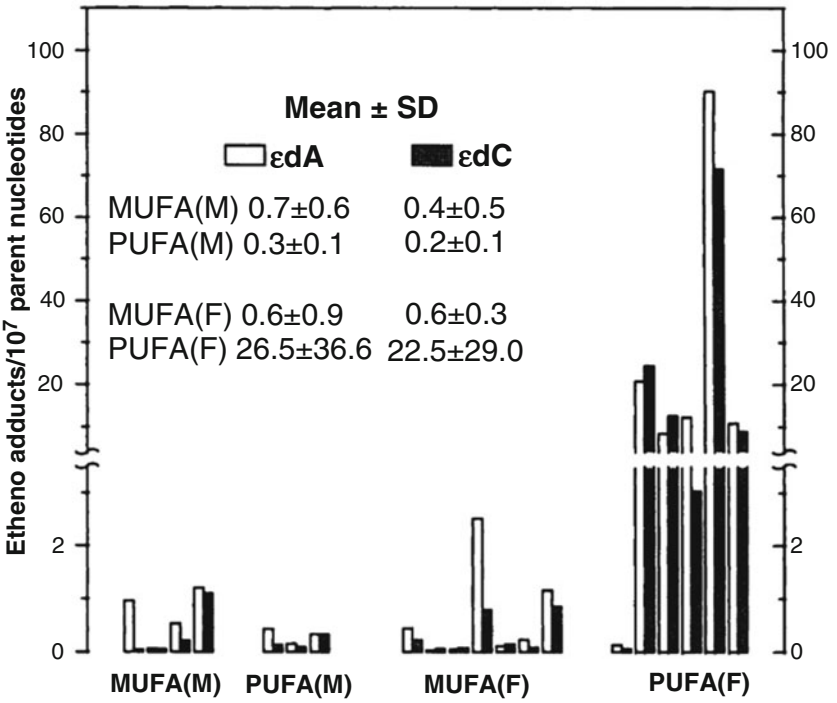

Fig. 9.2 *Column, individual and mean levels of etheno adducts/10^7 parent* nucleotides in human TWBCs. Individual volunteers on diets: MUFA, rapeseed oil; PUFA, sunflower oil, M, male; F, female [37]

Fig. 9.3 Incidence of invasive breast cancer in Israeli-Jewish and Israeli-Arab Women, 1990–2006 [39]

cancer initiation and a pathometabolic proliferation/progression trajectory, via both systemic and local breast tissue effects [38], while facing increased n-6 intake.

9.4 Pathometabolic Mechanisms

Various mechanisms by which diet and lifestyle may promote BC have been previously reviewed [41–43], suggesting that sedentary lifestyle, overweight, and a fat-rich diet are associated with insulin resistance and increased androgenic activity. Physical activity improves insulin sensitivity and decreases testosterone levels, and in the long term, insulin-like growth factor (IGF)-I levels. Insulin stimulates the synthesis of androgens in the ovary and the expression of growth hormone (GH) receptors, and inhibits the liver production of sex hormone-binding globulin (SHBG) and IGF binding protein (IGFBP)-1 and 2, thus increasing the bioavailability of both sex hormones and IGF-I. Alcohol intake increases the synthesis of androgens and estrogens [44]. Postmenopausal overweight is associated with increased peripheral conversion of androgens into estrogens, decreased SHBG, and increased insulin levels. Adipocyte production of adipokines may affect tumorigenesis through the upregulation of genes involved in proliferation, invasion, and metastasis [45]. Along with decline in steroid hormones with age, estrogen, testosterone and dehydroepiandrosterone sulfate (DHEAS) were higher and SHBG was lower in obese individuals, smokers (>15 cigarettes/day), and drinkers (≥20 g alcohol/day) [46]. Beyond postmenopausal estrogen sources from adipose tissue aromatization, 27-hydroxycholesterol (27HC) was recently shown to have an estrogen-like effect, acting as an endogenous selective ER modulator (SERM), which is potentially reduced by an anti-cholesterol synthesizing enzyme (3-hydroxy-3-methylglutaryl-CoA reductase [HMGCR]) compounds, including medications (i.e. statins) [47], further suggesting implications for dietary intervention.

9.5 Metabolic Syndrome (MetS)

Insulin resistance and hyperinsulinemia are a feature of the metabolic syndrome (MetS), defined by at least three of five metabolic factors, each of which has been found to be associated with BC incidence: high plasma levels of glucose (>110 mg/100 mL) [48], high levels of triglycerides (>150 mg/100 mL) [49], low levels of high-density lipoprotein (HDL) cholesterol (<50 mg/100 mL) [50], large waist circumference ([WC] >88 cm) [51], and hypertension (systolic blood pressure >130 mmHg or diastolic blood pressure >85 mmHg) [52]. A systematic review and meta-analysis (97,277 adult females) showed a modest positive association between MetS and BC risk (RR = 1.47) [53], confirmed for postmenopausal women (HR = 1.80) and for high blood glucose (HR = 1.89) in a large-scale case-cohort study, though a link was not observed for premenopausal women [54]. A connection to refined sugar intake was demonstrated by higher risk of developing MetS with

increased sugar-sweetened beverage (SSB) consumption [55, 56]. Cholesterol's connection was recently emphasized, through the observed agonist effect of its metabolite 27HC on estrogen receptors and postmenopausal BC pathogenesis [57]. Moreover, BC patients with MetS have shown worse prognoses, especially if associated with increased androgenic activity [58]; together suggesting that beyond individual factors association with BC, their combination may elevate the risk by activating molecular pathways through endocrine, metabolic, and immune functions, which together influence breast tumorigenesis [59].

9.6 Gender-Based Nutrition: Specificity for Women

Women's evolutionary advantage associated with body fat accumulation, which historically yielded health benefits for nurturing and longevity against scarcity, is now counteracted by greater and faster increasing risks of obesity and related diseases in the obesogenic environment. Women's differential body fat accumulation and distribution is exemplified during puberty/adolescence, with body fat percentage higher than men's even with equal BMI, lower abdominal and visceral fat accumulation vs. gluteal and subcutaneous accumulation, lower fat loss on weight-reduction diets, better response to high-protein vs. high-carbohydrate diets, higher risks with sedentariness vs. exercise benefits, and tendency toward delayed manifestation of central obesity, MetS, T2DM, CVD, and certain cancers until menopause, but accelerated thereafter, reflecting women's specific metabolic and chronological life cycle patterns [40].

Women's higher desaturase activity and enhanced eicosanoid synthesis, which in the western diet high in n-6 PUFA and n-6:n-3 ratio potentially yields greater concentrations of n-6 inflammatory and carcinogenic eicosanoids [38], may more readily induce cell proliferation shown to enhance DNA damage and mutations, which may in turn drive cancer initiation and recurrence [60].

Postmenopausal changes are associated with systemic declines in estrogen and progesterone that cause fat redistribution, with increased abdominal adiposity and homeostasis imbalances, including decreases in insulin sensitivity and leptin secretion and changes in glucose and lipid metabolism, resulting in reduced energy expenditure and increased weight gain and obesity. These factors may contribute to BC development through increased localized inflammation and estrogen production in breast tissue, and increased growth-factor secretion despite reduced ovarian estrogen [61].

The above suggests women's need for specific metabolic and chronological perspectives for prevention/intervention, especially for BC, which closely represents the essential female life cycle pattern of endocrine-metabolic related and diet-dependent risks [40].

9.7 Anthropometric Risk Factors

9.7.1 Body Fatness (BMI)

Overweight and obesity have yielded 'convincing' evidence of correlation to increasing risk of BC after menopause [41, 62], mostly associated with serum estrogens [63, 64] from aromatization of androgens to estrogens in the adipose tissue. Obese BC patients appear to have a higher risk of lymph node metastasis, larger tumors, and poorer prognosis [65, 66]. While body fatness (BMI) correlation with BC for all-ages showed inconsistent relationships, meta-analyses of cohort data showed an 8–13 % increased risk in post- and 15 % decreased risk in premenopausal women per increase of 5 kg/m^2 [1, 64, 67]. This paradoxical dichotomy could be related to the differences between premenopausal obesity, having less abdominal/central fat and more subcutaneous and gluteal/femoral fat—shown to be metabolically protective with regard to lipid and glucose metabolism, leptin, and cytokines—vs. the typical postmenopausal pattern of higher abdominal fat and related risks [68, 69].

9.7.2 Abdominal Fatness (WC)

Abdominal fatness, a 'probable' risk for BC, is most often represented by WC. All studies evaluating waist circumference and most of those evaluating waist-to-hip ratio have shown increased BC risk with increased abdominal fatness, and meta-analyses suggest 19 % increased risk per 0.1 increment in waist-to-hip ratio [1]. It directly affects levels of many circulating hormones, such as insulin, IGF-1, and estrogens, creating an environment that encourages carcinogenesis, discourages apoptosis [1], and stimulates inflammatory responses, which may contribute to the initiation and progression of several cancers [70].

9.7.3 Adult Weight Gain

Adult weight gain, a 'probable' cause of postmenopausal BC, has demonstrated correlation to increasing BC risk in nearly all related studies, with a meta-analysis of case control studies showing a 5 % increased risk per 5 kg added [1]. While total adult weight gain of ≥20 kg was shown to double BC risk [71], modest weight loss (5–10 %) either before or after menopause reduced the risk of postmenopausal BC by 25–40 % compared to women who continued to gain weight, and a 10 % weight reduction yielded 50 % lower BC risk after menopause in the Nurse's Health Study [72], the effect shown with and without a family history [67, 71, 73].

9.7.4 Large Birth Weight

Heavier birth weight showed 'probable' evidence for correlation to increased BC risk, with a meta-analysis of cohort data showing an 8 % increased risk per each 1 kg addition [1]. Birth weight represents prenatal conditions and endocrine-metabolic programming that could increase long-term patterns, including for insulin and IGF-1. The action of both estrogens and IGF-1 are important in fetal growth and mammary gland development, and play a central, synergistic role in the initiation and promotion of BC [74, 75], as shown by offspring being influenced by maternal dietary modifications [76].

9.7.5 Adult Tall Height

Greater adult attained height showed 'convincing' evidence for both pre- and postmenopausal BC. Meta-analyses of cohort studies showed an 11 % and a pooled analysis (7 cohort studies, n > 337,000 participants) showed a 7 % increased risk per 5 cm of added height, respectively [1, 67]. The causal factor is unlikely to be tallness itself, but rather factors that promote growth in childhood [1], including reduced insulin sensitivity, with compensatory increase in insulin secretion [12, 77], and increased lipolysis and fat-to-glucose oxidation ratio [13], all closely related to the insulin-GH axis and resultant increased height velocity and adult tallness, and predisposition to later pathometabolic risk of BC [25].

9.8 Lifestyle Factors

Lifestyle factors and their degree of assessed association with postmenopausal BC risk are summarized in Fig. 9.4.

9.8.1 Alcohol

There is ample, 'convincing' evidence of a dose-response, no-threshold relationship between alcohol consumption and BC, for both pre- and postmenopausal [1] and for both ER+ and ER− states [73], as well as differing statuses of progesterone receptors (PR) and human epidermal growth factor receptor (HER)2. The European Prospective Investigation into Cancer and Nutrition (EPIC), a Europe-wide cohort study of diet and cancer covering 3,670,439 person-years and 11,576 incident BC cases, confirmed a 4.2 % increase in BC risk per each 10 g/day increased alcohol

a **FOOD, NUTRITION, PHYSICAL ACTIVITY AND BREAST CANCER (PREMENOPAUSE) 2010**

	DECREASES RISK	INCREASES RISK
Convincing	Lactation	Alcoholic drinks
Probable	Body fatness	Adult attained height[1] Greater birth weight
Substantial effect on risk unlikely	None identified	

b **FOOD, NUTRITION, PHYSICAL ACTIVITY AND BREAST CANCER (POSTMENOPAUSE) 2010**

	DECREASES RISK	INCREASES RISK
Convincing	Lactation	Alcoholic drinks Body fatness Adult attained height[1]
Probable	Physical Activity[2]	Abdominal fatness Adult weight gain
Substantial effect on risk unlikely	None identified	

[1]Adult attained height is unlikely directly to modify the risk of cancer. It is a marker for genetic, environmental, hormonal, and also nutritional factors affecting growth during the period from preconception to completion of linear growth (6.2.13 – Second Expert Report)(9.4a, b).

