

EFFECTS OF DIETARY FAT AND OMEGA-3 FATTY ACIDS ON EICOSANOIDS,  
ENDOGENOUS SEX HORMONES AND THE INSULIN-LIKE GROWTH FACTOR  
PATHWAY

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## **Dedication**

*I dedicate this dissertation to my parents: Carol Orr, whose undying support has enabled me to follow my dreams, and in loving memory of my father, James Orr; and to my loved ones who inspired me to pursue a career in cancer prevention:*

*Kelli Whithorn, Duane Whithorn and Elaine Hoar.*

## Abstract

This dissertation details a clinical trial that investigated the effects of three controlled, 8-week duration test diets: a high fat diet (HF; 40% of energy from fat), a low fat diet (LF; 20% of energy from fat), and a low fat diet high in omega-3 (n-3) fatty acids (LFn3; 23% energy from fat including 3% of energy from n-3 fatty acids) on breast cancer risk markers including plasma and urinary sex hormones, urinary eicosanoids, and insulin-like growth factor (IGF) pathway endpoints in postmenopausal women.

Chapter 1 contains a review of the literature providing context for the clinical trial. Chapter 2 describes the effects of the three test diets on plasma phospholipid fatty acids (PLFA), urinary eicosanoids, and plasma sex hormones. The LFn3 diet significantly increased plasma n-3 PLFA and the HF diet significantly increased estradiol and urinary eicosanoids. These results indicate that high fat diet increases breast cancer risk markers, but are inconclusive with respect to n-3 fatty acids.

Chapter 3 describes the effect of the three test diets on urinary sex hormones and metabolites. Urinary excretion of estrone was significantly greater after the LF and LFn3 compared to the HF; however in the context of all the urinary hormones and metabolites measured, this indicates that no clinically significant alterations were observed following the test diets.

Chapter 4 details the effects of the test diets on IGF pathway endpoints. LFn3 increased IGF-I and IGF binding protein-3 (IGFBP-3) and the LF increased IGFBP-3. These results indicate that low fat diet may reduce free IGF-I while the addition of n-3 fatty acids to the low fat diet may increase free IGF-I concentrations. The impact on breast cancer risk mediated by the increase in IGF-I with the LFn3 is unknown, but an increase in circulating IGF-I may have an impact on reducing the effects of aging.

In conclusion, the test diets had pronounced effects on PLFA but modest effects on plasma and urinary sex hormones. The LFn3 unexpectedly increased IGF-I concentrations, which may demonstrate a role of n-3 in preventing the effects of aging.

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## List of Abbreviations

2-ME1: 2-methoxy estrone  
2-ME2: 2-methoxy estradiol  
4-ME1: 4-methoxy estrone  
4-ME2: 4-methoxy estradiol  
16 $\alpha$ OH-E1: 16 $\alpha$ -hydroxy estrone  
2OH-E1: 2-hydroxy estrone  
2OH-E2: 2-hydroxy estradiol  
4OH-E1: 4-hydroxy estrone  
4OH-E2: 4-hydroxy estradiol  
A: androstenedione  
AA: arachidonic acid  
AARP: American Association of Retired Persons  
ALA:  $\alpha$ -linolenic acid  
BBD: benign breast disease  
BMI: body mass index  
COMT: catechol-O-methyl transferase  
COX-1: cyclooxygenase-1  
COX-2: cyclooxygenase-2  
CYP450: cytochrome P450  
DHA: docosahexaenoic acid  
DHEA: dehydroepiandrosterone  
DHEAS: dehydroepiandrosterone sulfate  
DIANA: Diet and Androgens Randomized Trial  
DMBA: 7,12-dimethylbenz[ $\alpha$ ]anthracene  
DPA: docosapentaenoic acid  
E1: estrone  
E1-S: estrone sulfate  
E2: estradiol  
E3: estriol

