Date: September 8, 2005

Protocol: Adolescent Diet, Hormones and Breast Cancer Susceptibility

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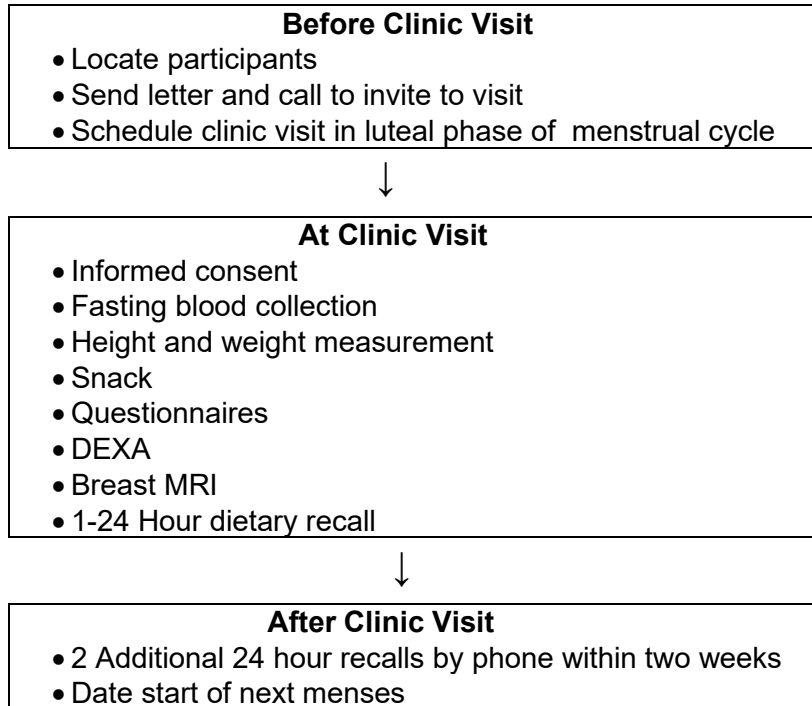Minneapolis, MN 55454-1015
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2.0 INTRODUCTION/RATIONALE

Breast cancer is the most common malignancy among women in the US and the second most common cause of death from cancer. Each year approximately 184,000 women in the US are newly diagnosed with breast cancer and 43,000 die from their disease [1]. Although animal studies, international comparisons, and migrant studies support the hypothesis that a high fat diet increases breast cancer risk, these findings generally are not confirmed by epidemiological studies that evaluate the association of adult diet with breast cancer. Adolescence is a time of rapid growth and maturation of the breasts, and a woman's diet as an adolescent could potentially affect her risk of developing breast cancer more than her diet as an adult. Because of difficulties remembering exposures in the distant past, there is considerable potential for misclassification bias in case-control studies of adult breast cancer that attempt to evaluate recalled adolescent diet. Cohort studies begun today to evaluate the effect of childhood and adolescent diet on breast cancer risk will not begin to yield results for 40-50 years. Alternative strategies are clearly needed.

The Dietary Intervention Study in Children (DISC) was a multicenter, randomized controlled clinical trial that evaluated the effect of a reduced fat dietary intervention during puberty on serum sex hormones in 301 girls who were healthy 8-10 year olds at randomization. After 5 years of participation, girls in the intervention group had significantly 30% lower estradiol and non-SHBG bound estradiol, 21% lower estrone, and 29% lower estrone sulfate levels during the follicular phase of their menstrual cycles compared to girls in the usual care group. After 7 years, differences in estrogens were no longer apparent, but girls in the intervention group had significantly 53% lower luteal phase progesterone levels compared to girls in the usual care group. These findings suggest that the DISC intervention altered function of the hypothalamic-pituitary-ovarian (HPO) axis. Although it is currently unknown whether these changes will ultimately influence participants' risk of developing breast cancer as adults, estradiol and progesterone are both breast mitogens that regulate breast development during puberty. We will evaluate in DISC participants, who are now in their twenties, the effect of the DISC intervention during puberty on biomarkers strongly associated with breast cancer

risk including serum hormone levels, breast density, and bone mineral density. This unique opportunity will greatly improve our understanding of breast development and possibly the origins of breast cancer.

2.1 Background

2.1.1 Diet, Hormones, and Breast Cancer

Animal models, international comparisons, and migrant studies strongly support a role of dietary fat in the etiology of breast cancer. Animals whose diets contain a higher percent of fat develop significantly more tumors [2]. International comparisons of breast cancer mortality rates suggest a strong positive association with per capita fat intake (r=0.72) [3]. When analyzed by fat source, a strong positive association is observed for animal fat (r=0.76) but not vegetable fat (r=0.18). Breast cancer incidence rates in Asian Americans born in Asia and the US are, respectively, 50% and 75% the rates of US-born Caucasians and twice the rate of women living in Asia [4]. The change in breast cancer incidence rates among Asian immigrants to the US could be related to the change from a typical Asian diet, low in fat and calories, to a typical US diet, high in fat and calories.

In contrast to animal and ecologic studies, results of observational epidemiological studies that have evaluated the role for dietary fat in breast cancer etiology are equivocal. In a combined analysis of 12 case-control studies, the relative risk for breast cancer for postmenopausal women in the highest vs. lowest quintile of saturated fat intake was 1.47 and statistically significant [5]. However, the association was no longer significant after adjusting for energy intake. Because of potential for recall bias in case-control studies, prospective studies are better suited to evaluating diet-cancer hypotheses [6]. In a pooled analysis of 7 prospective studies that included almost 5,000 cases, relative risks of breast cancer for women in the highest quintiles of energy-adjusted total fat and saturated fat were 1.05 and 1.07, respectively, and the trends were not statistically significant [7]. Two explanations have been proposed to explain discrepant findings on the association of dietary fat with breast cancer. One reasons that the variation in fat intake within populations is not large enough to observe significant associations with breast cancer. The other speculates that diet during childhood and adolescence, rather than adult diet, is related to breast cancer risk. The Women's Health Initiative clinical trial is evaluating the first hypothesis. Our past and proposed research in the DISC clinical trial is aimed at evaluating the second hypothesis.

Women with elevated serum estrogen and androgens are more likely to develop breast cancer [8-15]. In a pooled analysis of prospective studies of postmenopausal women [16], a doubling of serum estradiol concentration was associated with a significant 31% increase in risk. Although results were based on hormone measurements in a blood sample collected at a single time-point, the intraclass correlation coefficient of estradiol measurements in blood samples collected over a 3-year period was .68, suggesting that a single measurement in postmenopausal women can reliably categorize women, at least over the short term [14]. Although fewer studies have prospectively evaluated the associations of serum sex hormones with breast cancer development in premenopausal women, results are generally supportive of a positive association with estradiol and particularly bioavailable estradiol [13,17,18]. Luteal phase progesterone levels were also elevated in premenopausal women who developed breast cancer in 1 of 2 prospective studies, but the sample size was small and the association was not significant [13,19].

In premenopausal women, plasma estradiol and estrone are positively correlated with daily intake of total fat and saturated fat and inversely correlated with the ratio of polyunsaturated-to-saturated fat in the diet [8,20-22]. Wu [23] conducted a meta-analysis of 13 intervention studies that evaluated the association of dietary fat with serum estrogens. Although there was considerable heterogeneity among the studies, in combined analyses, with a 10%-25% reduction in percent of calories from fat, serum estradiol decreased significantly by 7.4% and 23% in premenopausal and postmenopausal women, respectively. Few studies before DISC have evaluated the association of adolescent diet with serum hormones. In contrast to our findings from DISC, Persky [24] reported that vegetarian girls who consumed less fat than non-vegetarian girls had significantly higher follicular phase estradiol levels. Dissimilarity in fat intake, populations studied, and study designs could have

contributed to the disparate results. No difference in hormone concentrations was reported in a second study that compared vegetarian and non-vegetarian girls, but fat intakes between the two groups also did not differ [25]. Thai girls have lower progesterone levels than British girls, which could potentially be related to dietary differences [26].

2.1.2 Adolescent Exposures and Breast Cancer

Breast development occurs primarily during puberty and is not complete until the first pregnancy. In the rat, susceptibility to mammary carcinogenesis depends on age and the corresponding stage of mammary gland development [27]. Administration of a carcinogen at puberty induces the greatest number of intraductal tumors. Once differentiation is complete following a pregnancy, tumor incidence induced by the same carcinogen dose is decreased by more than 50%.

The increased breast cancer incidence among women exposed to radiation at a young age for treatment of tuberculosis, thymic enlargement, and other health conditions and among survivors of the atomic bomb during World War II provide convincing evidence that exposures during childhood and adolescence are also related to breast cancer development in humans [28]. Breast cancer risk varies directly with radiation dose and inversely with age at time of exposure; in the cohort of atomic bomb survivors, relative risks of breast cancer for women who were less than 20, 20-39, and 40+ years old at the time of the bomb were 3.5, 2.5 and 1.1, respectively [29].

Because adolescence is a time of rapid growth and maturation of the breasts, a woman's diet as an adolescent may affect her risk of developing breast cancer. Mice fed a low fat/low calorie diet beginning at or before puberty have a longer mammary tumor latency and a lower mammary tumor incidence and multiplicity than mice fed a low fat/low calorie diet beginning after puberty that is related to longer exposure to the diet [30]. In recent analyses from the Nurses' Health Study, adolescent diet was significantly related to risk of benign breast disease and breast cancer. Adolescent energy intake was significantly positively associated with breast cancer risk [31], whereas vegetable fat and vitamin E were significantly inversely associated with risk of benign breast disease and breast cancer [31,32]. However, findings from studies in humans on the association between adolescent diet and breast cancer are inconsistent [33-35], possibly because these studies rely on recall of diet in the distant past, which may cause some individuals to be misclassified, leading to biased results. In the prospective Boyd Orr cohort study, each 1 MJ/day (239 calories) increase in energy intake during childhood was associated with a 10% increase in breast cancer mortality [36]. Although the association was not statistically significant, with only 26 breast cancer deaths, the study lacked power to detect small differences.

Difficulties retrospectively assessing childhood and adolescent diet have led researchers to evaluate relationships of surrogate markers of childhood and adolescent diet with breast cancer risk. Early age at menarche is an established risk factor for breast cancer, and diet is associated with the timing of menses onset [37-40]. Furthermore, adult height is influenced by childhood diet and is positively associated with breast cancer risk. Adolescent height is also positively associated with breast cancer risk. Using record linkage in Finland, Hilakivi-Clarke [41] found that girls who were taller at each age from 7-15 years were at a significantly increased risk of developing breast cancer as adults, with a relative risk of 1.9 for girls in the highest quintile. Herrinton [42] also found a significant positive association between height at 15-18 years of age and risk of breast cancer in an analysis of Kaiser Permanente medical records, but height at 9-11 years of age was not related to risk. In the Nurse's Health Study, high peak height velocity during adolescence was related to a 30%-40% increased risk of breast cancer [43].