[2]Physical activity of all types: occupational, household, transport and recreational. (9.4.b)

Fig. 9.4 (**a**, **b**) Lifestyle factors and degree of assessed association with premenopausal (**a**) and postmenopausal (**b**) breast cancer [182]

intake, across ER+/PR+, ER−/PR−, HER2− and ER−/PR−/HER2− tumors, with stronger effects in women who started drinking prior to first full-time pregnancy [78]. Other meta-analyses showed increases of 5–10 % risk per 5 drinks/week and 10 g ethanol/day [1].

The effects of alcohol may be mediated through the production of prostaglandins, lipid peroxidation, and free radical oxygen formation. Reactive metabolites of

alcohol, such as acetaldehyde, may be carcinogenic, interfere with estrogen metabolism and receptor function, downregulate the tumor suppressor gene BRCA1, increase estrogen and prolactin receptor activity, and induce DNA strand deletions, chromosomal aberrations, and DNA adduct formation, with drinking before the first term of pregnancy being particularly associated with cumulative risk [73]. Dietary fiber's potential modulation of the association between alcohol intake and risk of hormone-dependent cancers [79], with probiotic bacteria's potential contribution to restoration of bowel flora and for greater improvement in alcohol-induced liver injury [80], suggest potential for a protective dietary effect.

9.8.2 Breastfeeding Duration

Most studies showed decreased future BC risk with increasing duration of breastfeeding. Meta-analysis showed a 3 % decreased risk per 5 months of total breastfeeding [1], and pooled analysis showed a 4.3 % reduction per each 12 months of breastfeeding [81, 82]. The protective effect of lactation is known to be associated with increased differentiation of breast cells [83], lower exposure to endogenous sex hormone during lactation-amenorrhea, and with exfoliation of breast tissues during lactation and massive epithelial apoptosis at the end of lactation, which could together eliminate cells with potential DNA damage [84]. Postpartum breastfeeding also yield long term metabolic benefits, i.e. delay in onset of late T2DM to 12.3 years compared with 2.3 years in women who did not breastfeed (85), with the lowest T2DM risk observed in women who breastfed for >3 months, potentially also explaining the long-term metabolic protection against BC [85–87].

9.8.3 Physical Activity

A sedentary lifestyle was consistently associated with increased risk of BC, both before and after menopause [41], while women who regularly engage in physical activity could decrease their BC risk by 30–50 % or more [41, 45]. Nearly all cohort and case-controlled studies showed decreased risk with increased physical activity, and a meta-analysis of cohort data showed a 3 % decreased risk per 7 metabolic equivalent hours of recreational activity/week in postmenopausal women; though inconsistent correlation was shown for all-ages meta-analysis of case-control data and for premenopausal protection [1].

Potential anticancer effects of physical activity include reductions in endogenous sex hormone concentrations, insulin resistance, and chronic low-grade inflammation, which in turn affect oxidative stress, telomere length, global DNA hypomethylation, immune function, and increased gene expression of BRCA and other DNA repair

genes [88]. Physical activity, especially higher levels of occupational/household physical activity, were significantly associated with lower mammographic density and complete involution among postmenopausal women [89].

9.9 Dietary Factors

Increasing evidence shows that dietary factors can play an important role in both the development and prevention of BC [90]. Among the leading factors demonstrating contributory associations are consumption of 'well-done' red meat, total cholesterol and triglyceride levels, and glycemic load, while high intake of nutritionally dense, whole, fresh foods—such as fish and plant-based patterns, including whole grains, beans, fruits and vegetables, incorporating phytonutrients (i.e. fibers, polyphenols and isoflavones)—may have potential protective effects. Vitamin D supplements appear to be protective against BC development, as are other vitamins and oligo-elements [90], though their effects may vary according to endocrine-metabolic status. Some of the main dietary factors suggesting correlation to breast cancer are presented below.

9.9.1 Fat Intake

Despite the previous assumption that dietary fat affects BC similarly to other western diseases, there is only limited evidence overall suggesting effects on postmenopausal BC [1, 91]. While some case-controlled studies have suggested increased risk of BC with increased fat intake [92], this was not observed in most cohort studies [93, 94] or pooled analyses [95]. High fat intake can increase the bioavailability of sex hormones [96, 97], altering the gut microbiota composition–with increased intestinal permeability, leading to increased endotoxin levels in the intestinal lumen and in the plasma, and resulting inflammation thereby accelerating obesity [98], and potentially affecting BC risk. Among several types of fat, saturated fatty acid (SFA) and n-6 PUFA intakes are associated with increased risk of BC and n-3 PUFA with reduced risk, while the relationships between monounsaturated fatty acid (MUFA) intake and BC risk are conflicting.

9.9.2 Saturated Fat/Fatty Acids (SFA)

The positive association of SFA intake with BC risk has been suggested by several case-control and cohort studies, particularly in the etiology of hormone-sensitive rather than receptor-negative BC subtypes [99], and by a meta-analysis of 14 cohort studies [91], as well as in experimental animal models [100]. However, a pooled analysis of eight cohort studies has shown a weak elevation of risk (RR = 1.09) with

replacement of SFA intake by carbohydrate consumption in an isocaloric diet [95], while a rat model showed maternal consumption of high-SFA lard to be associated with decreased BC risk in offspring [76].

9.9.3 Monounsaturated Fatty Acids (MUFA)

Although MUFA would be expected to contribute to BC protection, due to its resistance to oxidation and known contribution to the success of the Mediterranean diet, the actual results of studies suggest that its effects depend on the source, i.e. whether it is from extra-virgin olive oil—shown to be protective—or from hydrogenated fat high in *trans*-fatty acids (as in margarine), the latter being linked to increased BC risk [101]. Though some large case-controlled studies [102] and a meta-analysis of 10 case-controlled studies [103] have shown roles for MUFA in the pathogenesis of BC, and a twofold increased risk in a large meta-analysis (10 case-controlled studies) [104], several cohort studies have shown an inverse association of MUFA intake and BC risk [105]. Beyond the MUFA component, epidemiological evidence has shown olive oil to have a protective effect [106], attributable to improvement of insulin resistance [107] and olive oil polyphenols, including hydroxytyrosol and oleuropein aglycone [108] (Fig. 9.5), have been shown to be highly effective against the viability of various human BC cells lines [109].

9.9.4 N-6 Polyunsaturated Fatty Acids (PUFA)

N-6 PUFA are precursors of proinflammatory and procarcinogenic eicosanoids. Epidemiological studies on populations consuming high amounts of n-6 PUFA—primarily LA as in the United States and Israel—have demonstrated an association with high prevalence of BC [38].

Increasing phospholipid ratio of n-6 long-chain PUFA (LCPUFA) arachidonic acid ([ARA] 20:4) to n-3 eicosapentaenoic acid ([EPA] 20:5) in plasma and adipose tissues was associated with a proinflammatory response and altered adiponectin secretion that could contribute to development of MetS [110]; and by competing with n-3 PUFA transformation to their eicosanoids, they may enhance cellular and DNA damage [111] and accelerate the oxidative and proinflammatory effects.

Though many case-controlled studies have reported positive relationships between n-6 PUFA and BC [111, 112], others have suggested inverse relationships [113]. Animal studies have confirmed a promoting effect of LA and ARA on mammary tumorigenesis, and inhibitory effect of marine-derived n-3 LCPUFA, including EPA and docosahexaenoic acid ([DHA] 22:6) [114, 115].

Aspirin, the cyclo-oxygenase (COX_2) enzyme inhibitor of n-6 LCPUFA conversion to its procarcinogenic/proinflammatory eicosanoids, was shown to be associated

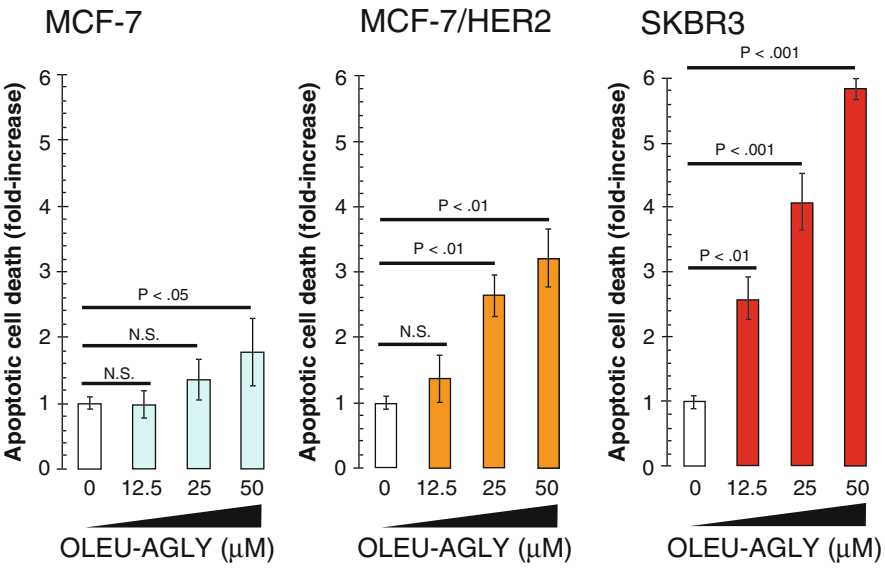

Fig. 9.5 Effects of oleuropein aglycone on breast cancer apoptotic cell death. Quantification of apoptosis-related cell death in MCF-7, MCF-7/(pBABE)HER2 and SKBR3 cells treated with increasing concentrations of oleuropein aglycone was determined by Cell Death ELISA as described in "Material and methods". The enrichment of histone-DNA fragments in oleuropein aglycone-treated cells was expressed as fold-increase in absorbance by comparing with control (vehicle-treated) cells using the following formula: $[A405 - A490]_{TREATED}/[A405\text{-}A490]_{UNTREATED}$. Data are the mean (columns) and 95% confidence intervals (bars) of three independent experiments performed in duplicate. One-factor ANOVA was used to analyze differences in the percentage of apoptosis between the various treatment groups and the control group. * P < .01; ** P < .001; N.S.: Not statistically significant (one-factor analysis of variance). All statistical tests were two-sided [108]

with improved BC survival and to reduce BC-specific mortality, all-cause mortality, and relapse/metastasis when taken before diagnosis [116], though taking after diagnosis was not significantly effective [117]. The effectiveness of aspirin against many western diseases, including BC, can be explained by inhibition of the deleterious impact of the very high n-6 PUFA western diet on their related eicosanoids-mediated risks.

9.9.5 N-3 Polyunsaturated Fatty Acids (PUFA)

Systematic review of cohort studies and meta-analyses showed an inverse association between BC and n-3 PUFA and n-3:n-6 ratio, especially when confirmed in biological samples, such as adipose tissue, erythrocyte membranes, serum and plasma [118, 119]. This is possibly due to n-3 PUFA-related alteration of estrogen

metabolism, reduction of oxidative stress, inflammation, and carcinogenic effects, and enhanced insulin sensitivity [120]. In a cohort of women with early stage BC, high EPA and DHA intakes (>73 mg/day) from foods (marine sources) for 7.3 years reduced BC events by approximately 25 % [122]. High intake of n-3 long-chain PUFA (LCPUFA, EPA and DHA) was recently shown to modulate BC risk biomarkers – both in pre-menopausal [123] and post-menopausal women [125], suggesting their potential contribution to primary BC prevention. A comprehensive review noted that n-3 PUFA may enhance the effectiveness of antiestrogenic therapy for prevention of BC [126].

In a rat model, n-3 PUFA's protective effect was shown in mammary glands of the offspring of pregnant rats consuming n-3 PUFA, which contained more lobules and were thus more differentiated [127]; and prepubertal exposure to a low-fat n-3 PUFA-rich diet had reduced later mammary tumorigenesis, though a very high-fat high n-3 PUFA diet increased subsequent BC risk [128].

9.9.6 Cholesterol

A high fat and cholesterol diet decreases latency and increases tumor growth and metastasis in animal models. Cholesterol was suggested to accelerate and enhance tumor formation, aggression, and angiogenesis, while its blood levels are reduced during tumor development [129].

A cholesterol metabolite—27HC—may increase the proliferation of ER+ BC cells [130]. The 27HC-producing enzyme, CYP27A1, which is expressed primarily in the liver and in macrophages, was significantly elevated within breast tumors, acting as an estrogen receptor agonist and stimulating the growth and metastasis of tumors in several models of BC [57]. 27HC demonstrated associations and upregulation of ER target genes and increases in cyclin D1 expression and in the number of cells entering S-phase; as it does not require aromatization, 27HC may have a potent impact on ER– mediated processes involved in BC cell growth [131]. Results of a 'window-of-opportunity trial' that included 50 patients with primary invasive BC have suggested that targeting the cholesterol synthesis enzyme HMGCR in BC cells *in vivo* by statins may also have an anti-proliferative effect in HMGCR-positive tumors [132].