EPA: eicosapentaenoic acid  
EPIC: European Prospective Investigation into Cancer and Nutrition  
ER: estrogen receptor  
ER+: estrogen receptor positive  
Estriol: 16 $\alpha$ -hydroxy estradiol  
FA: fatty acid  
FAME: fatty acid methyl esters  
FFQ: food frequency questionnaire  
FSH: follicle stimulating hormone  
GH: growth hormone  
GLA: di-homo- $\gamma$ -linolenic acid  
HRT: hormone replacement therapy  
HUFA: highly unsaturated fatty acid  
IGF-I: insulin-like growth factor I  
IGF-II: insulin-like growth factor II  
IGFBP: insulin-like growth factor binding protein  
IGF-IR: insulin-like growth factor-I receptor  
IGF-IIR: insulin-like growth factor-II receptor  
IR: insulin receptor  
IRS: insulin receptor substrate  
LA: linoleic acid  
LID: liver-specific IGF-I gene deleted  
LOX: lipoxygenase  
MAPK: mitogen-activated protein kinase  
MMTV: mouse mammary tumor virus  
MUFA: monounsaturated fatty acids  
Her-2/neu: human epidermal growth factor receptor-2  
n-3: omega-3  
n-6: omega-6  
NHS: Nurse's Health Study



NIH: National Institutes of Health

ORDET: Hormones and Diet in the etiology of Breast Tumors Study

PG: prostaglandin

PI3K: phosphatidylinositol-3' kinase

PLFA: phospholipid fatty acid

PR+: progesterone receptor positive

PUFA: polyunsaturated fatty acid

SDR: spontaneous dwarf rat

SFA: saturated fatty acids

SHBG: sex hormone binding globulin

TGF $\alpha$ : transforming growth factor alpha

T: testosterone

TG: triglyceride

Tx: thromboxane

WHEL: Women's Healthy Eating and Living Trial

WHI: Women's Health Initiative Randomized Controlled Dietary Modification Trial

WINS: Women's Intervention Nutrition Study

**Chapter 1:**  
**Literature Review**

## **I. Breast Cancer**

Aside from skin cancer, breast cancer is the most commonly diagnosed form of cancer in women with a projected 192,370 new cases and a projected 40,170 deaths in the United States in 2009 (1). Breast cancer rates are highest among Westernized nations, while rates in developing nations steadily rise as they become more socioeconomically developed (2).

### **A. Risk Factors**

Risk factors for breast cancer are attributes associated with an increased risk of developing the disease. A number of risk factors are unmodifiable such as age, genetics, family history of breast cancer, and female gender. Some risk factors are modifiable such as diet (3). Having one or more risk factors increases a woman's chance of getting breast cancer, but it is not inevitable that she will develop the disease.

#### **1. Estrogen Exposure**

Lifetime exposure to estrogens is an established risk factor for the development of breast cancer. A woman is exposed to estrogens in utero and throughout childhood, adolescence, and adult life. At each stage estrogens mediate various growth and differentiation processes. In neonates and children, gestational age, birth length and weight, childhood increase in height and body mass index (BMI) are positively correlated with breast cancer development and they are all surrogate markers for exposure to growth factors, including estrogens (4).

Factors that increase the duration that a woman is exposed to estrogens increase breast cancer risk. Among these factors are increased number of menstrual cycles, assessed by age of menarche and menopause. Early age of menarche and late age of menopause increase breast cancer risk. Exogenous estrogens such as oral contraceptives and hormone replacement therapy also increase risk (2). Childbearing and breastfeeding reduce risk because the woman is exposed to lower levels of endogenous estrogens during pregnancy and lactation (2).

Exposure to estrogens is potentially modifiable through dietary means. For instance dietary flax seeds, which contain plant estrogens, have been shown to increase luteal phase length of the menstrual cycle (5) when circulating estrogens are lower relative to the follicular phase.

## **2. Age**

Age is among unmodifiable risk factors for breast cancer development. As a woman ages her risk of breast cancer increases. Most (80%) breast cancer diagnoses occur in women over 50 years old (4, 6).