Because of the associations of childhood obesity with early puberty and early puberty with breast cancer, it has been hypothesized that women who were heavier as children would be at an increased risk of breast cancer [7,44,45]. However, two record linkage studies suggest that childhood and adolescent weight is inversely related to breast cancer risk [41,46], and in 2 prospective cohort studies, BMI at 18 years of age was inversely related to breast cancer risk [47,48]. The association of adiposity with breast cancer is exceedingly complex. Adult obesity is associated with a decreased risk of breast cancer before menopause, possibly due to an increased frequency of anovulatory menstrual cycles in obese women that result in lower serum estrogens and progesterone [49].

Conversely, adult obesity is associated with an increased risk of breast cancer after menopause when aromatization of androgens in adipose tissue is the major source of circulating estrogens [50]. Weight gain after 18 is positively associated with postmenopausal breast cancer risk independent of adult weight, and part of the increased risk of postmenopausal breast cancer among women who were leaner at a young age could be due to greater weight gain by these women [48]. In the prospective Malmo Diet and Cancer Study, each 10 kg weight gain since age 20 was associated with a 16% increase in postmenopausal breast cancer risk [51]. Similarly, weight gain from age 18 was significantly associated with postmenopausal breast cancer in the Women's Health Initiative [52].

2.1.3 Hypothalamic Pituitary Ovarian (HPO) Axis Programming

'Programming' is the process by which a stimulus or insult at a crucial, sensitive period of early life results in permanent effects on structure, physiology or metabolism [53]. Fetal life and puberty are critical times for maturation of the HPO axis (figure 1)[54], which via complex feedback loops maintains ovarian hormone concentrations in the normal range (figure 2). In-utero programming of the HPO by testosterone results in sex differences in the pattern of ovarian and testicular steroid

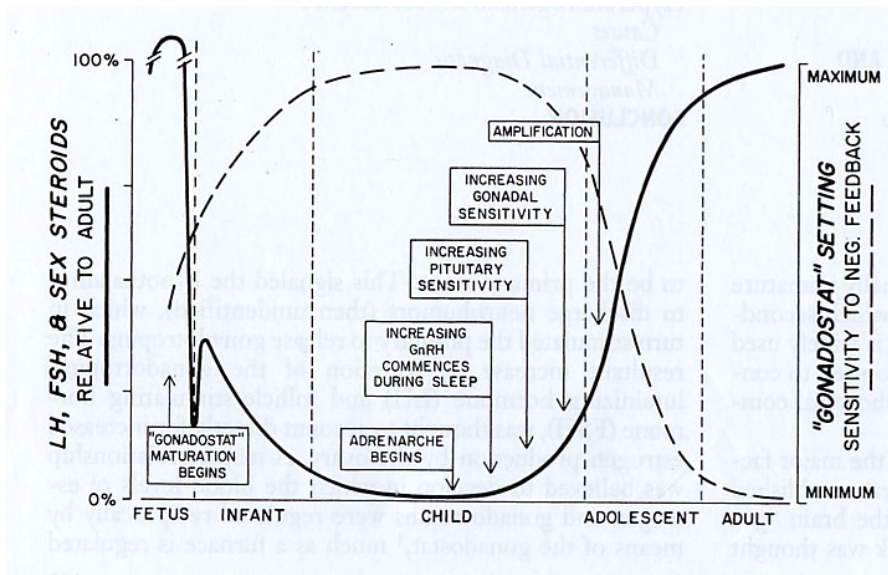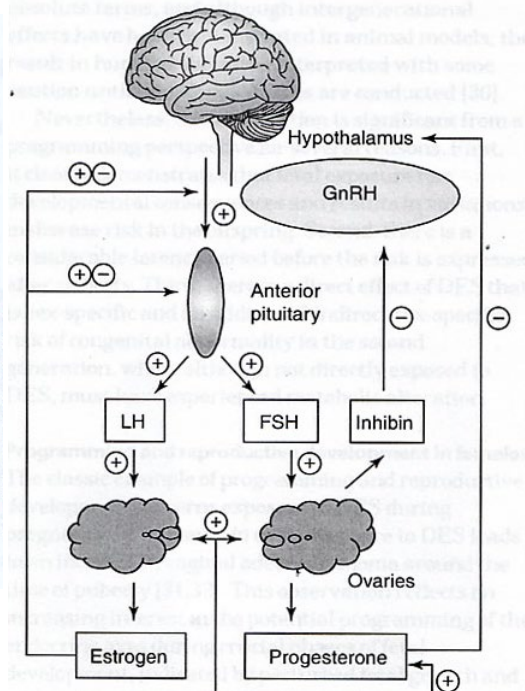**Figure 1. HPO Axis Development [53]**

Figure 2. HPO Axis Regulation

production [55]. Less is known about programming of the HPO during puberty, but treatment with an androgen receptor antagonist during puberty has been proposed to prevent the development of polycystic ovary syndrome (PCOS), a syndrome characterized by dysregulation of the GnRH pulse generator resulting in hyperandrogenemia and anovulation in adults [56]. Furthermore, valproate, a common anti-seizure medication, delays pubertal maturation, and when administered to pubertal but not adult mice delays GnRH cell maturation via effects on γ-aminobutyric acid (GABA) [57]. Progesterone also acts via GABA to suppress the GnRH pulse generator and modulate LH and FSH secretion [58]. We observed significantly lower serum progesterone concentrations in DISC intervention girls at their last visits, when dietary differences were no longer apparent and hypothesize that the DISC intervention during puberty may have differentially programmed the HPO axis during this critical time.

2.1.4 Bone Mineral Density

Estrogens are important determinants of bone mineral density, and several investigators have proposed using bone mineral density as an integrated marker of a woman's exposure to estrogens over time [59-61]. In prospective studies, postmenopausal women with high bone mineral density are significantly 2 – 3 times more likely to develop breast cancer compared to those with low bone mineral density [62-64]. Peak bone mass, which is achieved by 29 years of age, is one of the strongest predictors of bone density after menopause [65]. At least 90% of total adult bone calcium is acquired by the end of adolescence and approximately 60% is acquired during adolescence [66]. Heredity accounts for 60%-70% of variation in bone density with diet and other environmental factors accounting for 30% to 40% of this variation [67]. Because serum estrogen levels during adolescence are an important determinant of peak bone density and bone density later in life [68], associations of bone density with breast cancer in older women could be related to dietary and hormonal influences not only after menopause but also during adolescence. Because girls in the DISC intervention group had lower serum estrogens during a critical period for bone calcium deposition, we predict that they will have lower bone densities as adults. Beneficial effects of estrogens for bone health but detrimental effects for breast cancer are well established.

2.1.5 Breast Density

Unlike most organs, most breast development occurs after birth (Figure 3). A small network of ducts formed in-utero remains quiescent until puberty when these ducts grow out from the nipple into the mammary fat pad [69]. Under the influence of estrogens, the ducts elongate and bifurcate until

Figure 3. Mammary Gland Development [70]
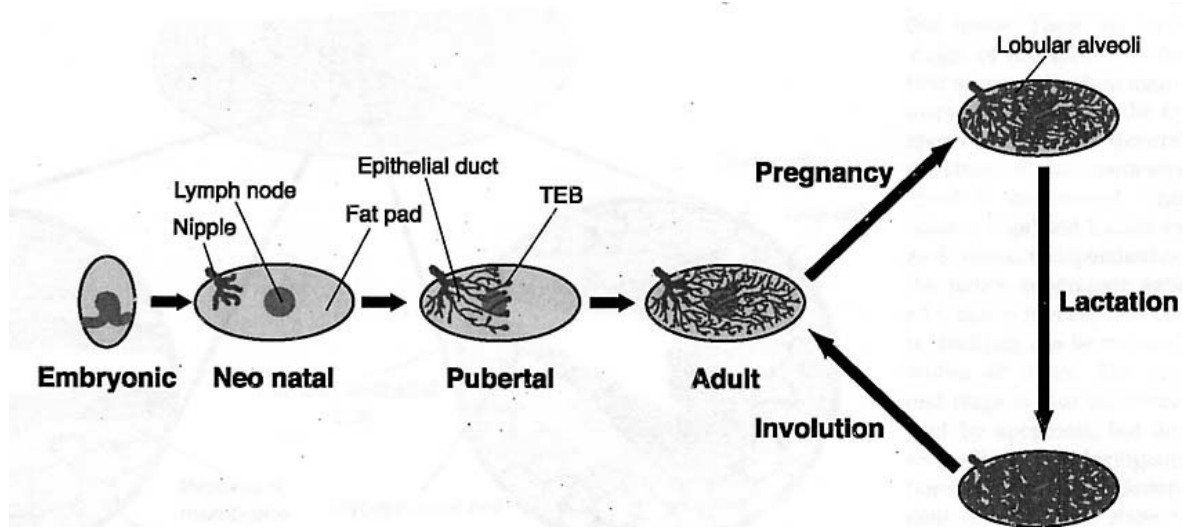
they reach the edges of the fat pad. During the luteal phase of the menstrual cycle and pregnancy, growth of side branches from existing mammary ducts occurs under the influence of estrogens and progesterone. Estrogens induce expression of the progesterone receptor in the mammary epithelium, and progesterone binds to its receptor to induce side branching of the mammary ducts by paracrine mechanisms [71]. Final development of the mammary gland occurs following pregnancy and lactation when reproductive hormones induce terminal differentiation of mammary lobular alveoli [72]. Interestingly, following completion of pregnancy and lactation the mammary gland involutes to its pre-pregnant state. Throughout this process there is cross talk between the mammary stroma and epithelium, and multiple proteins and growth factors contribute to regulation of breast duct formation and involution [70].

Given the central role of estrogens and progesterone in regulating the elongation and branching of mammary ducts, lower estrogens and progesterone in girls in the DISC intervention group during puberty when the ductal architecture of the breast is laid down could have long term

effects on breast cancer risk. In particular, we hypothesize that the intervention group will have less dense breasts as a consequence of less branching due to lower progesterone levels at this critical time in breast development. Breast density is one of the strongest risk factor for breast cancer. Women with dense breasts on mammography are significantly 4-6 times more likely to develop breast cancer [73-77]. Only age and BRCA1/2 mutations are stronger predictors of risk. Although it has been suggested that the association of breast density with breast cancer is an artifact created by the difficulty in detecting tumors in dense tissue, in prospective cohort studies the increased risk of breast cancer in women with dense breasts persists for 5-10 years, suggesting that the association is not due to detection bias [76,77].