9.10 Glycemic Factors

High dietary glycemic index (GI) or glycemic load (GL) may increase BC risk, i.e. through an effect on the insulin-IGF axis. High GL and carbohydrate intake were positively associated with increased risk of developing ER– and ER–/PR– BC among postmenopausal women, but no significant association was observed with ER+ BC [131]. A prospective cohort analysis (62,739 postmenopausal women, 1812 BC cases) showed no overall association between dietary carbohydrate or

fiber intakes and overall BC risk, but rather increased risk for BC with GI among overweight women, and with increased carbohydrate intake, GI, and GL in women with high WC (all RR ≈ 1.28–1.37); and a direct association for carbohydrate intake and GL in women with ER−BC [132]. A recent prospective study (879 BC cases, Italian EPIC) revealed increased BC risk to be associated with higher dietary GL but not GI and total carbohydrate intake [133]; while a meta-analysis of prospective cohort studies showed only modest association between BC risk and high GI or GL [134].

9.10.1 Refined Sugar Consumption

It has long been suspected that sugar sweetened beverages (SSB) play an etiologic role in the obesity epidemic. However, only recently have large epidemiological studies been able to quantify the relationship between SSB consumption and long-term weight gain, T2DM, and CVD risk. Experimental studies have suggested that SSBs contribute to weight gain by incomplete satiety compensation in subsequent meals following intake of liquid calories; high dietary glycemic load leading to inflammation, insulin resistance, and impaired beta-cell function; accumulation of visceral adiposity and increased hepatic *de novo* lipogenesis [135]. Frequent consumption of SSB was associated with MetS [55] and T2DM [56].

The impact of sucrose is primarily due to its fructose component, which—despite a moderate GI—has demonstrated an association with increased lipogenic and pro-inflammatory effects, particularly strongly expressed in the liver, due to transient ATP depletion by its rapid phosphorylation, potentially associated with non-alcoholic fatty liver disease (NAFLD) and resultant pathometabolic/endocrine outcomes [136]. Moreover, very recent study has shown that greater SSB consumption may reduce the age at menarche—a leading risk factor for BC—in every tertile of baseline BMI, while sugar-free ('diet') soda and fruit juice consumption were not associated with age at menarche [137].

9.10.2 Energy Density

Given the risks of body fatness, abdominal fat, and weight gain—all highly correlated with BC occurrence, recurrence and fatality—one of the leading recommendation has been to limit the intake of high energy density (ED) foods/meals. Limiting the intake of high ED foods is a well-known strategy to attain satiety for lower caloric intake [138], because portion size is more closely related to satiety than ED. Increasing the amounts of fruits and vegetables, starting the meal with soup or salad and/or with a low-calorie satiating pre-load, and/or a low-fat diet have all been shown to successfully contribute to reduce caloric intake and to support bodyweight management. However, recent research has shown that ED has remained quite

steady over the past decades, while increased body weight and caloric intake have been predominantly associated with increased eating occasions and the portion sizes of both solid and liquid items [139]. This further emphasizes the need for reducing ED, whose impact is currently being magnified by these developments. Thus, in addition to reducing ED, it would be advised to reduce eating occasions and especially intake of the types of fast foods and convenience snacks that are a popular but are often high ED and with relatively low satiating potential and nutritional value.

9.10.3 Fruit and Vegetables, Fiber and Phytonutrients

9.10.3.1 Vegetables

Total vegetable intake has been inversely related to BC risk (RR = 0.80), especially with high intake of allium vegetables and fresh legumes [140].

A meta-analysis has suggested that cruciferous vegetables may reduce the risk of breast cancer (RR = 0.77) [141]. These vegetables, especially broccoli, are high in sulforaphane, a potent inducer of detoxification enzymes such as NAD(P)H:quinone oxidoreductase 1 (NQO1) and glutathione-S-transferase (GST). NQO1 reduces the carcinogenic estrogen quinone metabolite, whereas GST detoxifies it, together preventing the estrogen-quinone mediated DNA damage and carcinogenesis [142].

A diet rich in raw vegetables and olive oil was shown to protect against BC, specifically against HER2+ cancers (RR = 0.25) that was much stronger than for HER2− cancers, suggesting foods exert differential protection against specific BC subtypes, which would require specificity of dietary approaches vs. the heterogeneity of BC genotypes [143]. Eating vegetables in a carbohydrate-rich meal was shown to reduce glycemic response, thus suggesting further explanation for their protection potential against pathometabolic IGF-I and insulin-related BC risk [144].

9.10.3.2 Fruit

Although no consistent association has been seen for total fruit intake with BC, high intake of citrus and Rosaceae fruits has demonstrated an inverse association [140]. Adding a polyphenol-enhanced fruit juice beverage to a high-fat meal was found to reduce the postprandial increase of circulating cholesterol, triglycerides, glucose, insulin, tumor necrosis factor (TNF)-α and interleukin (IL)-6, and related inflammation response [145]. Fruits have low ED and high nutritional density, contributing to the nutritional value of the diet, and can further partially curb the craving for sweets, which therefore could reduce intake of the latter and contribute to glycemic balance [146]. Moreover, fruit antioxidants—especially carotenoids and polyphenols—can reduce the oxidative stress following high GL, and thus are protective against their metabolic outcomes.

9.10.3.3 Fiber

Fiber intake has been linked to reduced risk of BC, with a 5 % risk reduction for every additional 10 g/day, potentially by reducing the reabsorption of estrogen and androgens in the bowel and hence their circulating levels. Soluble fiber appears to be the most protective, possibly through its high absorption capacity and beneficial effects on insulin sensitivity [147]. A prospective observational analysis recently showed that fiber intake significantly reduced the association between alcohol intake and risk of sex hormone-dependent cancer [79]. Through fermentation, grain fibers (i.e. from rye) reduce toxicity of free bile acids, produce short-chain fatty acids (SCFA) such as butyrate that yield anticancer effects—including against BC—and effectively absorb estrogen and reduce its re-absorption. Beans and whole grains, especially rye lignans, have antioxidative and anticarcinogenic potential, and contribute to satiety; and phytic acid, high in antioxidant activity is the likely contributor to rye's observed general anticancer properties [148] and BC protection in particular [149].

9.10.3.4 Carotenoids

A pooled analysis of eight cohort studies comprising most of the world's published prospective data on plasma or serum carotenoids and BC (3055 case and 3956 matched controls), suggested that women with higher circulating levels of α-carotene, β-carotene, lutein + zeaxanthin, lycopene, and total carotenoids may be at reduced risk for BC [150]. Though blood levels are more reliable and more strongly associated with risk indicator [151], in BC cells, carotenoids inhibited IGF-I-induced growth, estrogen-induced proliferation, and estrogenic activity [152]. Dietary α- and β-carotene are also inversely associated with the risk of BC, particularly among smokers and among women who do not use dietary supplements [153].

9.10.3.5 Polyphenols

Despite inconclusive epidemiological evidence for associations between polyphenols and BC, their leading antioxidative inflammatory and carcinogenic capacities suggest a potential protective contribution. Experimental trials have demonstrated synergistic interactions between polyphenols, i.e. green tea catechins, with conventional anti-BC agents such as tamoxifen or raloxifene in the treatment of ER+ and ER− BC, but no evidence of an interaction with aromatase inhibitors [154]. Peach polyphenols in a specific dose range equivalent to 2–3 whole peaches per day inhibited tumor growth and lung metastasis, through effects mediated by inhibition of metalloproteinase gene expression [155], and both peach and plum polyphenols have been shown to reduce BC cell viability and inhibit their proliferation [156]. Curcumin from turmeric root has also been shown to inhibit BC cells [157], an action further potentiated by green tea catechins to induce growth inhibition and apoptosis in resistant BC cells [158]. Additional compounds observed to exert

inhibitory actions against BC include bioactive punicalagins (from pomegranate) [159], carnosic acid (found naturally in rosemary), and silibinin (found in milk thistle seed) [157].

9.10.3.6 Soy Foods and Isoflavones

A recent meta-analysis summarized 14 studies showed that subjects who consume moderate amounts of soy throughout their life have a lower BC risk. Intake of 5 g of soy protein/day—yielding 10 mg of isoflavone, equivalent to 170 mL of soy milk—was associated with a 4 % risk reduction [160]. Soy isoflavone intake was shown to lower the risk of BC in both pre- and postmenopausal women in Asian countries; though for women in western countries, no association between soy and BC protection has been identified for either pre- /or menopausal status [161]. Moreover, several intervention studies using high doses of soy estrogens have shown changes in breast nipple fluid that would predict higher rates of breast cancer [162]. Interestingly, high dietary intake of soy isoflavones was associated with lower risk of recurrence among post-menopausal patients with BC ER/PR+ and those who were receiving endocrine therapy [163, 164].

The accumulated research may suggest that soy's protective potential functions in the context of the Asian diet—high in vegetables, seafoods (algae and animals), fermented foods, fresh and lightly cooked foods and soups, coconut oil, poultry and fish, and n-3 PUFA, while low in red meat, n-6 PUFA, and readymade foods and snacks. The effect of soy may be interrelated to other dietary components, not consistently protective in the western diet, or across life stages.

9.11 Meat, Fish, and Dairy Foods

9.11.1 Meat

Though vegetarian or vegan diets have not been shown to specifically reduce BC risk, women with a higher consumption of meat are at increased risk, with each additional 100 g/day of red meat increases risk by 4 %, and each additional 30 g/day of processed meat increases the risk by 3 % [165]. In the Shanghai BC study, high intake of total vegetables, certain fruits, milk, and eggs reduced the risk of BC, whereas high consumption of animal-source foods appeared to increase the risk, with no differences between ER/PR statuses [140]. Where a 'meat and potato' diet has been shown to increase BC risk, a 'whole food plant-based' diet may reduce the risk (164). Meat preparation has the potential to exert effects, with fried meat linked to increased risk of ER+/PR− BC in a large study (2952 cases, 17.4 years of follow-up), without an overall effect of red meat intake per-se [167].

A follow-up prospective study (44,231 women aged 33–52 years) showed that during adolescence, higher consumption of red meat was associated with increased

premenopausal BC risk but not postmenopausal; and substituting other dietary protein sources for red meat—including poultry, fish, legumes and nuts (one serving/day)–was associated with decreased overall (RR = 0.85) and premenopausal (RR = 0.77) BC risk [168].

9.11.2 Fish

Two portions of oily fish per week, providing 3.5 g of n-3 LCPUFA, are associated with the potential for a 25 % reduction in BC risk; each additional 0.7 g of marine n-3 LCPUFA per week reduces risk by 5 %. However, the vegetarian n-3 PUFA alpha-linolenic acid ([ALA] 18-3) does not appear to reduce risk [169]. Fish protein was recently suggested to reduce BC risk—similarly to poultry, legumes, and nuts—when substituting for red meat [168]. Moreover, fish is the main animal protein in the Mediterranean diet, a pattern associated with low western disease predisposition, including reduced BC risk.

9.11.3 Dairy

Though dairy foods contain the potentially cancer-promoting hormones estrogen and IGF-I, and despite observations that dairy protein can increase IGF-I, these concerns are not supported by epidemiological evidence. This may be due to the possibility that intake of the hormones in minute daily amounts—as compared to endogenous secretion in women—and with counteracting protective compounds—including SCFA and branched-chain fatty acids (BCFA), conjugated fatty acids, cysteine-rich whey proteins, calcium, and vitamin D—could explain the results from more than 40 case-control studies and 12 cohort studies that do not support an association of dairy products with BC risk [170]. Additionally, a recent meta-analyses incorporating 1,063,471 participants and 24,187 cases showed a 16 % lower rate of BC among high (>3 servings/day) vs. low (1 serving/day) dairy consumers [171]. The potential for milk fermented by probiotic bacteria like *Lactobacillus casei* to stimulate the immune response against BC, for inhibiting or delaying its growth, was recently suggested in an animal model [172] and in agreement with increasing understanding of the effects of gut microbiota on obesity and metabolic diseases.

9.12 Vitamin Supplements

A comprehensive, systematic bibliographic search of the medical literature suggests that the role of vitamin supplementation in prevention of BC is still unclear, despite biologic mechanisms supporting their anticancer effects [173], with the possible exception of vitamin D, in light of its widespread deficiency.