## **3. Family History and Genetics**

Family history and genetics are also unmodifiable risk factors. Women with no family history of breast cancer have lower risk of breast cancer development (7). A woman is at increased risk if her mother and/or sister had breast cancer and risk is greater if the family member is diagnosed young (< age 40) (7, 8). In families with four or more members with breast cancer, the heritable genetic mutations in BRCA1 and BRCA2 are likely the susceptibility factors (9). From estimations of twin studies, about 25% of breast cancer risk is attributable to genetic factors (10), although only a small percentage (5-10%) of these are explainable by high-risk genetic mutations such as the BRCA mutations (1, 11). Lower-risk alleles also affect breast cancer risk (2). Thus, polymorphisms, such as variants of metabolic enzymes that metabolize hormones or carcinogens, may account for a larger portion of breast cancer risk (12).

## **4. Dietary Factors**

Modification of diet and other lifestyle factors is one means by which an individual may reduce her risk of breast cancer. According to Doll and Peto, approximately one third of cancer risk is attributable to dietary factors (13).

## **5. Alcohol**

Breast cancer risk is increased in women who partake of one or more alcoholic beverages per day (14). In a pooled analysis of prospective cohort studies from four countries, breast cancer risk increased directly as daily consumption of alcohol increased (up to 60g/day) (14). Results from a recent case-control study indicated that lifetime alcohol consumption (>13g/day) is positively associated with development estrogen receptor positive (ER+) and progesterone receptor positive (PR+) tumors relative to hormone receptor negative tumors, which were not associated with alcohol intake (15). A similar result was observed in the Women's Health Study cohort, in which breast cancer risk was increased for women consuming >30g alcohol/day (16). This finding was valid only for those with ER+/PR+ tumors (16). This result suggests that the increase in breast cancer risk with alcohol consumption is mediated by sex hormones (17).

## **6. Fruit and Vegetable Intake**

Fruits and vegetables are a source of myriad phytochemicals, vitamins and antioxidants that have potentially cancer-preventative properties. Dark leafy green vegetables and brightly colored fruits and vegetables are purported to have the greatest potential. However, the association of fruit and vegetable intake with breast cancer risk in epidemiological studies is inconsistent. Intake of vegetables was associated with a decrease in breast cancer risk in a meta-analysis of 26 studies (18). Fruits were nonsignificantly associated with a decrease in risk (18).

## **7. Dietary Fat and Fatty Acids**

In the 1970's Armstrong and Doll concluded that total fat intake is a major risk factor for breast cancer due to its positive correlation with breast cancer incidence ( $r = 0.79$ ) and mortality ( $r = 0.89$ ) based on the per capita dietary fat consumption of 23 countries (19). As such, countries with a high per capita intake of dietary fat have higher rates of breast cancer. Since the time these figures were published, a multitude of studies has investigated the association of dietary fat and breast cancer risk. The

epidemiological data supporting an association of dietary fat and fatty acids (FA) with breast cancer risk is extensive, yet inconclusive. Studies in which a low fat dietary intervention was administered are also conflicting. This topic, to be developed further below, remains controversial to date.

## **Summary**

There are a number of modifiable and unmodifiable risk factors for breast cancer. Among the modifiable factors are dietary habits. Knowledge of the unmodifiable risk factors may lead a woman to seek measures to reduce her risk. A dietary recommendation that reduces breast cancer risk biomarkers, such as endogenous estrogens, would benefit women who are at risk for breast cancer.

## **II. Dietary Fat and Breast Cancer Risk**

The debate about whether dietary fat affects breast cancer risk has been highly contested over the past several decades (20). Animal studies from the 1940's showed a tumorigenic effect of high fat diet on mammary tissue (21). Since then numerous animal, cell, ecological, epidemiological and human intervention studies have been conducted investigating the effect of dietary fat on tumorigenesis and breast cancer risk.

### **A. Ecological Studies**

International correlation studies reinforced the results of early animal studies on enhancement of breast cancer risk with increased dietary fat consumption. Using data from several decades prior, ecological studies reported increased breast cancer mortality in countries with the highest per capita availability of dietary fat (22-24). Breast cancer rates in developing countries, such as Taiwan, increased in parallel as per capita dietary fat availability increased (24). Along similar lines, individuals who have emigrated from countries with low per capita dietary fat intake, such as Japan, have greater breast cancer risk after settling in a country with high dietary fat intake, such as the United States (25-27). Therefore, environmental factors, including diet, are thought to play a

larger role than genetics in the increase in risk experienced by migrants to a country with higher risk. Notably, there are a number of environmental factors in addition to diet and fat intake that are altered as a country transitions into a developed nation that could have an effect on breast cancer risk.