Only one study has evaluated the association of estradiol and progesterone with breast density and no association was observed [78]. However, day of the menstrual cycle was not taken into account in premenopausal women. In postmenopausal women, estradiol is frequently close to or below the assay limit of detection and progesterone is almost always below the limit of detection. Both the area and percent of breast tissue that is dense decrease with the decline of serum estrogens and progesterone after menopause [78]. Initiation of HRT use increases breast density and discontinuation of HRT decreases breast density [79,80]. Increases in breast density are more pronounced in women using combined estrogen plus progestin regimens compared to women using estrogen alone [79,81]. Tamoxifen blocks the estrogen receptor and decreases breast density [82] and breast cancer risk [83].

Although genetic factors are estimated to account for 63% of variation in breast density [84], environmental factors also are important. Cross-sectional studies of dietary fat intake and breast density have yielded inconsistent results [73,85-87]. Boyd [88] evaluated the effect of a low-fat diet in perimenopausal women on breast density in a randomized controlled clinical trial. The decrease in area of density during 2 years was significantly 3 times greater in the intervention group compared to the control group. Furthermore, among women who experienced menopause during the follow-up, percentage of density decreased significantly twice as much in the intervention group compared to the control group [87]. After adjusting for energy intake, decreases in saturated fat and cholesterol intake were significantly associated with reduction in percent density.

2.2 SUMMARY OF PRECLINICAL AND CLINICAL DATA TO BE COLLECTED

All data collection for an individual participant will be conducted on a single day. It is anticipated that the visit will take several hours to complete. Participants will be asked to fast for 12 hours before coming to the clinic. After a urine pregnancy test and venipuncture, the participant will be weighed and her height will be measured. She will be fed a standard snack, complete questionnaires, bone density will be measured by DEXA, breast density will be measured by MRI, and an in-person 24-hour dietary recall will be conducted. Two additional 24-hour dietary recalls will be conducted by telephone in the following 2 weeks. Trained and certified individuals who are masked to treatment assignment will perform all data collection.

Data to be collected at the follow-up visit includes the following: demographics (education/occupation, marital status, income, ethnicity), medical history (medications including oral contraceptives, serious or chronic illnesses, hospitalizations,), reproductive and menstrual histories (cycle regularity, usual cycle length, pregnancy, lactation); anthropometric measurements (height, weight), dietary assessment (three 24-hour recalls), physical activity assessment, serum hormones (progesterone, estradiol), bone mineral density, breast density, family history of cancer.

3.0 OBJECTIVES

3.1 Overall Hypothesis

We hypothesize that the DISC intervention to lower total fat and saturated fat intake during puberty will have long-term effects on serum sex hormones, bone mineral density, and breast density, characteristics that are strongly associated with risk of breast cancer development.

3.2 Primary Specific Aim

1. Determine the long-term effect of the DISC intervention on serum progesterone levels in early adulthood.
   Hypothesis: Luteal phase serum levels of progesterone in the DISC intervention group are significantly lower than in the usual care (control) group at 25-29 years of age.

3.3 Secondary Specific Aims

2. Determine the long-term effect of the DISC intervention on serum estradiol levels in early adulthood.
   Hypothesis: Luteal phase serum levels of estradiol in the DISC intervention group are significantly lower than in the usual care (control) group at 25-29 years of age.

3. Determine the long-term effect of the DISC intervention on bone mineral density in early adulthood.
   Hypothesis: Bone mineral density is significantly lower in the DISC intervention group than in the usual care (control) group at 25-29 years of age.

4. Determine the long-term effect of the DISC intervention on breast density in early adulthood.
   Hypothesis: Breast density in the DISC intervention group is significantly lower than in the usual care (control) group at 25-29 years of age.

This study focuses on the effect of the DISC intervention on the intermediate biomarkers most strongly associated with breast cancer risk - serum sex hormones, bone mineral density, and breast density. Blood for measurement of additional biomarkers and DNA will be collected and stored for future use.

4.0 SELECTION OF STUDY SUBJECTS

Participants for this study will include the 301 female participants in DISC.

5.0 REGISTRATION PROCEDURES

We will conduct a follow-up visit with 301 female participants in DISC, which will involve locating them, inviting them to participate, scheduling the visit to occur during the luteal phase of their menstrual cycles, and obtaining informed consent prior to conducting the visit.

5.1 Locating Participants

Retention in DISC was excellent; 89.4% of girls completed last visits, which were conducted on average 7 years after randomization. Extraordinary efforts will be undertaken to see all participants.

Because the participants were minors when they were active in DISC, informed consent was provided by the parents/guardians, and new HIPAA regulations may require that we initially contact participants through their parents/guardians or some intermediary (e.g. physician, school). Each center will follow the guidance provided by their IRB for contacting participants. In general, a letter will be sent to the participants' parents last known address informing them about the plans for a follow-up DISC visit, what would be involved, and asking them to have their daughter contact us. The envelope will be marked 'Forwarding Address Requested', so that the post office will provide us new addresses for individuals who have moved. A telephone call will be made to the last known telephone number within 2 weeks of mailing the letter. Whenever possible, a DISC staff member who knew the family will make the call. Because DISC was a family intervention and parents know the DISC staff, we are confident that parents will assist us in contacting their daughters.

Participants not found using the above procedure will be traced using several sources: 1) the two friends/relatives identified during the DISC trial as individuals who did not live in the household but would know where to locate the family will be contacted; 2) parents will be contacted at their place of

employment; 3) post offices will be asked to provide forwarding addresses; 4) participants' personal physicians will be contacted; 5) local telephone books will be searched; 6) internet based telephone, residential address and email address websites will be searched. Additionally, in some cases, reverse (criss-cross) directories may be used to obtain telephone numbers of neighbors to contact. The only information that will be disclosed to neighbors in this case is the participant's name, that she was a prior neighbor, and that we are trying to locate her because she previously participated in a research study of diet in healthy people and we would like to invite her to participate in another research study.

For participants who cannot be found locally, identifiers including names, last known address and telephone number, and contact information will be forwarded to a commercial search firm.

5.2 Scheduling Clinic Visits

All clinic visits will take place during the luteal phase of participants' menstrual cycles. Although follicular phase estradiol levels in the intervention group were significantly 28% lower than the usual care group at Year-5 visits, we did not observe any significant differences in estrogens or androgens in the follicular phase of the menstrual cycle at last visits. However, at their last DISC visits, the intervention group had significantly 53% lower serum progesterone levels during the luteal phase of their menstrual cycles compared to the usual care group. To maximize power to observe differences in progesterone and possibly estrogens at the proposed follow-up visit, all visits will be scheduled to take place during the luteal phase of the menstrual cycle.

Because time from ovulation to onset of next menses is more constant than time from previous menses to ovulation, we will time visits in relation to expected date of start of next menses. The participant in consultation with clinic staff will select a target date for the clinic visit based on when she usually gets her period. When the participant starts her last period expected before the target date, she will call the clinic. Clinic personnel will record the date the participant started her last period and then schedule the visit to occur 3-4 days ($\pm$ 2 day window) before her anticipated start of next menses based on date of start of last period and usual cycle length. We also considered timing the clinic visit in relationship to ovulation by asking participants to use urine ovulation kits. However, participants would have to come to the clinic in about a week after ovulating, which would give them little time for planning to take time off from school or work and in some cases fly to the clinical center. Additionally, using this approach we would include only ovulatory cycles, which would remove a source of variation in the hormone data.

6.0 STUDY DESIGN

A follow up study of women who participated in DISC during adolescence will be conducted.
- Number of groups: 2
- The sequencing of treatment: None
- Method of subject allocation: Random
- Type of control: Usual care
- Blinding procedure: All data collection personnel will be blinded to treatment group.
- The essential characteristics of the subject population: Females currently in their 20s who previously participated in the DISC study as adolescents
- The duration of exposure to individual subjects: Average 7 years in DISC trial. No additional interventions in current study.
- The total expected duration of the study: 4 years.
- The drug dosage/device usage schedule: The DISC diet was a balanced food pattern that achieved the following goals: limit intake of total fat to $\leq$28% calories, with <8% of calories from saturated fat, $\leq$9% of calories from polyunsaturated fat and the remainder from monounsaturated fat, limit cholesterol intake to 75mg/1000 calories not to exceed 150 mg/day, maintain protein intake at 14% calories (2/3 animal protein, 1/3 vegetable protein), and maintain carbohydrate intake at 58% calories.

7.0 <u>MEASUREMENT OF EFFECT</u>

Intervention and usual care group participants' serum progesterone and estradiol, bone mineral density and breast density will be compared as described under statistical considerations. Data on these endpoints and potentially confounding variables will be collected at a clinic visit. This section provides an overview of the clinic visit and a description of data collection procedures.

7.1 <u>Clinic Visit</u>

All data collection for an individual participant will be conducted on a single day. It is anticipated that the visit will take several hours to complete. Participants will be asked to fast for 12 hours before coming to the clinic. After a urine pregnancy test and venipuncture, the participant will be weighed and her height will be measured. She will be fed a standard meal, complete questionnaires, bone density will be measured by DEXA, breast density will be measured by MRI, and an in-person 24-hour dietary recall will be conducted. Two additional 24-hour dietary recalls will be conducted by telephone in the following 2 weeks. Trained and certified individuals who are masked to treatment assignment will perform all data collection.

We will pay transportation costs for participants who live out of town. For participants who have to fly, one night lodging will be provided. Participants who have moved and live closer to a different DISC clinical center will have the option of going to that center for evaluation. Participants who attend the clinic visit be reimbursed $500 for participating in this very involved study.

7.2 <u>Anthropometry</u>

Height will be measured by a stadiometer and weight will be measured by an electronic scale. Participants will be clothed in a hospital gown without shoes. Consistent with earlier DISC measurement acceptability criteria, if height measurements do not agree within .5 cm or if weight measurements do not agree within .2 kg, measurements will be performed a third time and the two closest values will be averaged.