Epidemiological studies have suggested an inverse association between vitamin D status and risk of BC [174, 175], and several etiological studies have identified a potential mechanism of action for vitamin D in cancer prevention, including antiproliferation [176], prodifferentiation [177], and cell cycle stabilization [178]; however, the available evidence had not been enough to conclude that a causal effect exists [179]. A recent meta-analysis of prospective studies correlating plasma 25(OH)D to BC risk and stratified by menopausal status showed a stepwise inverse association in postmenopausal women between 27 and 35 ng/mL (sufficiency level is ≥20 ng/mL), with no relationship observed for premenopausal women [180].

9.13 Recommended Changes for Breast Cancer Prevention

9.13.1 Lifestyle Factors

A study on the potential for BC prevention has highlighted the critical changes in lifestyle that need to be implemented [181]. Based on the available evidence [1, 182], the World Cancer Research Fund (WCRF) and American Institute for Cancer Research (AICR) issued eight general and two special recommendations on diet, physical activity, and weight management, including the following: (1) maintain adequate body weight; (2) be physically active; (3) limit the intake of high ED foods; (4) eat mostly plant foods; (5) limit the intake of animal foods; (6) limit alcohol intake; (7) limit salt and salt-preserved food intake; and (8) meet nutritional needs through diet; plus S1) breastfeed infants exclusively up to 6 months; and S2) after cancer treatment—follow the recommendations for BC prevention. Evaluation of the specific associations between each of these recommendations and BC risk [183–186] across tumor subtypes and considering hormonal receptors and the HER2 status [187], have demonstrated encouraging results, showing that adherence to only three vs. six or more recommendations increased risk by OR = 2.98 in the 'all-age group', OR = 2.66 in the 'premenopausal group', OR = 3.60 in the 'postmenopausal group', and OR = 4.23 in the HER+ group.

Non-adherence to the 'limit intake of high density foods' recommendation, strongly increased BC risk, with respective menopausal status-specific OR = 1.86, 2.24 and 1.52; non-adherence to 'eat mostly plant foods' OR = 1.65, 1.22 and 2.35; not-'limited alcohol drinking' OR = 1.35, 1.39 and 1.32; and not 'maintaining adequate body weight' OR = 1.24, 1.10, and 1.44, respectively. Together, these findings suggest that for postmenopausal women, the three leading recommendations—'eat mostly plant foods,' 'limit intake of high ED foods,' and 'maintain adequate body weight'—are highly interactive factors. Both low intake of high ED and high intake of plant-based foods, which are high in fiber and water and thus generally low-ED, are expected to be more satiating and contribute to body weight management; and not 'limiting intake of animal foods' yielded OR = 0.91 for postmenopausal women, suggesting a minimal disadvantage, though red meat was previously suggested to

increase the risk. In premenopausal women, not 'limiting high-ED food intake' increased the risk of BC (OR = 2.24), possibly reflecting the high ED foods' deleterious qualities—being mostly high-sugar, high-fat and high in refined flours—which more badly affected this group, as they can increase blood insulin, triglycerides, and cholesterol, and may potentially reduce essential dietary micronutrients. High sugar intake, particularly in SSB, has demonstrated a direct association with obesity, MetS, and diabetes [55, 56] as well as with early menarche [137], suggesting its deleterious potential for increasing BC risk. Alcohol intake was associated with a steady level of risk throughout the pre- and postmenopausal years (OR = 1.32–1.39), though much less than the ED effect. Several large cohort studies have also reported lower rates of BC among women who adhere to the WCRF/AICR guidelines. Five studies of postmenopausal women reported 16–60 % risk reductions, mainly linked to reduced body fatness and alcohol intake rather than specific differences in dietary patterns [184, 186, 188, 189]; and 31 % lower rates of BC in women who adhered to 'increased wholegrain products' and 'reduced meat and alcohol', rather than other lifestyle factors [183], independent of family history of BC [186, 190], and for both ER+ and ER− BCs [184, 189]. Similar increases in penetration of BRCA2 (1920–2000) and BC incidence in the general population suggests that both types share common risk factors [191], as well as potential benefits from BC-targeted nutritional prevention.

9.13.2 Healthy Dietary Patterns

Beyond the general recommendations for a healthy diet, personal adaptations according to anthropometric and biochemical measures can be harnessed to attain an anticarcinogenic diet—incorporating small meals, whole foods, high nutritional and phytonutritional densities, protectively prepared, with low ED and GL. Some balanced ethnic patterns where shown to be more easily translated to behaviors and successfully adapted and contributory to dietary prevention of western diseases—including potentially against BC—than the recommendations stemming from dietary analyses, which do not effectively translate to protective eating.

9.13.3 Whole Food Plant-Based Diet

A whole food plant-based diet is high in micronutrients, including vitamins, minerals, fiber and phytonutrients including antioxidants from vegetables, fruits and whole grains and beans [192] required for enabling eumetabolic patterns. Foods high in fiber and water like vegetables, fruits and cooked grains and beans are low-ED, potentially supporting body weight management, which are essential for BC prevention.

An inverse correlation between consumption of fruits and vegetables and the incidence of BC is also partially attributed to their bioactive compounds, with three

principal groups of phytonutrients—carotenoids, polyphenols, and isothiocyanates—having documented cancer-preventive activity. Carotenoids have been shown to inhibit IGF-I-induced growth, estrogen-induced proliferation, and estrogenic activity of BC cells [152]. A similar inhibitory action was exerted by polyphenols such as carnosic acid, curcumin, and silibinin, and the isothiocyanate sulforaphane [157]. Strong relationships between a phytochemical-rich diet and a reversal of epigenetic alterations and/or modulated signaling pathways of carcinogenesis (initiation, promotion, and progression) suggest a potential approach for BC prevention [193].

Plant-based diets may reduce BC risk especially ER/PR− [166], though the 'salad and wine' version increased the risk for ER/PR+ [194]. A 'high fruits and vegetables' diet, such as would be expected in Dietary Approaches to Stop Hypertension (DASH) score, and a diet high in plant protein and fat and moderate in carbohydrate—both were associated with a lower risk of ER− BC [195].

A diet rich in raw vegetables and olive oil has demonstrated protective potential against BC. In the ORDET study, only the salad vegetable pattern was associated with significantly lower BC incidence (RR = 0.66), with a linear trend [196]. Higher consumption of vegetables, fish, and olive oil, were independently associated with decreased risk; and the principal component analysis (PCA), showed combined vegetables, fruit, fish and legumes, significantly reduced adjusted risk of BC (OR = 0.67) [197].

9.13.4 Mediterranean Diet

The combination and range of foods included in the Mediterranean diet pattern have been independently associated with decreased BC risk [197]. They provide high amounts of antioxidants from phytochemicals such as flavonoids and carotenoids and antioxidant vitamins, additional phytonutrients such as phytoestrogens and fiber, adequate folate, and a favorable FA profile [65, 198].

The DIANA interventional trials demonstrated that Mediterranean and macrobiotic dietary principles can reduce body weight, insulin levels, and MetS factors, as well as the bioavailability of sex hormones and growth factors [199–201]. As a consequence of the highly satiating diet, women lost weight and reversed MetS, suggesting that there is room for proposing adjuvant dietary changes for both prevention and treatment of BC, especially in the context of the MetS epidemic, with increasing prevalence in western populations [45, 202]. A strong inverse association between measured adherence to the Mediterranean diet pattern, and trunk-to-leg ratio, the latter being lower specifically with increased legume intake and higher with increased red and processed meat, showing the Mediterranean pattern to be more contributory to both lower obesity and healthier fat distribution [203], and could thus help women reduce their BC risk [204].

Adherence to a Mediterranean diet-type pattern has not been specifically shown to protect against BC, even when alcohol intake is removed from the diet score [205]. Even after a BC diagnosis, adherence to a healthy diet was not linked to

reduced mortality from BC itself, but rather from other causes [204, 206, 207]. Therefore energy balance and reduced adiposity seem to be more important for preventing BC than the specific composition and qualities of the diet.

9.13.5 Okinawan Diet

The traditional diet of Okinawa consists of foods low in calories but rich in nutritional value [11]. It was also among the lowest in fat intake, particularly in terms of saturated fat; with carbohydrate sources being calorie-poor antioxidant-rich, orange-yellow root vegetables, such as sweet potatoes, and green leafy vegetables. Many of the traditional foods, herbs, or spices consumed on a regular basis could be labeled 'functional foods,' and indeed, are currently being explored for their potential health-enhancing properties [208]. It is very low-ED diet, high in fiber, micronutrients and phytonutrients, marine fish, whole grains, beans, fruits and vegetables, n-3 PUFA, fermented products, and fresh and lightly cooked foods, with very limited red meat and n-6 PUFA. Together, it has yielded the longest life expectancy of cultures studied, and given the increase in local BC mortality associated with non-adherence [11], as well as structural similarity to the Mediterranean diet [197, 208], may be highly relevant for BC prevention in other populations as well.

9.14 Summary and Conclusions

The present chapter presents the multivariate nature of diet association with BC. The most updated support of the potential for BC prevention comes from recent studies showing encouraging results of decreased risk across genetic types and menopausal statuses through adherence to 6–8 basic recommendations, particularly as compared to non-adherence. The dietary recommendations include low-ED, low-GL, and nutritious plant-based foods, with minimal intake of animal foods and alcohol; and other lifestyle recommendations include physical activity, body fatness management, including total body and abdominal fatness and adult weight-gain, and extended breast feeding duration. Research results strongly support the effectiveness of these recommendations for reducing BC risk.

The chapter further presents carcinogenic mechanisms as related to dietary factors involved—including pro-oxidants, PUFA, and estrogen metabolism—as a source of DNA-adduct and mutation production; and the endocrinological-metabolic trajectory of the high-calorie, high-fat, high-PUFA diet and the glucose-insulin-growth factor cascade as promoters of BC risks.

Given that the critical periods between menarche and the first full pregnancy/lactation period are where undifferentiated breast tissue is exposed to carcinogenic processes, early menarche represents early fat accumulation, fast growth rate, and puberty-related risks of impaired glycemia, and insulinemia, with high growth factor

and free estrogen levels. Though these and other perinatal pregnancy-related risks may be tempered by the potential protection of breastfeeding, such premenopausal risks are cumulative and may later exacerbated by postmenopausal factors to further increase BC risk.

Nutritional strategies—including food selection and meal planning for prevention of acute effects, i.e. as associated with overconsumption and oxidative stress—involves quantitative management of food intake for improved anthropometric measures, and further present the potential of dietary patterns, i.e. low ED and GL, plant-based, Mediterranean, DASH, and Okinawan patterns, which were found to be more descriptive regarding the eating patterns and more easily applied than the recommendations based on dietary analyses and composition.

Taken together, the existing science supports the potential for lifetime BC prevention, starting from early critical periods—*in utero*, puberty/adolescence, perinatal/pregnancy-lactation, and menopausal years—with nutritional prevention aiming both for primary and secondary cancer prevention, as well as for modifying the metabolic trajectory against the disease occurrence and recurrence, and improving survival and quality of life, throughout the life cycle.

Increasing incidence of BC, beyond 1 out of every 8 women, necessitates the support of the population by health authorities for life-long BC prevention.

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Chapter 10
Vaccination Against Breast Cancer and its Role in Prevention

Brian J. Czerniecki, Nadia Nocera, Lea Lowenfeld, Lori Showalter, and Gary Koski

Abstract The immune response against cancers, including breast cancer, are shown to play a critical role in survival. Vaccines have long been hailed as the most effective medical intervention to prevent a disease. While cancer vaccines have mostly been used therapeutically with little success in established breast cancer, their role in early breast cancer appears more promising, and primary prevention of breast cancer by vaccination is now being contemplated. The selection of vaccine targets is a critical issue, since unlike cancers with established viral etiology (e.g. cervical cancer), there is no single cause of breast cancer. Instead, there are multiple subsets of breast cancers including: Luminal A, Luminal B, HER-2, and subsets of basal-like cancer. Each of these types can be antigenically distinct, and present immune targets that may be phenotype-specific or to some degree overlapping between subsets. Three general categories of such targets are being developed as breast cancer vaccines. These include oncodrivers, breast tissue specific antigens, and cancer specific antigens. It is likely that combinations of these vaccine approaches may be best for treatment and prevention. Carriers of high-risk breast cancer mutations represent a potential target patient population for prevention. However, approximately 85 % of breast cancers occur in patients with no identified risk. Recent evidence suggests that a loss of natural immune responses against oncodrivers may identify patients at risk for early breast cancer. Devising tests to identify subjects at risk for breast cancer are needed since these will allow us to focus prevention efforts, including vaccination, on those individuals where such resources are most needed. Preventive breast cancer vaccines may be achievable with our improved understanding of breast cancer biology, and the immune response in breast cancer.