## **B. Case-control Studies**

Following the lead of ecological studies, a number of case-control studies were conducted to address the dietary fat and breast cancer risk hypothesis. Howe et al (28) published a combined analysis of 12 case-control studies of dietary factors related to breast cancer risk completed prior to 1986. In postmenopausal women, intake of saturated fat was significantly and positively associated with breast cancer risk. However, because cases may differentially recall diet relative to controls, case-control studies are criticized for leading to artificial associations (29). Results from prospective cohort studies are therefore preferred because dietary data is collected prior to disease onset and cases and controls assumedly make the same type of errors in reporting their dietary intake (29).

## **C. Prospective Cohort Studies**

Several prospective cohort studies are outlined in Table 1-1. In all of these studies, dietary fat intake was assessed by food frequency questionnaire (FFQ) and breast cancer incidence was an endpoint. Several of these studies indicate no significant associations between dietary fat intake and breast cancer incidence in postmenopausal women (30-32). No association between total dietary fat intake and breast cancer risk was found at 14 (30) and 20 (31) years follow-up, or in a subgroup of women with no history of benign breast disease (BBD) (32) in the Nurses Health Study (NHS) cohort. In contrast, intake of total fat and monounsaturated FA (MUFA) was found to be positively associated with breast cancer risk in a similar cohort of women with no history of BBD (33). A relatively early (1996) combined analysis of 8 prospective cohort studies totaling 337,819 women age 28-93 and 4980 cases by Hunter et al (34) indicated a null association between dietary fat and breast cancer risk for total fat,

saturated FA (SFA), MUFA, polyunsaturated FA (PUFA), and fat from animal and vegetable sources.

Other cohort studies have found positive associations between dietary fat and breast cancer incidence (20, 33, 35). Women in the Italian Hormones and Diet in the Etiology of Breast Tumors Study (ORDET) were at increased risk of breast cancer with increased total fat intake (35). Most recently, results from the National Institutes of Health (NIH)-American Association of Retired Persons (AARP) Diet and Health Study Cohort showed positive associations for total fat, SFA, MUFA, and PUFA and breast cancer incidence (20). Additionally, a more recent meta-analysis (2003) that included 14 cohort studies and 31 case-control studies indicated a weakly positive association between breast cancer incidence and total fat (RR 1.13 (1.03 – 1.25)) and SFA intake (RR 1.18 (1.04 – 1.34) (36). Also of note, the range of dietary fat intakes in many of the epidemiological studies is narrow relative to the international range (15 – 40% or more), which may have precipitated the null findings of many studies (29).

#### **D. Dietary Intervention Trials**

Many research dollars have been invested in settling the dietary fat and breast cancer debate. A few very large-scale randomized, controlled dietary intervention trials have been conducted that involved low fat dietary interventions in healthy women or breast cancer survivors using breast cancer incidence or recurrence as endpoints (see Table 1-2).

##### **1. Women's Health Initiative**

The Women's Health Initiative Randomized Controlled Dietary Modification Trial (WHI) was designed with a dietary intervention that aimed to reduce fat intake to below 20% of total energy and increase servings of fruit, vegetables and grains while a control group made no dietary changes (37). Dietary intake was reported by FFQ. After an average 8-year follow-up, women in the intervention group had a significantly lower reported fat intake, but breast cancer incidence did not differ significantly between the two groups. Yet the statistical significance for the comparison of breast



cancer incidence between the intervention and control groups was equivocal ( $P = 0.09$ ), and some experts interpret this result as clinically valuable since a 9% decrease in risk was reported for the overall study population and a 22% decrease in risk was reported for a subset of women with the highest intake of dietary fat at baseline (38).

Importantly, the intervention group of the WHI did not reach their goal of 20% of energy from fat and this is a major limitation of the study. Secondary analysis revealed that the hazard ratio for a subset of compliant subjects was lower, indicating reduced breast cancer risk relative to the control group for those that were more compliant to the low fat intervention.