7.3 <u>Dietary Assessment</u>

Three 24-hour dietary recalls will be collected within 2 weeks of the clinic visit by Nutrition Coordinating Center certified nutritionists using Nutrition Data System for Research (NDS-R). This is a Window's based direct data entry system that prompts the nutritionist performing the interview to ask appropriate questions regarding foods. For example, the nutritionist is prompted to ask the participant about the percent fat in milk consumed and serving size. This state of the art system calculates nutrient composition and is used extensively in nutrition research. The system also has the capability to calculate servings of subcategories of fruits, vegetables and sweetened beverages. Efforts to complete other categories are underway and are expected to be ready for use in the DISC follow-up analyses. Two recalls will be conducted on weekdays and one will be conducted on a weekend day. The first recall will be a face-to-face recall with the nutritionist. The second and third recalls will be by telephone. Two dimensional food models will be used to help participants estimate serving sizes. These procedures are the same as were used previously in DISC and participants will be familiar with the approach. A 20% sample of recalls will be randomly selected for evaluation at the Nutrition Coordinating Center for quality assurance.

7.4 <u>Physical Activity</u>

Physical activity will be measured using the Modifiable Activity Questionnaire developed by Dr. A. Kriska [126]. The questionnaire captures leisure time and occupational/school physical activity, is age appropriate and has excellent test-retest reliability (r=.92 for individuals 21-36 years of age) [126]. It has been validated against Caltrac activity monitor (r=.6-.8) [126] and doubly labeled water (r=.7)

[127]. A copy is included in Attachment B. We also will repeat the questions about usual physical activity used in the original DISC study for comparison (Attachment B).

7.5 Menses Dates

Participants will report date of last menses and record date of start of next menses after the clinic visit on a postcard and return it to the clinic. Because data on start of next menses will be important for interpreting serum hormone and breast MRI data, if a postcard is not received within 5 days of estimated start of next menses, clinic staff will call the participant to see if she started her period. If she has, the date will be recorded. If she has not, clinic staff will call her each week until she has her period.

7.6 Psychosocial Assessments

Because affective disorders can lead to alterations in HPO axis functioning [128-130], we will collect limited data on these characteristics to evaluate for potential confounding. Depression will be measured using the Center for Epidemiological Studies-Depression scale (CES-D) [131]. To reduce participant burden, we will use the 11-item short form [132]. The short-form reproduces the same four factor structure as the original CES-D (i.e., depressed affect, positive affect, somatic complaints, and interpersonal problems), has high reliability, and is strongly correlated with the original full scale. General levels of anxiety will be measured using the 20-item trait anxiety scale of the Spielberger State-Trait Anxiety Inventory (STAI) [133], which has been shown to have high internal consistency and is well correlated with other dispositional measures of anxiety. The STAI has proven to be effective in screening college students for anxiety problems and has been widely used with a variety of populations (e.g., normal, healthy adults; medical patients).

7.7 DISC Medical History Questionnaire

A DISC Medical History questionnaire (Appendix B) will be used to collect updated information on demographic characteristics (education, occupation, marital status, income, ethnicity); medical history (serious or chronic illnesses, hospitalizations,); medications (current medications and detailed current and past use of hormonal contraceptives including brand names, age started and duration of use); menstrual history (age at menarche (a few participants had not reached menarche at last visits), cycle regularity and length); reproductive history (number and dates of pregnancies and births; number, dates and duration of lactation); dietary supplements (supplements with hormonal properties, vitamins and minerals including calcium); alcohol use (frequency and amount, binge drinking); tobacco use (current, past, cigarettes/day); family history of cancer (update information on cancer among first degree relatives).

7.8 Blood Sample Collection and Processing

A total of 55 ml of blood will be collected by venipuncture using standard procedures with the participant in the supine position. Three 15ml red top serum separator tubes for hormones and storage and one 10 ml yellow top ACD tube for DNA extraction will be collected from each participant. Serum and lymphocytes will be separated and aliquoted at the clinical centers and stored at -80°C until shipped to the Fox Chase Cancer Center (FCCC) Repository. Shipments to FCCC will be made monthly by overnight Fed-Ex with samples packaged in dry ice. Each participant's specimens will be divided in half and sent to FCCC in 2 separate shipments so that if there is an accidental thaw, only half of samples are affected. Serum for hormone assays will be stored at FCCC at -80°C until shipped to the Central Hormone Laboratory for analysis at the end of data collection. Additional serum will be stored for future assays. High molecular weight DNA will be extracted from lymphocytes using standard phenol/chloroform techniques and stored at 4°C for future use.

7.9 Serum Hormones

All hormone assays will be conducted under the direction of Dr. Frank Z. Stanczyk at the Reproductive Endocrine Research Laboratory, Department of Obstetrics and Gynecology, University

of Southern California Keck School of Medicine. Dr. Stanczyk has been supervisor and director of this laboratory since 1972. The laboratory has extensive experience in measuring steroid, peptide and protein hormones using well-validated methodology that has been published. All hormone assays will be performed at the end of data collection. Serum samples for hormone assays will be batched at FCCC; batches will be balanced on treatment group. Masked quality control samples indistinguishable from participant samples will be included in batches. Samples will be shipped to the laboratory on dry ice. The hormones will be analyzed by specific immunoassays as described below.

7.9.1 Progesterone

Progesterone will be quantified by radioimmunoassay (RIA) following its extraction with ethyl acetate:hexane (2:3) and Celite column partition chromatography [136]. Approximately 1000 d.p.m. of ^{3}H-progesterone will first be added to each serum sample to follow procedural loss. The RIA of purified progesterone will use iodinated progesterone in conjunction with a specific antiserum against progesterone. After an appropriate incubation period, a second antibody is used to separate antibody-bound progesterone from unbound progesterone. The resulting progesterone values will be corrected for procedural loss. Sensitivity of the assay is 0.2 ng/ml; intraassay and interassay coefficients of variation (CVs) are 8% and 9%, respectively.

7.9.2 Estradiol

Estradiol will be measured by RIA following extraction and Celite column partition chromatography [137]. The extraction step will utilize ethyl acetate:hexane (2:3). Following evaporation of the organic solvents, the extract will be applied on a column of Celite impregnated with ethylene glycol. Estradiol will be separated by elution with 15% ethyl acetate in isooctane and 40% ethyl acetate in isooctane, respectively. After evaporating the eluates, the residues will be redissolved in assay buffer, and appropriate aliquots will be taken for RIA. Each RIA uses a highly specific antiserum in conjunction with an iodinated radioligand. Following an appropriate incubation period, antibody-bound and unbound estradiol is separated using a second antibody. The antibody-bound fraction is then counted after centrifugation. Sensitivity of the estradiol assay is 5 pg/ml; intraassay and interassay CVs are 9.3% and 11.4%, respectively.

7.10 Breast MRI

While the majority of studies showing an association between breast density and breast cancer risk are based on mammographic measurements, mammography is generally not recommended for young women because of its reduced effectiveness of cancer detection in dense breast tissue, and the radiation exposure, which is quite low but cumulative. In this study of young women, we propose to use MRI to measure breast density. MRI is not impaired by high parenchymal breast density, making it especially effective for younger women with dense breast tissue. MRI can easily distinguish fibroglandular and fatty breast tissue and gives three-dimensional information not provided by mammography.   Additionally, MRI does not use ionizing radiation.   In studies using MRI to stage the extent of carcinoma of the breast, MRI has demonstrated significantly greater accuracy than mammography in defining tumor size when compared to pathology [140-143].

7.10.1 MRI Procedure

Each subject will be imaged in a whole body 1.0 Tesla or higher field strength MRI scanner using a dedicated breast imaging radiofrequency coil. The subject will lie prone on the table with both breasts hanging freely in the coil. The following pulse sequences will be performed in the transaxial orientation with a 32-40 cm field-of-view for bilateral coverage:

1. 2D or 3D spin echo or gradient echo localization sequence; 32-40 cm field of view (FOV), 5 cm slice thickness, number of locations to cover both breasts in I/S direction.

2. <u>2D T2-weighted fast spin echo sequence</u>; TR/TE = 5500 ms/85 ms, 24 slices, 3-4 mm slice thickness to cover both breasts in I/S direction, echo train length 8, 32-40 cm field of view (FOV), 512x256 matrix, Frequency A/P.
3. <u>3D fast gradient echo sequence without fat-saturation</u>; TR/TE = 20 ms/minimum TE with Fat/Water in phase, 30 degree flip angle, FOV 32-40 cm, 60 slices, 1-2 mm slice thickness to cover entire breast in I/S direction, 512-256 matrix, frequency A/P.
4. <u>3D fast gradient echo sequence with fat-saturation</u>; Repeat #3 with chemical saturation fat-suppression.

No contrast agent will be administered for this examination. Additional pulse sequences including diffusion-weighted and 3D steady state free procession sequences may be acquired depending on availability of these techniques on the MRI systems at the participating sites. The total exam time, including patient positioning will be less than 1 hour. Subjects are given the opportunity to lie in the magnet before scanning begins. Those subjects experiencing claustrophobia are counseled by a technologist or physician and will not be pressured to finish the exam. Earplugs are provided to each subject to reduce noise levels.

All MRI exams will be performed during the luteal phase of the menstrual cycle. Although diagnostic breast MRI is usually scheduled in the follicular phase of the menstrual cycle because it is easier to detect tumors, we will be using MRI to measure physiological characteristics of the breast, not to detect tumors or other abnormalities. Because non-contrast MRI scans to be used are not diagnostic, we do not anticipate detecting abnormalities. However, if abnormality is detected, the participant will be informed and the results will be sent in writing by the clinical center PI to the physician identified by the participant on the consent form.

7.10.2 Reproducibility of measurement of breast tissue total volume
In order to quantify the variability in breast volume measurement due to patient re-positioning, two normal volunteers, one with low and one with high mammographic breast density, were each scanned twice on the same day. Two different users delineated breast tissue regions as described above and the overall volume of fibroglandular tissue was calculated on the whole MR data set. The intra-user variability with patient repositioning was 2% for the dense breast and 8% for the fatty breast. The inter-user variability for the dense breast was 7% and for the fatty breast was 18.3%. The subjects in the DISC study will each receive only 1 MRI exam and therefore variability due to repositioning will not be a factor. Because of the young age of the study population, we anticipate that the majority of subjects will have dense breast tissue, the category in which the best reproducibility was measured. The same investigator will analyze all breast MRI studies.

7.10.3 Image Transfer
Data will be archived with patient identifying information replaced by study ID number. Image data will be sent by optical disk to University of California at San Francisco (UCSF) for breast density measurements.

7.10.4 Image Analysis
Image data will be processed at UCSF using customized image processing software to identify the chest wall/breast tissue boundary and skin surface, and to separate breast fibroglandular and fatty tissue. Total volumes of fibroglandular and fatty tissue will be computed separately for each breast. In problematic cases such as those with incomplete or failed fat-saturation, manual delineation will be used.