Keywords Vaccines • Breast cancer • Primary prevention • Oncodrivers • Tumor Immunity • Breast cancer stem cells • Dendritic cells

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10.1 Background: What's at Stake?

Due to the significant public health burden, breast cancer is a particularly appealing target for preventative therapy. Breast cancer is the second most common cancer and the second most common cause of cancer death among American women. The incidence and mortality rates associated with breast cancer remain stubbornly stable and high, with more than 230,000 new cases diagnosed annually and more than 40,000 breast cancer related deaths each year [1]. Worldwide about 500,000 deaths from breast cancer occur each year [2]. Furthermore, the success of standard treatment, though prolonging survival, often results in significant disfigurement. Finally, the annual cost of breast cancer treatment in the United States is estimated at $16.5 billion [3], making it one of the most expensive malignancies.

Given the complex interaction between the immune system and cancer, immunotherapy in general and vaccine prevention in particular, is an appealing option for dealing with cancer. Vaccination against specific pathogens that are known to cause cancer (e.g. EBV and lymphoma [4] or gastric cancer [5], HPV and cervical, anogenital, and oropharyngeal cancer [6], HBV/HCV and hepatocellular carcinoma [7]) prevent infection, and therefore subsequent tumorigenesis. However, only an estimated 12 % of human cancers are at this time attributable to viral infections [8] and therefore susceptible to this strategy of prevention.

The majority of cancers, including breast cancer, are not directly caused by a single pathogen, but, nonetheless, vaccines developed against over-expressed or mutated cancer associated proteins can be used to target these malignancies. The first type of anti-cancer vaccine, like their anti-microbial counterpart, is **preventative** in nature. These can be further sub-divided into two categories. Vaccines aimed at **primary prevention** are administered to patients prior to the development of disease. Ideally, these will block the development of malignancy, and the patient will never develop cancer. Vaccines aimed at **secondary prevention** are administered to patients who have a history of cancer that has been eliminated or reduced to undetectable levels through conventional therapy. These vaccines protect against later recurrence of disease. **Therapeutic vaccines**, on the other hand, are administered to patients who possess measurable tumor burdens. The aim of this approach is to generate sufficient anti-tumor immunity to favorably alter the course of existing disease, either alone or in conjunction with conventional therapy.

10.2 Breast Cancer and Immune Response

The breast, by virtue of its communication with the outside world through the nipple, is by necessity endowed with complex immune populations. Breast lobular units contain dendritic cells, CD4+ and CD8+ T cells, B cells, and NK cells [9] (Fig. 10.1). These immune cells located in breast tissue defend against microbes, but also play a role during breast involution following lactation [10] and may play a

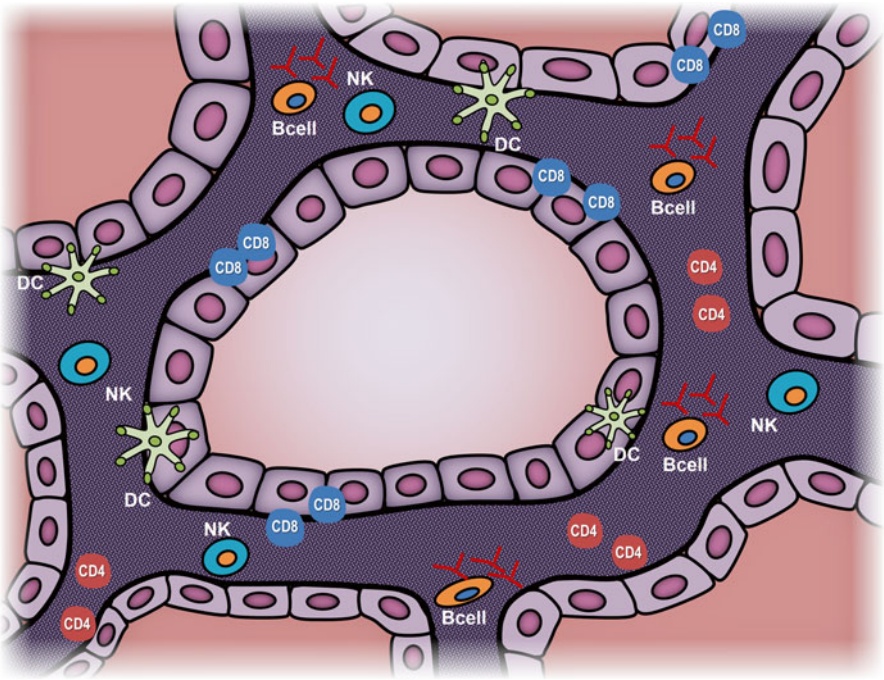

Fig. 10.1 Protective immune players in the normal breast lobule: B cells, Natural Killer cells (NK), Dendritic cells (DC), CD4+ (CD4) and CD8+ (CD8) T-cells. Myeloid and lymphoid cells are localized to the lobules with CD8s and DCs intimately integrated in the breast epithelium

role in tumor immunosurveillance [11]. While locally regulated inflammation may control tumor proliferation, chronic inflammation has been associated with cancer development, including breast cancer [11–13]. In addition, immune suppression may increase the risk of breast cancer development, most notably in transplant patients [14] or those on immunosuppressant medications. Clearly, the immune response can play a complex role in both promoting and preventing breast cancer development. It may suppress tumor growth by destroying cancer cells, but may also select for cancer cells that are more adept to survive in an immunocompetent host [15]. This immune selection favors the development of less immunogenic tumors, allowing these tumors to escape immune surveillance—otherwise known as cancer "immunoediting" [12]. As tumor cells evade the immune system, a more aggressive phenotype is selected for and the surviving tumor cells that do not express recognized antigens will continue to evade the immune system. The complexity of the immune response to breast cancer is such that any attempts at prevention will need to be cautiously undertaken to induce only a gamma interferon (IFN) producing anti-tumor immune response. A preventative vaccine should avoid chronic inflammation and type II immune responses, which may be tumor-promoting [16]. Shifting the inflammatory response in the tumor environment can change the environment from tumor promoting to tumor eradicating [12,17] with Th1 and type

I macrophages. For example, increasing the presence and response of cytotoxic T cells (CTLs) and decreasing the presence of type II macrophages would promote a tumor eradicating environment in breast tissue.

10.3 The Problem of Developing a Preventive Breast Cancer Vaccine: Selection of target Antigens

The hallmark of the adaptive immune responses is specificity—immunity directed against individual proteins, or antigens. This allows the immune system to distinguish, based on the differential expression of proteins, between entities that should be attacked and eliminated, versus normal, healthy cells of the body, which are to be spared. In the case of vaccines against infectious agents, this is a comparatively easy task, since the evolutionary divergence between humans and microbial pathogens is so great that many of their proteins do not share significant sequence homology. These differences are easily perceived by the immune system, and vaccines directed against microbial pathogens usually elicit strong immune responses against the microbe with high specificities that do not cross-react with proteins on normal host cells. In the case of breast cancer, however, there is usually a relatively small subset of proteins that distinguish a malignant cell from its normal, healthy counterpart.

Breast cancer is a complex, multifactorial disease that develops from the normal host breast tissue. Therefore, developing preventive vaccines relies on identifying and targeting normal over-expressed, mutated, or cancer-specific targets. Three potential vaccination targets emerge including (1) oncodriver over-expressed proteins, (2) tissue specific antigens, and (3) cancer specific antigens (Fig. 10.2). We will discuss the utility of targeting each of these three groups of cancer-associated molecules in breast cancer, realizing that the best preventive vaccines may draw from a combination of these different targets.

10.4 The Case for Targeting Oncodrivers in Breast Cancer Prevention

The breast matures in distinct stages that are related to sexual development and reproduction. These stages are embryonic, prepubertal, pubertal, pregnancy, lactation and involution [19]. During early telarche, initial breast bud development occurs, however, the terminal end buds (TEB) do not complete maturation until pregnancy and lactation [20]. Following completion of lactation, a complex involution occurs causing terminal breast buds to die and the breast to return to a pre-pregnancy state.

The growth and invasion of TEB mimics cancer invasion of the breast stroma and is driven by the same oncodrivers found in many breast cancers [21]. These

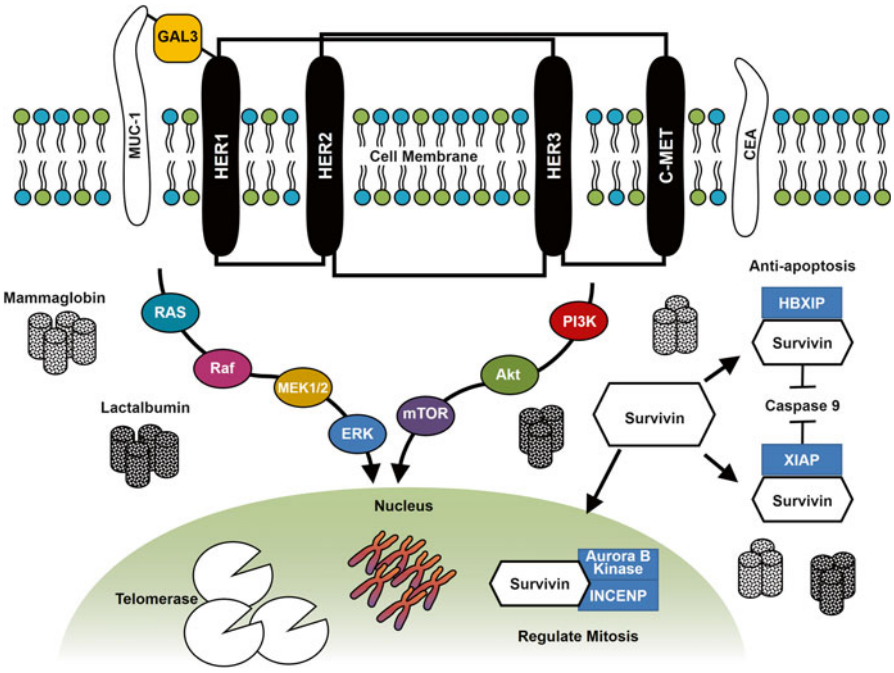

Fig. 10.2 Antigenic targets in breast cancer vaccination. Oncodrivers (HER-1, HER-2, HER-3, C-MET—*black*); Tissue Specific Breast Proteins (Mammaglobin, Lactalbumin—*grey*); Cancer Specific Proteins (Telomerase, Survivin, MUC-1, CEA—*white*)

TEBs are rapidly proliferating masses of epithelial cells that invade into stromal tissue, displaying properties associated with tumor progression-invasion, re-initiation of cell proliferation, resistance to apoptosis, and angiogenesis [20]. Carcinoma with epithelial growth factor receptor (EGFR) mutations, p53 mutations, or BRCA1 defects, such as (adeno) myoepithelial carcinoma, medullary carcinoma, metaplastic carcinoma, and ductal invasive basal carcinoma, have expression patterns similar to stem cells. In contrast, tubular, lobular, and grade 1–3 ductal invasive carcinoma have an immunophenotype similar to glandular cells. Basoluminal and ductal invasive grade 3 carcinoma with HER2 amplification fall in the intermediary cell category [21].

HER family members—HER-2, HER-3 and HER-1 (EGFR) as well as hepatocyte growth factor (c-MET), are expressed during breast development and growth [22]. HER-2 is also expressed during TEB growth during pregnancy. These same drivers have also been shown to be overexpressed in many breast cancers, suggesting their potential role in breast tumorigenesis. HER-2 is the classic example of a tumorigenic protein, and is overexpressed in both DCIS lesions and 20–30 % of invasive breast cancers (IBC) [23]. HER-3 has been found to promote HER-2-induced changes in breast epithelium before, during, and after tumor formation [24], and is expressed in numerous triple-negative cancers. HER-3 overexpression

is associated with worse outcomes and increased recurrence in several cancers, including breast cancer [25]. EGFR or HER-1 is also overexpressed in half of triple negative or basal breast cancers [26]. C-Met has been found to be expressed in triple-negative and some estrogen-expressing cell lines. The targeting of HER-3 and HER-1 is beginning to be explored using antibodies and kinase inhibitors to block the down-stream signaling pathways [26].

We have shown many of these oncodrivers to be expressed in early breast cancers, such as DCIS. DCIS is a proliferation of malignant epithelial cells confined within the basement membrane of mammary ducts, and appears to be a precursor lesion to IBC. These oncodrivers expressed in early DCIS lesions may be ideal targets for breast cancer prevention because of their key role in driving growth, invasion, and metastatic spread. Since these proteins are normally expressed in mammary development, innate immune responses may exist for controlling these oncodrivers. If these immune responses exist, then these oncodriver proteins may be suitable targets for breast cancer vaccination.