## **2. Women's Intervention Nutrition Study**

In contrast to the WHI results, the interim outcome of the Phase III Women's Intervention Nutrition Study (WINS) suggest that a low fat diet is effective in prolonging relapse-free survival in breast cancer survivors (39). The aim the WINS was to reduce fat intake to 15% of energy with the assumption that participants would reach a target of 20% of energy from fat. The WINS cohort was successful in reducing dietary fat intake to 20% of energy and maintaining the reduction for five years of follow-up. Intervention participants lost an average of six pounds, which may have confounded the results (40). Breast cancer relapse was significantly reduced in the intervention group relative to the control group. Moreover, with exploratory analysis the authors discovered that the intervention was more effective in patients with hormone receptor negative tumors. Alternatively, others note that this finding may be due to the higher rate of recurrence in hormone receptor negative tumors within the first 5 years and that a longer follow-up period may yield a greater proportion of hormone-positive tumors (41).

## **3. Women's Healthy Eating and Living Trial**

Counter to the WINS, another study in breast cancer survivors, the Women's Healthy Eating and Living Randomized Trial (WHEL), had non-significant results (42). The WHEL study aimed to reduce dietary fat to 15-20% of energy and increase servings

of fruit, vegetable juice, and vegetables through telephone counseling sessions, newsletters and cooking classes in the intervention group while the control group received printed materials related to the “5-A-Day” guidelines. After an average seven year follow-up, no difference was detected in disease recurrence, new primary tumors, or all-cause mortality between the intervention and control groups. One distinguishing point is that the WHEL intervention group did not maintain a reduction in fat intake after the initial first year’s drop to 21% of energy from fat and the group ended the study at the same percentage of fat intake at which it started (29%). Dietary fat intake at 29% of energy is already reduced compared to the average American population, which may have affected the results. Some experts argue that the WHEL trial was not suitable as a test of the outcome of the WINS (43).

### **III. Omega-3 Fatty Acids**

While the hypothesis that a diet low in total fat reduces breast cancer risk has received inadequate validation from epidemiological and large-scale intervention studies, attention has turned toward investigating the specific type of fat in the diet in relation to breast cancer risk. Increasing omega-3 (n-3) FA and decreasing omega-6 (n-6) FA has largely been the focus of these investigations. Not only do n-3 FA hold promise for primary prevention of breast cancer through hormonal and metabolic means, but they also have shown potential for prevention of breast tumor recurrence and as an adjuvant therapy to chemotherapy (44).

#### **A. Omega-3 and Omega-6 Fatty Acids**

Dietary fat is one of the three macronutrient sources of energy for human beings, the other two being carbohydrates and proteins. Nutritional FA vary in carbon chain length and have either no double bonds (saturated) or one or more double bonds (unsaturated). Unsaturated FA can be either cis-MUFA (one double bond), cis-PUFA (2 or more double bonds) or trans-unsaturated FA (one or more double bonds)(45).

Structurally, the kinked FA chains of PUFA due to multiple cis-double bonds are instrumental in maintaining cellular plasma membrane fluidity (46).

Under normal circumstances, PUFA includes two classes: the various n-3 FA and n-6 FA, so named because the first double bond from the methyl (or “omega”) end of the FA chain is at the 3<sup>rd</sup> or 6<sup>th</sup> carbon, respectively. Omega-7 fatty acids also exist, and under n-6 fatty acid deficiency, a polyunsaturated omega-9 (n-9) FA (20:3n-9) accumulates, but PUFA will refer to n-3 and n-6 FA in this paper.

Linoleic acid (LA, 18:2n-6) and  $\alpha$ -linolenic acid (ALA, 18:3n-3) cannot be synthesized de novo in humans because humans do not possess the enzymes necessary to desaturate at the n-3 and n-6 positions, respectively (45). Linoleic acid and ALA are nutritionally essential because they are necessary for a number of basic physiological processes including those necessitated by lipid mediators, such as eicosanoids, and for plasma membrane structure. Deficiency of LA results in impaired wound healing and dermatitis (46) and n-3 deficiency results in reduced visual acuity and impaired cognition (47) .