7.10.5 Quality Assurance Program
Prior to the start of subject enrollment, a training session for all site MRI technologists will be conducted at UCSF. Hands-on instruction will be provided to address patient positioning, breast positioning in coil, scan prescription, and data acquisition. In particular, technologists will be trained to recognize and correct failures due to incomplete fat-suppression, motion artifact, and inadequate

breast coverage. Each site will be provided with 2 breast tissue mimicking phantoms consisting of doped-water and oil compartments that will be used to periodically test bilateral breast MR imaging sequences at each site. In addition, before any participant exams, breast MRI data from 5 volunteers will be acquired from each site and sent to UCSF for review. Image quality will be evaluated and acceptability will be required for site certification.

7.11 Bone Densitometry

We will measure bone density using dual x-ray absorptiometry (DEXA). The entire DEXA examination is very low dose radiation and typically less than the daily effective dose from natural sources (cosmic radiation, environmental isotopes in our bodies and surroundings). When compared to other radiological procedures, this protocol is approximately 10 times less radiation than a chest x-ray and 10 times less radiation than received when flying round trip coast-to-coast.

7.11.1 Scan Protocol

The participant will be asked to disrobe and wear a hospital gown. (Buttons, heavy fabrics, bra clasp, etc., can all cause inaccurate results.) They will then lie down on the table of the DEXA device. This is typically a simple padded tabletop. The x-ray arm will make several passes over the patient for each scan mode. The following scans will be acquired:

1. Lumbar Spine: A/P or P/A view, default scan speed, L1 – L4.
2. Proximal Femur: A/P or P/A view, default scan speed.
3. Whole body: default scan speed

The scan combination of dedicated proximal femur, lumbar spine (L1-L4), and whole body DEXA scans is important for this study for several reasons. Dedicated scans of the lumbar spine (L1-L4) and of the proximal femur are the most widely used combination for diagnosing osteoporosis and assessing fracturing risk since fractures at these sites (1) are best predicted by site-specific scans, and (2) these fractures have the highest morbidity. Bone density measures at these two sites will allow for direct comparison of these study results to most of the osteoporosis literature. The spine contains a high percentage of trabecular, or spongy, high turnover bone (66%) while the proximal femur contains more of a mix of trabecular and cortical (compact) bone. The whole body is comprised of 80% cortical bone. It is a measure of systemic bone health and responds slower to changes in bone turnover. Thus, it may contain more "memory" of the intervention than the localized spine and femur sites. The entire examination should take less than 30 minutes. Any of the currently manufactured DEXA devices from Hologic, Inc., or GE/Lunar are appropriate.

7.11.2 Reproducibility of Bone Density Measurements

DEXA provides precise composition analysis with a low radiation exposure (< 0.1 μGy). The precision error (1 SD) for total body bone mineral density is less than 0.01 g/cm^2 and the coefficient of variation for this SD is 1.8% [140]. The same investigator will analyze all DEXA scans.

7.11.3 Image Data Transfer

Data will be archived with patient identifying information replaced by study ID number. Image data will be sent by super disk to UCSF for bone density measurements.

7.11.4 Image Analysis

Image data will be processed at UCSF using customized image processing software. Bone mineral content (BMC) and bone area (AREA) will be measured and bone mineral density (BMD) will be calculated. BMC is the bone mass in grams of the region being measured and AREA, in square centimeters, is the area projected by the bone in a 2-dimensional x-ray image. BMD is the value most reported as DEXA bone density. It is found by dividing the BMC/AREA and is in units of grams per square centimeters, or g/cm^2. Bone density as measured by DEXA techniques is an areal density, not a true physical density (mass per unit volume). This is because the DEXA measure is derived from 2-

dimensional x-ray projections. Thus, DEXA bone density is affected by bone size as well as physical density, and areal BMD will always be confounded by bone size (e.g. participant height) unless corrected. There are several techniques to accomplish this. Carter [141] derived "bone mineral apparent density" or BMAD that takes into account that the spine vertebral body is close to cylindrical in shape and this information can be used to estimate the missing dimension. It was shown that BMAD = BMC/(Bone projected area)$^{1.5}$. The UCSF investigators have also derived a whole body apparent density that effectively removes bone size from whole body BMD[142]. We will derive these apparent density variables in our analysis to reduce the data scatter caused by participant differences. Any abnormalities will be reported to the participant's physician.

In addition to bone density, the whole body scan provides body composition. DEXA is the most precise method to estimate whole body percent fat, and the CV for repeated scans is ~1% [143]. This is part of the whole body protocol and requires no additional radiation or time. Because breast density and bone density are strongly correlated to body composition, the %fat will be used in multivariate analyses of these two outcome variables.

It is not anticipated that abnormalities will be detected by DEXA. However, if abnormality is detected, the participant will be informed and the results will be sent in writing by the clinical center PI to the physician identified by the participant on the consent form.

7.11.5 Quality Assurance Program

Since the study needs to be sensitive to even small changes bone density, a comprehensive quality assurance program has been included. DEXA devices can have calibration drifts or major service issues over the course of the study. However, it is possible to correct for these calibration drifts posthoc if appropriate phantoms are scanned as part of the protocol. The phantoms of choice for this study are the device-specific spine phantoms (come with each device and different for each manufacturer) and the Hologic Whole Body phantom. These phantoms accurately predict the in vivo calibration of the device such that changes in the phantom measures are used to directly correct participant data collected during that interval. The spine phantom is typically scanned once a day as standard practices at most clinical DEXA sites. The whole body phantom is critical since previous work by the UCSF investigators has shown that the typical spine quality control program does not predict the whole body calibration. The whole body phantom would be scanned 3 times a week. The UCSF investigators will use Cumulative Sums (CUSUM) statistics to determine when a change in the calibration has occurred [144].

DEXA participant scans will be acquired at each of the 6 clinical sites. To pool scan results from different DEXA devices, static calibration differences must also be corrected. Ideally, this is done by scanning the same individuals on all devices. However, this is impractical. We will circulate a single set of calibration phantoms to all sites. This set of phantoms is more complete than the daily phantoms and made available by the UCSF investigators. Machines of the same make and model will be readily cross calibrated using this phantom data. Devices from different manufacturers will be calibrated with in vivo calibration equations from the literature. The calibration and monitoring activities will be coordinated by one of our investigators from UCSF (Shepherd) who is an expert in this area. The DEXA cross calibration protocol will take about 1 hour, and be done once during the course of the study.

To insure data accuracy and conformity to the protocol, the following training and procedures will be used. One of the UCSF investigators will travel to each site and train local DEXA personnel on the protocol including any special patient positioning, data transfer issues, and phantom scanning procedures. Each site will then recruit five volunteers who will be scanned according to the study protocol. The scans will be sent to UCSF for evaluation. The clinical center will not recruit study participants until their test data meets study standards. After site certification, participant scans will be sent to UCSF for central analysis to ensure there are no clinic specific inaccuracies due to placing regions of interest slightly differently. Participant scans that are found to be inaccurate due to artifacts, motion, or poor positioning will be rejected from the study data and coded for the type of problem found. The UCSF investigators find approximately 5% of scans received from trained clinical sites have some type of quality issue.

7.12 Training and Certification

A centralized data collection training session will be held during the first year of the grant to train and certify individuals responsible for the different types of data collection except DEXA and breast MRI. Dr. Shepherd will perform DEXA training locally at each site as described above. Breast MRI training will be performed at the Univ. of California at San Francisco to allow adequate hands on time on the magnet. A technician from each center will be sent to San Francisco for this training. Each Clinical Center will have at least one person centrally trained and certified to collect each type of data who will train others at their center locally. Requirements for certification are:

• Height, Weight	Repeat measurements within acceptable limits
• Diet Assessment	NCC Training and Certification
• Other Questionnaires	Receive passing grade on exam about procedures
• Blood Collection	Receive passing grade on exam about procedures
• Breast MRI	Completion of 5 acceptable breast MRI's on volunteers
• DEXA	Completion of 5 acceptable DEXA measurements on volunteers

8.0 STUDY PARAMETERS

- Serum hormone levels
- Breast density
- Bone mineral density

9.0 PHARMACOKINETIC STUDIES

Not applicable.

10.0 OFF-STUDY CRITERIA

Not applicable.

11.0 DRUG FORMULATION AND PROCUREMENT INFORMATION

Not applicable.

12.0 STATISTICAL CONSIDERATIONS

12.1 Data Management

All data will be processed at Maryland Medical Research Institute (MMRI), the original Data Coordinating Center (DCC) for the DISC study. MMRI currently uses a multi-access data entry system based on the Teleform® data entry software. The Teleform® software allows a clinical center to send data forms to the DCC either through a fax machine, through the mail (to be scanned at the DCC), or through a Web-based data entry system. If the forms are sent through a fax machine or through the mail for scanning, the data are read from the form using OCR/ICR software and verified. A preliminary edit is performed prior to insertion into a transaction database. A comprehensive edit of the data in the transaction database is performed and sent via e-mail to the clinical center prior to acceptance of the form into the main study database. MMRI is currently using MS Access as the main database system for studies the size of the DISC Follow-up Study with edits and other procedures written in Visual Basic. The DISC database will be housed on the main MMRI server running Windows 2000.

The preliminary edit compares the study participant ID number (using the same number as in the main DISC study) and namecode (the same as used in the main DISC study) to the main study database, which will have the original information available. If the identifying information is verified as

correct, the other data are scanned to verify that the data are within preset ranges and are of the correct type. The comprehensive edit will look for consistency in the responses on the form and for consistency across forms (and against the previous DISC data). Data entry will have quality control checks built into the system. Each month 10% of forms will be verified manually against the main database. A test set of forms with multiple errors will be run through the data edit system routinely to verify that errors are caught and correct items are not. Timeliness of responding to edit queries will be tracked, as will the time between completion of the visit for a particular woman and when her forms are submitted.

A back-up of the databases and data management programs is performed every night with a weekly archive sent off-site to an environmentally controlled and protected storage facility. All user computers are fully backed up every week and, for the main statisticians on a study, every night. Access to the main study database is through multilevel password protection and enforcement of user privileges on the database. The main MMRI network is not accessible from the outside or from the Web except through passwords and a firewall. Access to any particular PC on the MMRI network can only be obtained through the granting of specific privileges to a particular individual.

12.2 Data Analysis

All analyses will be performed using analysis files created by adding follow-up data to the main DISC database with verification by manual checking. SAS (SAS Inst., Cary, NC) will be the primary software used for analysis supplemented with S-Plus (Insightful Corp., Seattle, WA) and Stata (Stata Corp., College Station, TX).