10.5 Evidence for Immune Responses Against Oncodrivers

HER-2 has been the focus of numerous immune interventions. Peptides derived from this molecule can be recognized by CD8+ T cells in MHC class I molecules. One of the most studied immunogenic peptides derived from HER-2 is E75 or (369–377). E75 is a peptide that binds HLA-A2 and has been administered as a vaccine in numerous clinical studies [27–29]. It has generated CD8+ T cell responses when administered to patients with HER-2 expressing breast cancers [29]. HER-2 derived peptides have also been identified that bind MHC class II molecules and activate anti-HER-2 CD4+ cells [30–32]. These peptides have been used to successfully prevent recurrence in patients with HER-2 positive breast cancer [30–32] and cause regression of DCIS lesions [33–36]. Other forms of anti-HER-2 vaccination are also being tested in trials including DNA vaccines, protein and RNA vaccines to drive anti-HER-2 immunity for treatment [30, 37, 38].

We observed that healthy individuals have surprisingly high frequencies of circulating anti-HER-2 CD4+ Th1 cells that secrete INF-γ and TNF-α [39]. This anti-HER-2 CD4+ Th1 response is lost during HER-2 breast tumorigenesis [40] beginning very early in the process during DCIS and more profoundly at the time of invasion [39] (Fig. 10.3). Furthermore, in patients with HER-2+ IBC, low anti-HER-2 immune responses are associated with increased risk of recurrence and lack of achieving complete responses to neoadjuvant chemotherapy [39]. Additional situations where the anti-HER-2 immune response is lowered may increase susceptibility to breast cancer development. For example, nulliparous women who have higher HER-2 gene expression and lower anti-HER-2 immune responses compared with parous women also have an increased risk of breast cancer [41]. Post-partum, when it is known that pre-menopausal women

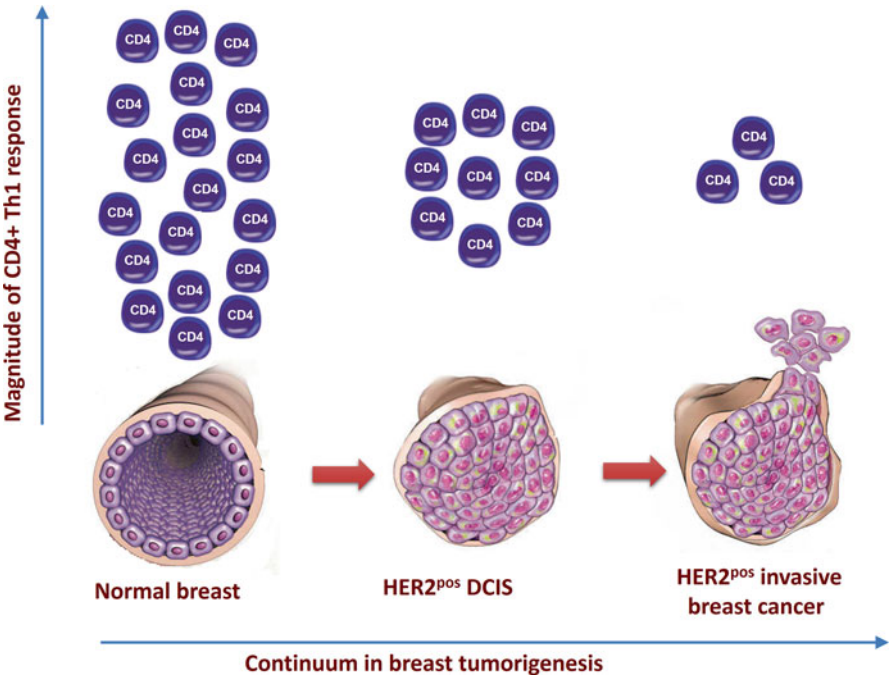

Fig. 10.3 Progressive loss of the anti-HER2 Th1 immune response along the breast cancer continuum

have some increased risk of breast cancer development in the 5-year window following pregnancies [42,43], women may display similarly high HER-2 gene expression and low anti-HER-2 immunity. We have developed a simple blood test that can measure anti-HER-2 CD4+ Th1 responses. Identifying patients with depressed anti-HER-2 CD4+ Th1 immune responses may be particularly useful in capturing those patients with HER-2 DCIS or IBC that are not detected with screening mammograms. Finally, although this deficient immune response is not corrected by surgery, radiation, or chemotherapy, we have shown that HER-2 peptide pulsed dendritic cells (DC1) activated to secrete high levels of IL-12 can be used in vaccination to augment and restore the anti-HER-2 immune response [39].

There are similar losses in CD4+ Th1 immunity being identified in HER-3 and other oncodrivers, suggesting this may be a common theme in breast cancer development. The ability to identify patients with suppressed immune responses against oncodrivers and correct the defective response prior to the development of breast cancer may be a feasible approach to breast cancer prevention. The benefits of targeting oncodrivers using vaccines are that these drivers are over-expressed in cancer cells, so only the aberrant cells would be targeted. This process may be a natural surveillance mechanism that the immune response uses to control proliferating cells during growth and development.

10.6 The Case for Targeting Tissue Specific Breast Proteins

The breast contains several tissue-specific proteins that are found in very few other organs, making these proteins breast-specific. Mammaglobin and lactalbumin are two important examples [44, 45]. Mammaglobin (MAM) is a member of the uteroglobin gene family that is highly expressed in the mammary epithelium and is over-expressed in up to 80 % of breast cancers [44]. Lactalbumin is conditionally produced only during lactation, but is expressed in over 60 % of breast cancers [46]. Immune responses have been generated against both of these proteins [45, 47, 48]. Both CD4+ and CD8+ T cell as well as antibody responses develop as a consequence of vaccination [46, 47]. Clinical trials have already shown that these immune responses are reproducible in human patients, with an increase in CD8+ T cells capable of lysing MAM+ breast cancer cells [48]. Murine models vaccinated against lactalbumin have been shown to prevent breast cancer development along with increased CD4+ and CD8+ T cell response [45, 49]. Mammaglobin may be useful in preventing a broad range of breast cancers as it is expressed in up to 80 % of estrogen receptor (ER) positive cancers and up to 40 % of triple-negative cancers [48]. Lactalbumin appears to be more highly expressed in triple-negative breast cancer [50], suggesting that vaccinating against lactalbumin may be most useful in preventing triple negative breast cancers.

The benefit of tissue-specific antigen vaccination is the low likelihood of life-threatening autoimmune pathologies, since expression of these antigens is limited to the breast. These antigens are also only minimally expressed on healthy and non-lactating tissue; therefore, when cells over-expressing these antigens arise, they are easily recognized. In this setting, vaccination acts as an immunologic mastectomy, eliminating duct cells that express lactalbumin or mammaglobin. Vaccination would be restricted to women who do not wish to lactate, have completed lactating, or are post-menopausal, since inducing these responses in lactating breasts can cause tremendous mastitis as seen in mouse models [51]. Nonetheless, there is continued progress in developing these vaccines against lactalbumin and mammaglobin in triple negative cancer.

10.7 The Case for Targeting Cancer Specific Proteins for Breast Cancer Prevention

In addition to oncodriver targets and breast tissue-specific targets, cancer-specific proteins that are abundantly expressed in transformed cells represent a third potential class of vaccine target. Examples of these proteins include telomerase, survivin, MUC-1 and differentiation antigens, such as carcinoembryonic antigen (CEA). All of these antigens are over-expressed in different types of breast cancers and are essential in transformation of normal cells to tumor cells. Telomerase is expressed in most tumors and prevents loss of telomeric DNA during the rapid cell division

characteristic of tumor growth [52]. Survivin is nearly undetectable in most normal adult tissues, but is highly expressed in some breast cancers, participating in the control of apoptosis, angiogenesis and proliferation [53]. MUC-1 is a mucoglycoprotein that is upregulated and hypoglycosylated in breast cancer. CEA is also a glycoprotein molecule that is overexpressed in many cancers, mainly gastrointestinal cancers, but has also been found in up to 50 % of breast carcinomas [54]. Targeting these antigens early, when they are initially over-expressed, is important in preventing IBC.

Immune responses have been induced against each of these cancer-specific molecules. In fact, clinical studies have already shown that vaccination against telomerase induces a peptide specific CD8+ immune response, increases progression free, and increases overall survival [55, 56]. Survivin has been shown to produce a CD8+ T cell response in vitro [57]. Clinical trials with survivin vaccination against prostate cancer have shown disease remission and regression [58], but clinical trials with survivin vaccination against breast cancer have yet to yield significant results [59]. Because MUC-1 is a glycoprotein, it tends to be weakly immunogenic. To ameliorate its weak immunogenicity, clinical trials in patients with breast cancer have coupled MUC-1 with Bacillus Calmette-Guerin (BCG) and tetanus toxoid in vaccines, which have been shown to have a selective immune response against MUC-1 [60–62]. Like MUC-1, CEA is also a glycoprotein and has been used in a recombinant vaccine with vaccinia. The MUC-1-vaccinia combination has been tested against many cancers, including breast cancer, with a good immune response and even showing a pathologic complete response [54].

Cancer-specific antigens are good targets for vaccination, thus group of antigens may be best utilized in secondary prevention of early lesions for primary prevention of invasive cancer. An example would be vaccination of DCIS, which is considered a precursor to IBC, this although secondary prevention would truly be primary prevention of invasive breast cancer. Eradicating DCIS at a pre-invasive stage with a continued immune response to the causal antigen would prevent future breast cancer. Telomerase, survivin, and MUC-1 are all expressed in DCIS [63–65], making these antigens good candidates for primary prevention and treatment against breast cancer. In fact, clinical studies have applied a MUC-1 vaccine in the setting of patient with a history of advanced colon adenoma—a precursor lesion to colon cancer—and found that these patients were able to exhibit long lasting immunity to the MUC-1 antigen [66].

10.8 The Special Case for Targeting Breast Cancer Stem Cells in Prevention

Breast malignancies may arise from specialized breast cancer stem cells (BCSC), or cancer initiating cells [67]. BCSC have been associated with late recurrences, and may very well be early precursor cancer initiating cells. Numerous groups are now focusing efforts to grow out stem cells that are CD44 high and CD24 low or ALDH1 positive as a means to develop strategies to target these cells. While oncodrivers,

such as HER-2, have been shown to be expressed on BCSC [68], there may be additional unique BCSC antigens [69]. As described below, we have developed vaccines against HER-2, clearly a BCSC associated molecule [68]. Once identified, additional unique BCSC antigens could be similarly targeted by vaccination.

10.9 The Lack of Evidence to Target Viral antigens

Some malignancies have been shown beyond doubt to have a strong viral component in their etiology. The best examples include the association of human papilloma virus types 16 and 18 with cervical, anogenital, and head and neck cancers, hepatitis B virus with hepatocellular carcinoma, EBV with Burkitt's lymphoma, Hodgkin's disease, and undifferentiated nasopharyngeal carcinoma, and Human Herpesvirus 8 (HHV8) with Kaposi's sarcoma. Vaccines have been developed against both HPV and HBV. Gardasil and Cervarix target the major capsid protein L1 of HPV and the hepatitis B vaccine is based on the major surface antigen of the virus (HBsAg). Targeting viral antigens to protect against breast cancer is dependent upon the extent to which viruses play a role in breast carcinogenesis. The causal relationship between viruses and human breast cancers remains controversial. Nonetheless, there are several suspect viruses that are being actively investigated. Three of the most prominent are briefly discussed here.

The first indication that breast cancer could have an infectious etiology came from studies initiated by Bittner in the 1930s [70]. The apparent filterable agent was later identified as a retrovirus designated mouse mammary tumor virus (MMTV). This virus could integrate into the genome of adult mice and be transmitted vertically through the endogenous route, or alternatively be transmitted to offspring through milk during nursing. The discovery of this virus, which was found to cause breast tumors in both captive-bred and wild mice, spurred vigorous investigations into the possible viral causes of breast cancer in humans.

Subsequently, MMTV, or a closely related virus (about 95+% sequence homology with MMTV) [71] has been discovered in some human breast cancers and designated human mammary tumor virus (HMTV) [72]. Viral gene sequences have been reported in 38 % of breast cancers, but only 1 % of normal breast tissues [73]. Interestingly, correlations have been reported between geographical regions of low breast cancer incidence and prevalence of detectable viral sequences [74], as well as more frequently detected viral genes in certain breast cancer subsets like gestational and inflammatory breast cancer [75, 76].