Polyunsaturated FA are primarily produced in plants and marine phytoplankton and accumulate in fish and animals that feed on these sources. Cold-water fish (sources of eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]), walnuts (ALA) and flax seed (ALA) are sources of dietary n-3 FA, while corn (LA) and sunflower (LA) oils are high in n-6 FA. Soybean and canola oils are sources of both n-3 FA (ALA) and n-6 FA (LA).

Dietary n-3 and n-6 FA follow the same course of metabolism and compete for the same enzymes of elongation, desaturation and oxidation. Several long-chain highly unsaturated FA (HUFA) including arachidonic acid (AA, 20:4n-6), EPA (20:5n-3), DHA (22:6n-3), and docosapentaenoic acid (DPA, 22:5n-3) are formed by elongation and desaturation of dietary LA and ALA or they are obtained directly from the diet. Highly unsaturated FA remain n-3 or n-6 FA following the desaturation and elongation process. Following desaturation and elongation, AA and EPA are integrated into cellular membrane phospholipids. Di-homo- $\gamma$ -linolenic acid (GLA, 20:3n-6), also derived from LA, is incorporated into plasma membrane phospholipids as well (48).

Dietary intake of n-3 and n-6 FA is reflected in plasma membrane composition of phospholipid FA.

Omega-3 and n-6 FA modulate cellular responses by metabolism to 20-carbon intermediates, known as eicosanoids (eicosa = 20), derived from AA and EPA (49). Phospholipid-bound AA and EPA act as second messengers when they are liberated from the cell membrane by a phospholipase upon stimulation by extracellular signaling molecules (50). Liberated AA is metabolized by cyclooxygenase-1 (constitutive, COX-1) and cyclooxygenase-2 (inducible, COX-2) to form two-series prostaglandin (PG) G<sub>2</sub>, which is subsequently converted to PGH<sub>2</sub>. Prostaglandin H<sub>2</sub> is then converted into a variety of inflammatory lipid mediators (PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2α</sub>, PGI<sub>2</sub>, and thromboxane [Tx] A<sub>2</sub>) the type of which is dependent upon what enzymes are expressed in a given cell (51). Arachidonic acid is also metabolized by lipoxygenase (5-, 12- or 15-LOX) to form leukotrienes (four-series) (52). Prostaglandins and leukotrienes diffuse out of the cell and bind G-protein-coupled cell surface receptors in an autocrine or paracrine fashion, which results in changes in cellular cyclic-AMP or calcium, which in turn affects cellular signaling. Eicosapentaenoic acid, relative to AA, is poorly metabolized by COX and LOX enzymes and forms the less inflammatory eicosanoids (i.e. 3-series prostaglandins) than those derived from AA (52). Through competition for COX, LOX, enzymes of desaturation and elongation and incorporation into cell membrane phospholipids, EPA attenuates the inflammatory effects of AA (50).

## **B. Eicosanoids and Breast Cancer Risk**

Cell studies indicate that eicosanoids may be involved in breast cancer progression (53, 54, 54). Prostaglandin E<sub>2</sub>, derived from n-6 AA, has been shown to increase the activity of aromatase, the enzyme that converts androgens to estrogens (55, 56). Three-series PGE<sub>3</sub>, derived from n-3 EPA, does not have this effect. Concentrations of estrogens are higher in breast tumor tissues than in other tissues, possibly due to increased conversion of estrogens to androgens by the aromatase enzyme or decreased turnover of estradiol in the tumor cells (57). Prostaglandin E<sub>2</sub> may stimulate expression of aromatase in breast tissues including breast stromal and

epithelial cells and mesenchymal (stem cell) adipose cells, thus increasing estrogen concentrations (53, 58). Aromatase expression in adipose tissue is greater at sites abutting a breast tumor than at sites in the breast further from the tumor (59). Prostaglandin E<sub>2</sub> may be the factor generated by tumor cells that leads to increased estrogen production in the adipose adjacent to breast tumors (58, 59). The PGE<sub>2</sub> generated locally at the tumor site may come from infiltrating macrophages or tumor epithelial cells (58). Because PGE<sub>2</sub> is derived from n-6 FA, estrogen levels would be expected to decrease with increased consumption of n-3 FA in proportion to n-6 FA (60).