Because DISC was a clinical trial, analysis of the specific aims will be performed by randomized treatment assignment. Mean serum progesterone will be compared between the intervention and usual care groups without regard to compliance with the intervention, attendance at intervention sessions, or attendance at earlier DISC clinic visits. Because hormonal contraceptives contain progesterone, only women not using hormonal contraceptives will be included in analysis of treatment group differences in serum progesterone levels. The distribution of serum progesterone values will be examined to determine whether transformation is appropriate prior to analysis. In previous DISC analyses, we ln transformed hormone concentrations to improve normality and decrease dependence of the variance on concentration. We anticipate that ln transformation of progesterone data collected at the proposed follow-up visit will also be appropriate, but a final decision will be made after examining the data. The analytical approach will depend on the distributions of age, race, and menstrual cycle day when blood was collected at the clinic visit in the two treatment groups. These characteristics are potential confounders that would not be affected by the intervention. If the treatment groups are balanced on these characteristics, means can be estimated directly and statistical significance of the treatment group differences can be tested using a Student's t-test. If the participants in the treatment groups are not balanced on these characteristics and there is evidence for confounding, we will estimate adjusted means and test for statistical significance using linear models. We used analysis of covariance to compare adjusted treatment group means in previous DISC hormone analyses.

The effect of missing progesterone data on observed treatment group differences will be evaluated using the multiple imputation method of Rubin [89], in which multiple datasets are constructed, analyzed, and results combined so that the underlying variability of the outcome is preserved.

In secondary analysis of the primary aim, we will determine whether treatment group differences in progesterone are related to differences in several individual characteristics including age at menarche, BMI, and % body fat by including appropriate covariates in linear models. We will investigate if there is a particular component of the diet intervention (energy, total or saturated fat, fiber) responsible for observed differences in progesterone levels. BMI and diet at time of the follow-up visit or earlier, during puberty, could be responsible for observed treatment group differences in progesterone at the follow-up visits. To evaluate if puberty is a critical time for exposure, we will estimate the mean level of exposure at earlier DISC visits and use linear models to evaluate whether

average pubertal exposure can explain treatment group differences in progesterone levels at the follow-up visit. Furthermore, we will evaluate potential confounding and effect modification of treatment group differences in progesterone levels at follow-up visits by changes in characteristics such as diet, anthropometry, and physical activity between the last DISC visit and the follow-up visit and by intervening events such as pregnancy, lactation, and use of hormonal contraceptives, by including terms for main effects in models to evaluate confounding and by including cross-product terms in models that include main effects to evaluate effect modification.

Analysis of secondary aims will be performed using the same approach as described for the primary specific aim except breast MRI analysis will use statistical techniques for paired organs as described below. Similar to progesterone, women using hormonal contraceptives will not be included in analysis of serum estradiol. We will, however, perform breast MRI and bone densitometry on these women. In analysis of treatment group differences in breast density and bone density, we will evaluate confounding by past and present hormonal contraceptive use. We also will test for interaction of hormonal contraceptive use with treatment group effects by including cross-products terms in models. Analyses will be stratified by hormonal contraceptive use if cross-product terms are significant.

We will use the generalized estimating equations (GEE) approach to analyze the breast density data. GEE will be used to adjust for the inherent correlation between the density measures of the two breasts within the same woman. This modeling can be done as either a nested model with breast nested within women or as an explicit factor in the model with appropriate interaction terms. Although other data reduction techniques could be used (such as averaging the breast density measures for both breasts so that we would use the average breast density for a women in the analysis), the GEE approach would preserve the information about the density for each breast so that we can investigate variation in density between the two breasts.

12.3 Sample Size and Power

Participants in the proposed study are the 301 female DISC participants. Because this is an existing cohort and sample size is set, we estimated detectable differences in luteal phase progesterone levels (our primary specific aim). For information, we also provide detectable differences for our secondary specific aims. Detectable differences in treatment group means were calculated using S-Plus 2000 (Insightful, Seattle WA).

12.3.1 Primary Specific Aim

We used data from the DISC last visit on serum progesterone levels in 9 usual care group participants whose blood was collected 1-6 days before the start of their next menses. This time frame is the window during which follow-up visits will take place in the proposed study. Earlier DISC analyses were performed on ln transformed hormone values. We also used ln transformed values to improve normality of distribution for these power calculations. The mean transformed progesterone concentration adjusted for age and cycle day was 6.76 (sd = 0.52). Hormones will be measured only in participants not using hormonal contraceptives. CDC estimates that 40% of women 20-24 years old use hormonal contraceptives [146]. In our pilot study of DISC participants, 48% of women reported using hormonal contraceptives and 10% were pregnant. Ninety-three percent of participants in our pilot said they would be willing to come to a follow-up visit. We estimated detectable differences in progesterone concentrations at 80% (conservative) and 90% participation rates assuming 50-60% of participants are using hormonal contraceptives or pregnant and cannot be included in these analyses (Table 1). Depending on participation rate, hormone use, and pregnancy we will be able to detect

Table 1. Minimal Detectable Differences For Luteal Phase Progesterone and Estradiol at α=.05 (2-sided) and 80% Power

Participation Rates	Hormonal Contraceptive Use or Pregnant	Number per Group	Detectable Difference Progesterone		Detectable Difference Estradiol	
			ln	**%**	**ln**	**%**

80%	50%	60	.266	23.3%	.164	15.1%
	60%	48	.297	25.7%	.183	16.7%
90%	50%	68	.250	22.1%	.154	14.3%
	60%	54	.280	24.4%	.173	15.9%

22%-26% lower progesterone levels in the intervention group compared to usual care (($e^{6.76}$– $e^{6.76-.266}$)/$e^{6.76}$). At last DISC visits the intervention group's mean progesterone level was 52.9% lower than the usual care group. We will have ample power to detect a difference that is less than half as large as this.

12.3.2 Secondary Specific Aims

Luteal Phase Serum Estradiol: We used data from the DISC last visit on serum estradiol levels in 9 usual care group participants whose blood was collected 1-6 days before the start of their next menses. The mean ln transformed estradiol concentration adjusted for age and cycle day was 2.62 (sd=.32). We estimated detectable differences in estradiol concentrations at 80% and 90% participation rates and assuming 50% -60% of participants are using hormonal contraceptives or pregnant and cannot be included in these analyses (Table 1). We will have 80% power to detect 14%-17% lower estradiol levels in the intervention compared to the usual care group.

Bone Mineral Density: We used data from Teegarden [147] to estimate the detectable differences in total body bone mineral density. Teergarden used DEXA to measure total body bone mineral density in 215 women 18-31 years old (mean age = 23.8 years). Their mean total body bone mineral density ranged from 1.00-1.37 gm/ cm^2 with a mean of 1.16 gm/cm^2 (sd = .08). This standard deviation is comparable to the population standard deviation of .087 in the Hologic Reference Data. With 80%-90% participation rate and 10% excluded because of pregnancy, we will have DEXA data on 108 – 122 women per group. This yields 80% power to detect treatment group differences in total body bone mineral density of .029 - .030 gm/cm^2.

Breast Density: We used data from Lee [95] to estimate detectable differences in percent dense (fibroglandular) breast tissue in intervention vs. usual care group DISC participants. Lee measured fatty and fibroglandular tissue volumes by MRI in the breasts of 40 women 20-83 years old. Because breast density changes with age and menopausal status and all DISC participants at follow-up visits will be 25-29 years old and premenopausal, we only used data from 15 women 20-45 years of age. Their percent dense tissue ranged from 18% - 83% with a mean of 45.1% (sd = 17.7%). With 80%-90% participation rate and 10% excluded because of pregnancy, we will have MRI data on 108 – 122 women per group. This yields 80% power to detect treatment group differences in percent dense breast tissue of 6.3% – 6.7 %.

12.3.3 Implications for Breast Cancer
Data are not available on the relationship of serum hormones, breast density, and BMD at 20-30 years of age and subsequent breast cancer risk. We extrapolate from data in older women, which gives an indication of possible implications of our findings for breast cancer risk. In postmenopausal women, each doubling of serum estradiol increases breast cancer risk by 31% [148]. Therefore, a difference in estradiol of 14-17% could potentially translate into a 4-5% difference in risk. Prospective data are not available on serum progesterone and breast cancer risk. However, risk of breast cancer associated with HRT use that includes estrogens plus progesten is significantly 50% higher compared to use of HRT that includes estrogen alone [149]. Incrementing the risk of 4-5% for estradiol alone by 50% yields a 6-8% difference in breast cancer risk. Yaffee and Boyd [150] estimated that each 1% increase in percent breast density translates into approximately a 2% increase in breast cancer risk. Therefore, a 6-7% difference in breast density could translate into as much as a 12-14% difference in

breast cancer risk. In the SOF, a 1 SD increase in BMD increased breast cancer risk by 30-50% [63]. A difference in BMD of .029-.030 is approximately one-third of 1 SD (Hologic Reference Data SD=.087), which could translate into a 10-17% difference in breast cancer risk. A difference of .029 - .030 gm/cm^2 is a 2.5- 2.6% difference (.029/1.16). In the MORE study, Raloxifene increased BMD by 2.4-2.7% and significantly decreased risk of vertebral fractures [151].

13.0 ADVERSE EVENT REPORTING

Adverse events could occur related to the measurements proposed. Risks to participants include loss of privacy and confidentiality of data collected by questionnaires; feeling dizzy, fainting, or bruising following venipuncture; feelings of claustrophobia during the MRI; and exposure to very low dose radiation by the DEXA (less than 0.1 μGy). Even at low doses radiation can cause a slight increase in cancer risk. The dose of radiation exposure during the DEXA is typically less than the daily effective dose from natural sources (cosmic radiation, environmental isotopes in our bodies and surroundings). This protocol exposes the individual to approximately 10 times less radiation than a chest x-ray and 10 times less radiation that received when flying round trip coast-to-coast. None of the risks to participants is life threatening or should have a major negative impact on the participants' physical, mental, or emotional well-being. Because radiation from DEXA could adversely affect the fetus and the effect of MRI on the fetus is not known, pregnant women will not have these exams.

Adverse events will be reported by the clinical center PI to his/her IRB, the data coordinating center, and the PI for the proposed study. Adverse events will be reviewed and written summary reports will be sent to PI's at all collaborating centers to forward to their respective IRBs.

14.0 PATHOLOGY REVIEW

Not applicable.