Also implicated in breast cancer etiology are the human papilloma viruses (HPV). High-risk human papilloma viruses type 16, 18 and 33 cause cellular transformation through early gene products (particularly E6 and E7), which act as oncoproteins that inhibit apoptosis and dysregulate cell cycle. HPV infection also induces a particular cytopathic effect in squamous epithelial cells that leads to the formation of a koilocyte, which is characterized by an enlarged, darkly-staining nucleus with pronounced cytoplasmic perinuclear clearing (e.g. "halo"). A number of studies

have used standard PCR to detect high-risk HPV strains in breast tumors, but not in the surrounding normal breast tissues [77–81]. More recently, Heng and co-workers performed in situ, as well as standard, PCR (with sequencing) and histopathology to assess presence of koilocytes in breast cancer. The in situ assay was designed to minimize the possibility of contamination by localizing the viral DNA to the nucleus. The investigators found evidence of high-risk HPV in breast cancer lesions, but also detected it in surrounding normal tissues and in the tissues of some healthy breasts (although frequency was higher in cancerous tissue). This was explained by the fact that even in the well-established relationship with cervical cancer, HPV infection precedes the development of malignancy, but does not guarantee eventual cancer development. Interestingly, koilocytes were observed in 18 of 28 (66 %) breast cancer specimens, and all of these were shown HPV-positive by in situ PCR [82, 83]. Taken together, these data suggest a possible link between HPV and breast cancer.

There is also a possible link between Epstein Barr virus (EBV) and breast cancer. EBV is a γ-Herpesvirus that has a strong tropism for B lymphocytes and epithelial cells. EBV principally manifests as infectious mononucleosis. The virus infects most individuals by young adulthood, and establishes a state of latency that lasts for the lifetime of the individual. During latency, only a subset of EBV genes is expressed. Certain triggers can reactivate the virus leading to re-establishment of lytic infection. Studies to determine an association between EBV and breast cancer have sought to detect viral genetic material via qPCR [84], PCR plus tissue microarray [85], and in situ hybridization [86], and to detect expressed viral proteins, such as Epstein-Barr nuclear antigens (EBNAs) and Latent membrane protein-1 (LMP-1), via immunohistochemistry [87]. These studies detected evidence of EBV genes or gene products in a subset of breast cancers.

In summary, it should be reiterated that a viral etiology for breast cancer remains highly controversial, and whereas we have cited a number of studies purporting to demonstrate the presence of viral products in breast tumors, a considerable body of work from numerous laboratories have reported either failure to find any association of these viruses with human breast cancer [88–90] or have attributed detection to contaminating, virally-infected but non-cancerous cells [87] or cross-reaction of detecting reagents with non-viral products [91]. Further studies are clearly necessary to settle this issue, and if a consensus is reached that certain viruses promote breast carcinogenesis, the associated viral antigens should be included in breast cancer vaccines.

10.10 Making Immunization More Effective: Vaccine Adjuvants

Immune response evolved primarily to deal with microbial infection. Therefore, elements of the innate immune system (such as dendritic cells) sense pathogen associated molecular patterns (PAMPs), become activated, and present pathogen-derived protein antigens complexed with MHC molecules to T lymphocytes, which are the agents of adaptive immunity. Since neither tumors nor pure protein antigens (such

as synthetic peptides) derived from tumors contain PAMPs, vaccine preparations including only tumor-derived proteins are unlikely to be strongly immunogenic and will poorly activate innate immunity. Adjuvants are compounds that amplify the immunogenicity of vaccines. Such adjuvants were originally developed to enhance vaccines against infectious diseases, but they are likely to be necessary for generating effective anti-tumor immunity. Adjuvants are thought to act by two general mechanisms. The first is the "depot" effect, which conserves the antigen at the site of injection for an extended period of time, where it is released slowly to provide long-term stimulation to the immune system. The second is through direct or indirect activation of components of the innate immune system. The natural adjuvant is an activated dendritic cell (DC), which can be prepared from peripheral blood DC precursors including monocytes [92]. For secondary prevention, as discussed later, this may be an ideal vaccine adjuvant; however, for primary prevention the harvesting of personalized DC is cumbersome and not cost-effective. An alternative simplified vaccine adjuvant must be selected.

The first adjuvant to gain wide use was aluminum salt (alum). Precipitation of vaccine immunogens with alum, and the attendant enhancement of immunity using this mixture, was first observed with diphtheria toxoid [93]. Alum has subsequently been employed in a variety of vaccines against infectious agents licensed for use in the United States. Despite its extensive use, the mechanisms by which alum amplifies immune responses are still uncertain. Alum has an excellent safety record spanning decades, but, unfortunately, it is probably unsuitable for generating powerful anti-cancer immunity. Alum largely induces Th2-dominated immunity [94]. Th2-responses are characterized by strong antibody production, and IL-4 and IL-5 producing T cells. Effective anti-tumor immunity, on the other hand, requires robust cell-mediated immunity characterized by IFN-gamma secreting "Th1"-polarized T cells and cytotoxic T cells.

Freund's adjuvant consists of paraffin oil that is mixed with an aqueous solution of the vaccine antigens to form an emulsion. Freund's "complete" adjuvant adds a killed preparation of bacteria (e.g. Mycobacterium) to enhance immunity, while the "incomplete" adjuvant contains only oil. It is likely that some of the adjuvant effect of Freund's complete adjuvant is derived from the PAMP molecules provided by the Mycobacteria. This adjuvant has been used for decades to induce powerful immunity in experimental animals; however, it is not suitable for humans, largely due to toxicity—i.e. severe inflammatory responses at the site of injection.

The success of Freund's in animal models has led to the search of other, less toxic, oil/water emulsion adjuvants that might be useful for humans. These include MF59 and AS03, manufactured by Novartis and Glaxo Smith Klein, respectively. Both adjuvant preparations are based on squalene, a 30-carbon lipid molecule originally derived from shark liver oil, but also obtained from a number of plant sources. Although not yet licensed in the United States, both of these adjuvants are utilized in Influenza vaccine preparations in Europe.

Synthetic or chemically-modified Toll-like receptor (TLR) agonists represent another highly promising avenue of investigation. TLR agonists directly activate dendritic and other antigen-presenting cells of the innate immune system through

their associated PAMP receptors. There are approximately ten known TLRs in humans, each identifying a different restricted set of possible ligands common to broad classes of potential pathogens. Ligation of TLR receptors induces enhanced antigen-presenting function of dendritic cells, and stimulates the secretion of cytokines and chemokines, which enhances the adaptive immune responses. Several of these receptors are being targeted by candidate adjuvants.

For example, Monophosphoryl lipid As (MPL) is a chemically altered, detoxified form of cell wall lipopolysaccharide from Salmonella Minnesota strain R595. Despite its chemically altered nature, it retains recognition by TLR4 and TLR2 and activates a MyD88-dependent signaling pathway that triggers secretion of pro-inflammatory cytokines and chemokines [95]. It is also associated with the generation of Th1 immunity [96]. A licensed HPV vaccine (Cervarix; GSK) contains MPL as an adjuvant, and a similar adjuvant formulation has been tested in vaccines in clinical trials against other viruses—including Herpes Simplex and Norovirus [97, 98], and cancers—including melanoma and colorectal cancer [99, 100]. Other TLR ligands being investigated in clinical trials as vaccine adjuvants include the double-stranded RNA mimic and TLR3 agonist, poly-ICLC for ovarian cancer and glioma [101, 102], and CpG DNA oligonucleotides (TLR9 agonists) and imiquimod (TLR7 agonist) for melanoma [103].

10.11 Enhancing Effector Function: Checkpoint Inhibitors

Ideally, cancer vaccines would work as stand-alone prevention for cancer as they so effectively do for a variety of infectious diseases. While this may be achieved in primary prevention, in the case of pre-existing disease, where the goal is either therapy or secondary prevention, vaccination will almost certainly have to be combined with other interventions to achieve maximal effect. This is largely because the presence of pre-existing disease either presents the immune system with too large a disease burden to eliminate without additional help, or because the tumors themselves exert regulatory influences on the immune system that may partially blunt or attenuate anti-tumor immunity.

One highly promising field of investigation is “checkpoint inhibitors”. T lymphocytes, which are largely responsible for dealing with both infection and cancer, are able to receive a variety of input signals that regulate their functional activity. Some of these signals activate the lymphocytes. Such signals are necessary to set immune responses in motion against microbial or malignant threats. Other signals are inhibitory, and are often referred to as “checkpoint” signals [104, 105]. Checkpoint signals are also important for maintaining homeostasis, because immune responses should not continue after the challenge has been eliminated, and normal, healthy tissues need to be spared from off-target immune attack. Receptors that receive these inhibitory signals represent the checkpoints that govern the limits of immune responses. These checkpoints become highly relevant for anti-tumor immunity because cells comprising malignant tumors often subvert these systems

of inhibitory control as a means of escaping the immune response. Checkpoint inhibitor drugs interfere with this strategy of subversion.

There are many possible receptor/ligand interactions forming checkpoints that tumors may use to evade immunity, but the two that are most studied, and that are being developed as therapeutic targets are the CTLA-4/B7 interaction and the Programmed death receptor (PD-1)/Programmed death receptor ligand (PD-L1 and PD-L2) interaction [106, 107].

CTLA-4 (i.e.CD152) is a surface receptor on T lymphocytes. CTLA-4 competes with another receptor, CD28, for interaction with B7-family co-stimulatory molecules (CD80 and CD86), which are expressed by dendritic cells and other antigen-presenting cells. One of the earliest steps in T cell activation occurs when dendritic cells present processed peptide antigen to T cells in the context of self MHC molecules. This antigenic signal is sensed by T cells through the T cell receptor. A second signal provided by the dendritic cell is through expression of surface co-stimulatory molecules, including CD80 and CD86. Resting T cells express high levels of CD28 (relative to CTLA-4), the counter-receptor for these co-stimulatory molecules. This interaction supplies an important second signal to the T cells that allows them to proceed to an activated state and avoid a state of chronic inactivation (anergy). Following T cell activation, levels of CTLA-4 begin to rise [108]. In contrast to CD28, ligated CTLA-4 supplies an inhibitory signal to the T cells that limits the scope of their effector function [109]. Monoclonal antibody-based drugs such as Ipilimumab have been developed that block signaling through CTLA-4 [110], preventing activated T cells from receiving feedback signals that will limit their activity. These drugs maintain T cells in a prolonged state of high effector activity, thereby improving the anti-tumor immune response. Ipilimumab has been tested in a number of clinical trials alone [111], in conjunction with chemotherapy [112], or in combination with vaccination [113]. Improved clinical responses have been observed in a subset of patients, but a relatively high rate of adverse effects has been reported, including diarrhea, colitis and dermatitis, and occasional more serious off-target toxicities to the liver and thyroid gland. These side-effects have limited the use of CTLA-4-blocking therapy, but the cases of improved clinical responses have spurred the search for other checkpoint inhibitors.

Programmed death receptor-1 (PD-1) is a transmembrane protein that is expressed on T lymphocytes. The ligands are PD-L1 and PD-L2. PD-L1 is expressed by activated dendritic cells, macrophages, B cells, and a variety of normal tissues. PD-L2 was initially thought to be found only on antigen-presenting cells, but it has now been identified in a number of immune and non-immune cell types, depending on a certain environmental factors [114]. When effector T lymphocytes are signaled through PD-1 by PD-L1 or PD-L2, they are negatively regulated in their activation, proliferation and expression of effector function. Consequently, transgenic mice lacking PD-1 suffer from several chronic inflammatory pathologies, indicating that this molecular interaction is critical for avoiding autoimmunity [115–117]. Also of significance, most tumor lines express PD-L1 or PD-L2, suggesting that tumors are subverting this system of autoimmune avoid-

ance to escape anti-tumor immunity. Consequently, PD-1 has come under scrutiny as a target for improving anti-tumor immune responses, and several monoclonal antibody-based therapeutics that interfere with PD-1 signaling (e.g. Pembrolizumab; Merck, Nivolumab; Bristol-Myers Squibb) are being developed and tested for treatment of solid tumors, including malignant melanoma and breast cancer [118–120]. Many of these studies provide evidence of objective responses and improvements in progression-free survival. The toxicity profile of these agents appears to be more promising than anti-CTLA therapy.