15.0 RECORDS TO BE KEPT

1. DISC Questionnaire (demographics, medical history, menstrual and reproductive history, medications, diet supplements, alcohol use, tobacco use, family history of cancer)
2. Anthropometric measurements (height, weight)
3. Dietary assessment (three 24-hour recalls)
4. Physical activity assessments
5. Psychosocial assessments (CESD, STAI)
6. Menses data
7. Serum hormones (progesterone, estradiol)
8. Bone mineral density
9. Breast density

16.0 INFORMED CONSENT

Written informed consent will be obtained before specimen/data collection at the beginning of the clinic visit. The study coordinator or other knowledgeable personnel at the clinic will describe what is involved in participating in the study and encourage the participant to ask questions. The informed consent statement will include a statement that participation is entirely voluntary; and provide information on the number of women being invited to participate, why they were selected, why the study is being done, what participation involves, the risks, benefits and costs of participation, how confidentiality will be protected, what happens if she is injured, who to contact if she has questions or problems with the study, what her rights are as a participant (including withdrawal), how samples and images (DEXA and MRI) will be used, and how results will be reported. Signed informed consent will be kept in the participant's file. Documentation that informed consent was obtained will be included in the study database.

17.0 REFERENCES

1. Ries LAG KC, Hankey BF, Miller BA, Clegg LX, Edwards BK (eds.). SEER Cancer Statistics Review, 1973-1996,. Vol NIH Pub. No. 99-2789. Bethesda, MD: National Cancer Institute; 1999.
2. Welsch CW. Relationship between dietary fat and experimental mammary tumorigenesis: a review and critique. Cancer Res 1992;52:2040s-8s.
3. Rose DP, Boyar AP, Wynder EL. International comparisons of mortality rates for cancer of the breast, ovary, prostate, and colon, and per capita food consumption. Cancer 1986;58:2363-71.
4. Stanford JL, Herrinton LJ, Schwartz SM, Weiss NS. Breast cancer incidence in Asian migrants to the United States and their descendants. Epidemiology 1995;6:181-3.
5. Howe GR, Hirohata T, Hislop TG, Iscovich JM, Yuan JM, Katsouyanni K, Lubin F, Marubini E, Modan B, Rohan T, et al. Dietary factors and risk of breast cancer: combined analysis of 12 case-control studies. J Natl Cancer Inst 1990;82:561-9.
6. Mannisto S, Pietinen P, Virtanen M, Kataja V, Uusitupa M. Diet and the risk of breast cancer in a case-control study: does the threat of disease have an influence on recall bias? J Clin Epidemiol 1999;52:429-39.
7. Hunter DJ, Spiegelman D, Adami HO, Beeson L, van den Brandt PA, Folsom AR, Fraser GE, Goldbohm RA, Graham S, Howe GR, et al. Cohort studies of fat intake and the risk of breast cancer--a pooled analysis. N Engl J Med 1996;334:356-61.
8. Dorgan JF, Longcope C, Stephenson HE, Jr., Falk RT, Miller R, Franz C, Kahle L, Campbell WS, Tangrea JA, Schatzkin A. Relation of prediagnostic serum estrogen and androgen levels to breast cancer risk. Cancer Epidemiol Biomarkers Prev 1996;5:533-9.
9. Dorgan JF, Longcope C, Stephenson HE, Jr., Falk RT, Miller R, Franz C, Kahle L, Campbell WS, Tangrea JA, Schatzkin A. Serum sex hormone levels are related to breast cancer risk in postmenopausal women. Environ Health Perspect 1997;105 Suppl 3:583-5.
10. Toniolo PG, Levitz M, Zeleniuch-Jacquotte A, Banerjee S, Koenig KL, Shore RE, Strax P, Pasternack BS. A prospective study of endogenous estrogens and breast cancer in postmenopausal women. J Natl Cancer Inst 1995;87:190-7.
11. Berrino F, Muti P, Micheli A, Bolelli G, Krogh V, Sciajno R, Pisani P, Panico S, Secreto G. Serum sex hormone levels after menopause and subsequent breast cancer. J Natl Cancer Inst 1996;88:291-6.
12. Gordon GB, Bush TL, Helzlsouer KJ, Miller SR, Comstock GW. Relationship of serum levels of dehydroepiandrosterone and dehydroepiandrosterone sulfate to the risk of developing postmenopausal breast cancer. Cancer Res 1990;50:3859-62.
13. Thomas HV, Reeves GK, Key TJ. Endogenous estrogen and postmenopausal breast cancer: a quantitative review. Cancer Causes Control 1997;8:922-8.
14. Hankinson SE, Willett WC, Manson JE, Colditz GA, Hunter DJ, Spiegelman D, Barbieri RL, Speizer FE. Plasma sex steroid hormone levels and risk of breast cancer in postmenopausal women. J Natl Cancer Inst 1998;90:1292-9.
15. Cauley JA, Lucas FL, Kuller LH, Stone K, Browner W, Cummings SR. Elevated serum estradiol and testosterone concentrations are associated with a high risk for breast cancer. Study of Osteoporotic Fractures Research Group. Ann Intern Med 1999;130:270-7.
16. Group EHaBCC. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst 2002;94:606-16.
17. Kabuto M, Akiba S, Stevens RG, Neriishi K, Land CE. A prospective study of estradiol and breast cancer in Japanese women. Cancer Epidemiol Biomarkers Prev 2000;9:575-9.
18. Rosenberg CR, Pasternack BS, Shore RE, Koenig KL, Toniolo PG. Premenopausal estradiol levels and the risk of breast cancer: a new method of controlling for day of the menstrual cycle. Am J Epidemiol 1994;140:518-25.
19. Helzlsouer KJ, Alberg AJ, Bush TL, Longcope C, Gordon GB, Comstock GW. A prospective study of endogenous hormones and breast cancer. Cancer Detect Prev 1994;18:79-85.

20. Goldin BR, Adlercreutz H, Gorbach SL, Woods MN, Dwyer JT, Conlon T, Bohn E, Gershoff SN. The relationship between estrogen levels and diets of Caucasian American and Oriental immigrant women. Am J Clin Nutr 1986;44:945-53.

21. Kaneda N, Nagata C, Kabuto M, Shimizu H. Fat and fiber intakes in relation to serum estrogen concentration in premenopausal Japanese women. Nutr Cancer 1997;27:279-83.

22. Nagata C, Takatsuka N, Kawakami N, Shimizu H. Total and monounsaturated fat intake and serum estrogen concentrations in premenopausal Japanese women. Nutr Cancer 2000;38:37-9.

23. Wu AH, Pike MC, Stram DO. Meta-analysis: dietary fat intake, serum estrogen levels, and the risk of breast cancer. J Natl Cancer Inst 1999;91:529-34.

24. Persky VW, Chatterton RT, Van Horn LV, Grant MD, Langenberg P, Marvin J. Hormone levels in vegetarian and nonvegetarian teenage girls: potential implications for breast cancer risk. Cancer Res 1992;52:578-83.

25. Gray GE, Williams P, Gerkins V, Brown JB, Armstrong B, Phillips R, Casagrande JT, Pike MC, Henderson BE. Diet and hormone levels in Seventh-Day Adventist teenage girls. Prev Med 1982;11:103-7.

26. Wilson DW, Turkes A, Jones R, Danutra V, Read GF, Griffiths K. A comparison of menstrual cycle profiles of salivary progesterone in British and Thai adolescent girls. Eur J Cancer 1992;28A:1162-7.

27. Russo IH, Koszalka M, Russo J. Human chorionic gonadotropin and rat mammary cancer prevention. J Natl Cancer Inst 1990;82:1286-9.

28. Preston DL, Mattsson A, Holmberg E, Shore R, Hildreth NG, Boice JD, Jr. Radiation effects on breast cancer risk: a pooled analysis of eight cohorts. Radiat Res 2002;158:220-35.

29. Tokunaga M, Land CE, Tokuoka S, Nishimori I, Soda M, Akiba S. Incidence of female breast cancer among atomic bomb survivors, 1950-1985. Radiat Res 1994;138:209-23.

30. Silverman J, Powers J, Stromberg P, Pultz JA, Kent S. Effects on C3H mouse mammary cancer of changing from a high fat to a low fat diet before, at, or after puberty. Cancer Res 1989;49:3857-60.

31. Frazier AL, Li L, Cho E, Willett W, Colditz G. Adolescent Diet and Risk of Breast Cancer (United States). Cancer Epidemiol Biomarkers Prev 2003.

32. Baer HJ, Schnitt SJ, Connolly JL, Byrne C, Cho E, Willett W, Colditz G. Adolescent Diet and Incidence of Proliferative Benign Breast Disease. Cancer Causes and Control 2003.

33. Hislop TG, Coldman AJ, Elwood JM, Brauer G, Kan L. Childhood and recent eating patterns and risk of breast cancer. Cancer Detect Prev 1986;9:47-58.

34. Potischman N, Weiss HA, Swanson CA, Coates RJ, Gammon MD, Malone KE, Brogan D, Stanford JL, Hoover RN, Brinton LA. Diet during adolescence and risk of breast cancer among young women. J Natl Cancer Inst 1998;90:226-33.

35. Pryor M, Slattery ML, Robison LM, Egger M. Adolescent diet and breast cancer in Utah. Cancer Res 1989;49:2161-7.

36. Frankel S, Gunnell DJ, Peters TJ, Maynard M, Davey Smith G. Childhood energy intake and adult mortality from cancer: the Boyd Orr Cohort Study. Bmj 1998;316:499-504.

37. Meyer F, Moisan J, Marcoux D, Bouchard C. Dietary and physical determinants of menarche. Epidemiology 1990;1:377-81.

38. de Ridder CM, Thijssen JH, Van 't Veer P, van Duuren R, Bruning PF, Zonderland ML, Erich WB. Dietary habits, sexual maturation, and plasma hormones in pubertal girls: a longitudinal study. Am J Clin Nutr 1991;54:805-13.

39. Merzenich H, Boeing H, Wahrendorf J. Dietary fat and sports activity as determinants for age at menarche. Am J Epidemiol 1993;138:217-24.

40. Koprowski C, Ross RK, Mack WJ, Henderson BE, Bernstein L. Diet, body size and menarche in a multiethnic cohort. Br J Cancer 1999;79:1907-11.

41. Hilakivi-Clarke L, Forsen T, Eriksson JG, Luoto R, Tuomilehto J, Osmond C, Barker DJ. Tallness and overweight during childhood have opposing effects on breast cancer risk. Br J Cancer 2001;85:1680-4.

42. Herrinton LJ, Husson G. Relation of childhood height and later risk of breast cancer. Am J Epidemiol 2001;154:618-23.
43. Berkey CS, Frazier AL, Gardner JD, Colditz GA. Adolescence and breast carcinoma risk. Cancer 1999;85:2400-9.
44. de Waard F, Trichopoulos D. A unifying concept of the aetiology of breast cancer. Int J Cancer 1988;41:666-9.
45. Colditz GA, Frazier AL. Models of breast cancer show that risk is set by events of early life: prevention efforts must shift focus. Cancer Epidemiol Biomarkers Prev 1995;4:567-71.
46. Le Marchand L, Kolonel LN, Earle ME, Mi MP. Body size at different periods of life and breast cancer risk. Am J Epidemiol 1988;128:137-52.
47. Huang Z, Hankinson SE, Colditz GA, Stampfer MJ, Hunter DJ, Manson JE, Hennekens CH, Rosner B, Speizer FE, Willett WC. Dual effects of weight and weight gain on breast cancer risk. Jama 1997;278:1407-11.
48. Barnes-Josiah D, Potter JD, Sellers TA, Himes JH. Early body size and subsequent weight gain as predictors of breast cancer incidence (Iowa, United States). Cancer Causes Control 1995;6:112-8.
49. Stoll BA. Essential fatty acids, insulin resistance, and breast cancer risk. Nutr Cancer 1998;31:72-7.
50. Bray GA. The underlying basis for obesity: relationship to cancer. J Nutr 2002;132:3451S-5S.
51. Lahmann PH, Lissner L, Gullberg B, Berglund G. A prospective study of adiposity and all-cause mortality: the Malmo Diet and Cancer Study. Obes Res 2002;10:361-9.
52. Morimoto LM, White E, Chen Z, Chlebowski RT, Hays J, Kuller L, Lopez AM, Manson J, Margolis KL, Muti PC, Stefanick ML, McTiernan A. Obesity, body size, and risk of postmenopausal breast cancer: the Women's Health Initiative (United States). Cancer Causes Control 2002;13:741-51.
53. Davies MJ, Norman RJ. Programming and reproductive functioning. Trends Endocrinol Metab 2002;13:386-92.
54. Rosenfield RL. Puberty in the Female and Its Disorders. In Sperling MA, editor. Pediatric Endocrinology. Second ed. Philadelphia, PA: Saunders; 2002. p. 455-518.
55. Grumbach MM. The neuroendocrinology of human puberty revisited. Horm Res 2002;57 Suppl 2:2-14.
56. McCartney CR, Eagleson CA, Marshall JC. Regulation of gonadotropin secretion: implications for polycystic ovary syndrome. Semin Reprod Med 2002;20:317-26.
57. Illig AM, Melia K, Snyder PJ, Badura LL. Sodium valproate alters GnRH-GABA interactions during development in seizure-prone mice. Brain Res 2000;885:192-200.
58. Chabbert-Buffeta N, Skinner DC, Caraty A, Bouchard P. Neuroendocrine effects of progesterone. Steroids 2000;65:613-20.
59. Browner WS, Malinow MR. Homocyst(e)inaemia and bone density in elderly women. Lancet 1991;338:1470.
60. Kuller LH, Cauley JA, Lucas L, Cummings S, Browner WS. Sex steroid hormones, bone mineral density, and risk of breast cancer. Environ Health Perspect 1997;105 Suppl 3:593-9.
61. Lippman ME, Krueger KA, Eckert S, Sashegyi A, Walls EL, Jamal S, Cauley JA, Cummings SR. Indicators of lifetime estrogen exposure: effect on breast cancer incidence and interaction with raloxifene therapy in the multiple outcomes of raloxifene evaluation study participants. J Clin Oncol 2001;19:3111-6.
62. Zhang Y, Kiel DP, Kreger BE, Cupples LA, Ellison RC, Dorgan JF, Schatzkin A, Levy D, Felson DT. Bone mass and the risk of breast cancer among postmenopausal women. N Engl J Med 1997;336:611-7.
63. Cauley JA, Lucas FL, Kuller LH, Vogt MT, Browner WS, Cummings SR. Bone mineral density and risk of breast cancer in older women: the study of osteoporotic fractures. Study of Osteoporotic Fractures Research Group. Jama 1996;276:1404-8.

64. Buist DS, LaCroix AZ, Barlow WE, White E, Cauley JA, Bauer DC, Weiss NS. Bone mineral density and endogenous hormones and risk of breast cancer in postmenopausal women (United States). Cancer Causes Control 2001;12:213-22.

65. Sowers MF. Lower peak bone mass and its decline. Baillieres Best Pract Res Clin Endocrinol Metab 2000;14:317-29.

66. Bailey AJ, Sims TJ, Ebbesen EN, Mansell JP, Thomsen JS, Mosekilde L. Age-related changes in the biochemical properties of human cancellous bone collagen: relationship to bone strength. Calcif Tissue Int 1999;65:203-10.

67. Prentice A. The relative contribution of diet and genotype to bone development. Proc Nutr Soc 2001;60:45-52.

68. Grumbach MM. Estrogen, bone, growth and sex: a sea change in conventional wisdom. J Pediatr Endocrinol Metab 2000;13 Suppl 6:1439-55.

69. Daniel CW, Silberstein GB, Strickland P. Direct action of 17 beta-estradiol on mouse mammary ducts analyzed by sustained release implants and steroid autoradiography. Cancer Res 1987;47:6052-7.

70. Wiseman BS, Werb Z. Stromal effects on mammary gland development and breast cancer. Science 2002;296:1046-9.

71. Brisken C, Park S, Vass T, Lydon JP, O'Malley BW, Weinberg RA. A paracrine role for the epithelial progesterone receptor in mammary gland development. Proc Natl Acad Sci U S A 1998;95:5076-81.

72. Russo J, Hu YF, Yang X, Russo IH. Developmental, cellular, and molecular basis of human breast cancer. J Natl Cancer Inst Monogr 2000:17-37.

73. Brisson J, Verreault R, Morrison AS, Tennina S, Meyer F. Diet, mammographic features of breast tissue, and breast cancer risk. Am J Epidemiol 1989;130:14-24.

74. Wolfe JN, Saftlas AF, Salane M. Mammographic parenchymal patterns and quantitative evaluation of mammographic densities: a case-control study. AJR Am J Roentgenol 1987;148:1087-92.

75. Saftlas AF, Hoover RN, Brinton LA, Szklo M, Olson DR, Salane M, Wolfe JN. Mammographic densities and risk of breast cancer. Cancer 1991;67:2833-8.

76. Boyd NF, Byng JW, Jong RA, Fishell EK, Little LE, Miller AB, Lockwood GA, Tritchler DL, Yaffe MJ. Quantitative classification of mammographic densities and breast cancer risk: results from the Canadian National Breast Screening Study. J Natl Cancer Inst 1995;87:670-5.

77. Byrne C, Schairer C, Wolfe J, Parekh N, Salane M, Brinton LA, Hoover R, Haile R. Mammographic features and breast cancer risk: effects with time, age, and menopause status. J Natl Cancer Inst 1995;87:1622-9.

78. Boyd NF, Stone J, Martin LJ, Jong R, Fishell E, Yaffe M, Hammond G, Minkin S. The association of breast mitogens with mammographic densities. Br J Cancer 2002;87:876-82.

79. Greendale GA, Reboussin BA, Sie A, Singh HR, Olson LK, Gatewood O, Bassett LW, Wasilauskas C, Bush T, Barrett-Connor E. Effects of estrogen and estrogen-progestin on mammographic parenchymal density. Postmenopausal Estrogen/Progestin Interventions (PEPI) Investigators. Ann Intern Med 1999;130:262-9.

80. Rutter CM, Mandelson MT, Laya MB, Seger DJ, Taplin S. Changes in breast density associated with initiation, discontinuation, and continuing use of hormone replacement therapy. Jama 2001;285:171-6.

81. Marugg RC, van der Mooren MJ, Hendriks JH, Rolland R, Ruijs SH. Mammographic changes in postmenopausal women on hormonal replacement therapy. Eur Radiol 1997;7:749-55.

82. Brisson J, Brisson B, Cote G, Maunsell E, Berube S, Robert J. Tamoxifen and mammographic breast densities. Cancer Epidemiol Biomarkers Prev 2000;9:911-5.

83. Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov N, Atkins J, Daly M, Wieand S, Tan-Chiu E, Ford L, Wolmark N. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 1998;90:1371-88.

84. Boyd NF, Dite GS, Stone J, Gunasekara A, English DR, McCredie MR, Giles GG, Tritchler D, Chiarelli A, Yaffe MJ, Hopper JL. Heritability of mammographic density, a risk factor for breast cancer. N Engl J Med 2002;347:886-94.

85. Vachon CM, Kushi LH, Cerhan JR, Kuni CC, Sellers TA. Association of diet and mammographic breast density in the Minnesota breast cancer family cohort. Cancer Epidemiol Biomarkers Prev 2000;9:151-60.

86. Nordevang E, Azavedo E, Svane G, Nilsson B, Holm LE. Dietary habits and mammographic patterns in patients with breast cancer. Breast Cancer Res Treat 1993;26:207-15.

87. Knight JA, Martin LJ, Greenberg CV, Lockwood GA, Byng JW, Yaffe MJ, Tritchler DL, Boyd NF. Macronutrient intake and change in mammographic density at menopause: results from a randomized trial. Cancer Epidemiol Biomarkers Prev 1999;8:123-8.

88. Boyd NF, Greenberg C, Lockwood G, Little L, Martin L, Byng J, Yaffe M, Tritchler D. Effects at two years of a low-fat, high-carbohydrate diet on radiologic features of the breast: results from a randomized trial. Canadian Diet and Breast Cancer Prevention Study Group. J Natl Cancer Inst 1997;89:488-96.

89. Rubin DB. Multiple Imputation for Nonresponse in Surveys. New York: John Wiley & Sons; 1987.

18.0 <u>MULTICENTER TRIALS</u>

Clinical Centers
Northwestern University, Chicago, IL
Johns Hopkins Children's Center, Baltimore, MD
Kaiser Permanente Center for Health Research, Portland, OR
Children's Hospital of New Orleans, New Orleans, LA
University of Iowa, Iowa City, IA
New Jersey Medical School, Newark, NJ

Data Coordinating Center
Maryland Medical Research Institute, Baltimore, MD

DEXA Coordinating Center
Department of Radiology, University of California, San Francisco, San Francisco, CA

MRI Coordinating Center
Magnetic Resonance Science Center, University of California, San Francisco, San Francisco, CA

Nutrition Coordinating Center
University of Minnesota School of Public Health, Minneapolis, MN

19.0 <u>APPENDIX</u>

APPENDIX A. INFORMED CONSENT

APPENDIX B. LETTERS TO PARTICIPANTS

APPENDIX C. TELEPHONE SCRIPT

APPENDIX D. QUESTIONNAIRES