10.12 DCIS as a Model for Prevention

The immunogenicity of breast cancer that has been described above makes breast cancer a particularly promising candidate for vaccination designed to generate "secondary cancer prevention". Specifically, breast cancer tumor antigens have been observed to initiate a tumor-specific adaptive immune response. [121, 122] and lymphocytic infiltration is associated with improved survival [123, 124].

Early vaccine trials have focused on later stages of disease when standard treatments have failed. Under these conditions, cancer vaccines have had limited success—even vaccines that were able to stimulate an immune response did not demonstrate corresponding clinical improvement [125].

With the introduction of screening mammography, pre-invasive lesions are increasingly diagnosed. Ductal carcinoma in situ (DCIS) represents greater than 20 % of breast cancer cases diagnosed. Pre-invasive or early stage disease may be a better suited target for vaccination and cancer prevention for a variety of reasons [126]. These include:

- Patients with pre-invasive or early stage breast cancer may be more adept at responding to vaccination as they are usually otherwise healthy.
- Patients with pre-invasive or early stage breast cancer do not require adjuvant cytotoxic treatment which may induce immunosuppression via immunosuppressive cytokines, anergy, lymphopenia, impaired antibody production, inhibition of immune effector function, reduction of MHC expression, or inhibition of co-stimulatory proteins [127–132] (There are some chemotherapies, like cyclophosphamide and 5-Fluorouracil, that may induce immunogenic cell death and eliminate regulatory immune subsets, which would actually enhances the immune response [133]).
- The slow progression from DCIS to invasive breast cancer gives time for the patient to receive neoadjuvant booster vaccinations and develop a robust immune response,
- The smaller tumor burden of early disease may be more amenable to penetration and destruction by the immune effector cells
- Both immune and clinicopathological responses to neoadjuvant treatment can be assessed rapidly at the time of surgical resection.

Treatment of DCIS may (1) prevent the progression to invasive disease, (2) decrease the extent of surgical resection or the need for radiation therapy, thereby reducing the associated morbidity resulting from current treatments, and (3) lower the risk of subsequent recurrence and the associated psychological fear.

DCIS is a non-obligate precursor to invasive breast cancer—this means not all patients with DCIS will progress to invasive breast cancer. DCIS is frequently present on routine autopsy, suggesting that up to 15 % of DCIS lesions may be clinically insignificant [134, 135]. Therefore, ideal treatment of DCIS should be provided preferentially to higher risk patients. High-risk patients have an increased risk of invasive disease, subsequent recurrence, and require more aggressive treatment (e.g. mastectomy or lumpectomy with radiation).

Conventional predictors of high risk DCIS include patient, tumor, and treatment factors, including: younger age, family history of breast cancer, tumor size, tumor grade, and resection margin [136]. More recently, molecular markers that are prognostic in invasive breast cancer have also been shown to be expressed in DCIS [137]. In fact, HER-2/neu is overexpressed in DCIS (56 %) as compared to invasive breast cancer (11 %) [138], and HER-2 positivity is significantly associated with a higher rate of invasive disease [139, 140] and increased risk of recurrence [141] in patients with DCIS. This association suggests that HER-2 may have a critical role in cancer progression, or at least represent as a biomarker for increased risk of invasive disease. Therefore, HER-2-targeted therapy in DCIS may be of particular benefit in preventing the development of invasive breast cancer, or alternatively eliminate HER-2 expressing cancer stem cells. The latter would leave behind less harmful non-cancer stem cells with favorably less malignant phenotypes.

We have taken this approach in patients with HER-2-expressing DCIS in two neoadjuvant studies using HER-2 pulsed type I activated dendritic cell (DCI) vaccines. The advantages of this approach are that the DC are activated ex vivo where they cannot be further influenced by tumor factors, and that there is no adjuvants including aluminum compounds as the DC1 are the adjuvant themselves. The drawback to this personalized approach is that DC precursors must be obtained from each individual subject. In our first clinical trial of our anti-HER2 dendritic cell vaccine, we vaccinated patients who were diagnosed with $HER2^{pos}$ DCIS (either HER-2 2+ or 3+). Patients underwent leukapheresis with elutriation of blood product to provide monocytes (DC precursors) for vaccine preparation. Monocytes were cultured overnight in GM-CSF and IL-4-containing culture medium (to induce DC differentiation), pulsed with six HER-2/neu MHC class II binding peptides, and rapidly matured using IFN-γ and LPS. If the patient was HLA-$A2^{pos}$, the monocyte pool was divided in half and pulsed with either MHC class I binding peptide 369–377 or 689–697. Four to six weekly injections were administered into bilateral groin lymph nodes. In the second study, we randomized patients to injections in the groin nodes, the breast in the region of DCIS, or both sites.

The vaccine was well tolerated with only grade 1 and 2 toxicities observed and no cases of unacceptable toxicity. Vaccination with HER-2/neu peptide pulsed DC1s induced both $CD4^{pos}$ and $CD8^{pos}$ HER-2/neu-reactive T-cells, infiltration of

lymphocytes into the breast around the DCIS tumor (Fig. 10.4), and durability of the response >48 months. Additional complement-dependent, tumor lytic antibodies were induced in some subjects, suggesting an additional effector role. Clinical response (i.e. no evidence of disease found in the breast at the time of surgical resection) occurred in about 30–35 % of ER^{neg} $HER\text{-}2^{pos}$ subjects, but only 4 % of ER^{pos} $HER\text{-}2^{pos}$ patients experienced no residual disease [34,36]. Combining anti-estrogen therapy with vaccines in this latter group resulted in complete response rates of 30 %, similar to the ER^{neg} $HER\text{-}2^{pos}$ subjects (*submitted for publication*). Further studies combining HER-2 pulsed DC1 vaccines with HER-2 targeted blockade are underway in an effort to further increase pathologic complete response rates, decrease the extent of surgical and cytotoxic therapy used to treat for high risk DCIS lesions, and prevent subsequent breast cancer events.

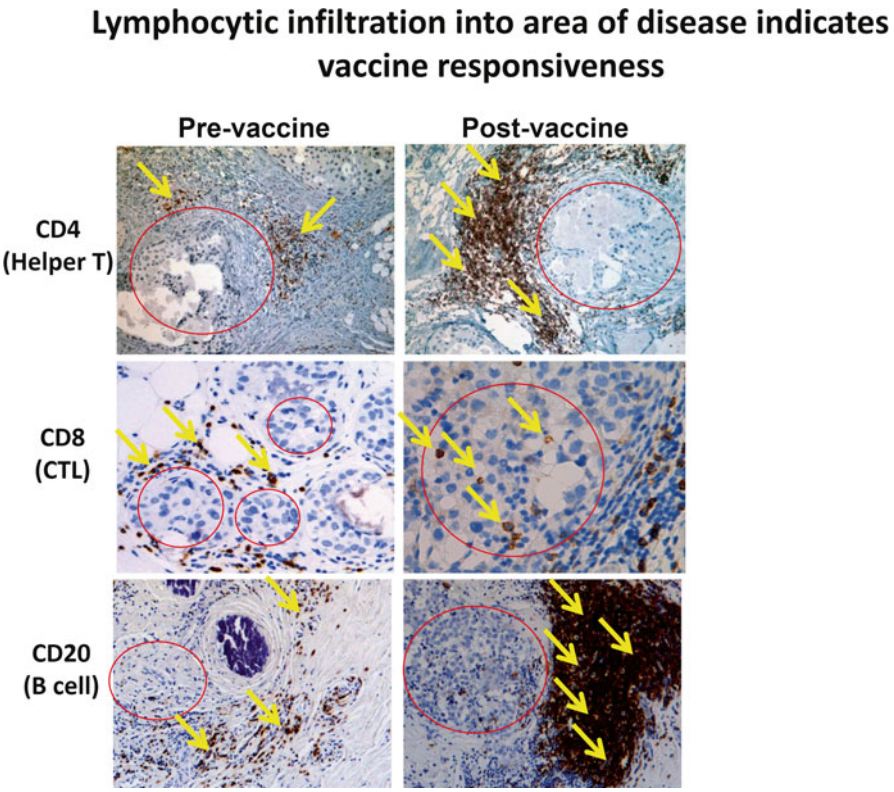

Fig. 10.4 Pre-vaccine biopsies were compared to post-vaccine surgical specimens by staining thin tissue sections for $CD4^{pos}$ "helper" T cells, $CD8^{pos}$ cytotoxic T cells, and $CD20^{pos}$ B lymphocytes. Areas of DCIS are subtended by *red circles*, lymphocytic infiltrates are stained *dark brown* and highlighted with *yellow arrows*. Note large increase in CD4 T cells in periductal areas after vaccination. $CD8^{pos}$ cells typically did not increase as dramatically, but often were observed entering the diseased duct. Somewhat unexpectedly, $CD20^{pos}$ B cells dramatically increased for some subjects in periductal regions after vaccination

10.13 Identifying High Risk Groups

10.13.1 Genetic Predisposition

Patients that are at high risk for developing breast cancer can be divided into those with genetic predisposition and those with acquired risk. While we can identify those with genetic predisposition quite readily, these patients account for only 10–15 % of all breast cancer patients. Despite the development of whole genome sequencing, genetic mutation identification has outstripped our ability to offer numerous treatment alternatives/ for prevention. Surgery remains the most effective treatment, but does so at an enormous cost to the patient. Other prevention techniques, such as anti-estrogens or surgical oophorectomy, are modestly risk reducing, but also with substantial side effects. Vaccination of women who have a genetic predisposition to develop breast cancer is a particularly appealing strategy for prevention.

For example, BRCA1 carriers are at increased risk of developing triple-negative breast cancers [142]. These patients present with a highly aggressive tumor at a young age, can be offered only limited available treatment options, including highly toxic chemotherapy, and still often succumb to recurrent disease. Oncodrivers, including HER-3, EGFR, and c-MET are overexpressed on triple negative tumors, and therefore represent potential targets for vaccination. Similarly, the lactalbumin protein is also over-expressed in triple negative tumors, and presents another potential target for vaccination. Cancer-specific targets, including MUC-1, telomerase, and survivin, could also be targets of vaccination therapy in this setting. These high-risk patients and their potential vaccine targets are both readily identifiable and are therefore well-suited to be treated with preventative vaccination. Because of the high lifetime risk of developing invasive breast cancer (60–80 %). This group is particularly well-suited for breast cancer preventive vaccines.

10.13.2 Acquired Risk

Identifying the patients with acquired risk is more difficult, but not impossible. For example, pre-menopausal women have been shown to be at increased risk for developing breast cancer in the 5 years following pregnancies [143–145]. Some of these patients have diminished immune response gene expression related to dendritic cells and T cell function [146]. With the rapid blood immune tests that we have developed, we can identify a diminished anti-HER-2 immune response or a transient loss of immune responses against oncodrivers, such as HER-2, HER-3 and c-MET. Women with a decreased immune response may be at increased risk of developing post-partum breast cancer. Additionally, this deficit can be corrected by vaccination.

Many of the acquired risk-associated breast cancers have HER-2, HER-3, and c-MET oncodrivers in early DCIS since these are the main oncodrivers involved in

breast elongation. In contrast, HER-2-expressing breast cancer rarely are associated with patients with BRCA1 or BRCA2 mutations. Vaccinations targeting HER-2 may be a very effective way to prevent non-hereditary breast cancer, and clinical trials are in progress [34–36] and being developed in large scale Phase III trials in patients with DCIS.

Finally, there is an increased risk of breast cancer development in patients taking immunosuppressive medications, particularly following organ transplantation [14]. Vaccination against oncodrivers, tissue specific antigens, or cancer specific antigens may be able to augment the immune responses and reduce breast cancer risk in these populations where immune suppression needs to be maintained.

10.14 Realizing the Potential

Clearly the immune response can determine the outcome and influence survival in invasive breast cancer [39, 95]. The loss of immune responses against oncodrivers early during tumorigenesis further suggests a crucial role of the immune response against protection for the development of breast cancer [39]. Vaccinations against oncodrivers to restore immunity may help to prevent breast cancer. Blood tests to measure the immune responses may be used to identify individuals at risk of developing breast cancer, and allow for vaccination prior to developing invasive disease. Developing vaccines against oncodrivers, breast tissue specific antigens, and cancer specific antigens will be useful to develop in the armamentarium for breast cancer prevention in those with identified risk including those with genetic predisposition. Since many acquired breast cancers have HER-2 involved even vaccinations to correct the anti-HER-2 immunity may be a good starting point for prevention in these patients. The time is nearing that we can now begin to realize the potential of using vaccines to prevent breast cancer and in the next decade will begin to realize this potential.

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Index

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