Arachidonic acid-derived PGE<sub>2</sub> has also been implicated in the process of angiogenesis, the recruitment of new blood vessels by a tumor in order for it to receive adequate oxygen and nutrients (44). Angiogenesis is necessary for continued growth and invasion by a tumor and for metastasis via infiltration of systemic blood flow (44).

### **1. Human Studies: Omega-3 Fatty Acids and Eicosanoids**

It is well established that long-term consumption of foods high in marine n-3 FA imparts relatively high levels of tissue long-chain n-3 FA as seen in the serum of coastal Japanese fishing village inhabitants (61) and in the Canadian Inuit population whose traditional diets are high in n-3 FA (62). Whether the inclusion of n-3 FA in the diet via high n-3 foods or fish oil supplements confers a decrease in systemic inflammatory eicosanoids (i.e. those produced from n-6 precursors) or an increase in less-inflammatory eicosanoids (i.e. those produced from n-3 precursors) was the focus of several investigations (See Table 1-3). Much of the work in this area focused on the prospect of heart disease prevention by shifting prostaglandin synthesis from the two-series prostanoids to the three-series prostanoids. Early studies by Fischer and Weber (63) aimed to identify 3-series prostaglandins derived from EPA in human platelets following cod liver oil ingestion and to test the aggregation of the platelets by collagen stimulation. Following ingestion of 20 mL cod liver oil 2x/week by 8 males, TxB<sub>3</sub> (metabolite of TxA<sub>3</sub>, a less aggregatory eicosanoid than TxA<sub>2</sub>) was formed in the platelets following collagen stimulation, and platelet aggregation was reduced (63).

Fischer and Weber went on to demonstrate the production of PGI<sub>3</sub> in a males consuming 40mL/day cod liver oil (containing 4g EPA) for 24 days or 750g mackerel for 3 days (64). They later determined a dose-response in incorporation of EPA and DHA into plasma phospholipids and free fatty acids relative to differential n-3 fatty acid (cod liver oil) ingestion (65). A urinary metabolite of PGI<sub>3</sub> increased from non-detectable concentrations to about 50 ng/mg creatinine after cod liver oil ingestion, while a urinary metabolite of PGI<sub>2</sub> did not change throughout the study (65).

Ferretti et al conducted a number of studies on n-3 FA and prostaglandin metabolism (66-69). Prostaglandin E<sub>3</sub> production was demonstrated in a woman who had been supplementing her diet with large doses of fish oil (containing 1.8 – 9.0 g/day EPA and 1.3 – 6.5 g/day DHA) for four years (66). Following that study, a number of cross-over design trials were conducted examining the effects of fish oil or a salmon diet on PGE<sub>1</sub>/PGE<sub>2</sub> and TxB<sub>2</sub> metabolism. Ferretti et al investigated the effects of altering the n-6:n-3 ratio of men's diets from 19.5 to 3.6 using a controlled salmon diet and a reference diet (both had composition of 16% energy from protein, 54% energy from carbohydrate, and 30% energy from fat) on urinary PGE<sub>1</sub> and PGE<sub>2</sub> turnover (67). The salmon diet reduced the urinary PGE metabolite by 24%. In another intervention, Ferretti et al supplemented a reference diet with 15g/day placebo oil, then 15g/day fish oil (7.5g/day n-3), then the fish oil capsules plus 200mg/day  $\alpha$ -Tocopherol (68). The fish oil supplement significantly reduced PGE<sub>1</sub> + PGE<sub>2</sub> turnover relative to the placebo oil intervention, but there was no difference between the fish oil and the fish oil plus  $\alpha$ -Tocopherol intervention. 11-dihydro-TxB<sub>2</sub> was also reduced as a result of the previous fish oil intervention (69). Although n-3 FA were not included, Ferretti et al demonstrated reduced urinary metabolites of PGE<sub>1</sub> and PGE<sub>2</sub> following a low fat, high LA dietary intervention (19% energy from fat; PUFA:saturated fat ratio: 1.31; MUFA:SFA ratio: 1.48) compared to a high fat intervention (41% energy from fat; PUFA:SFA ratio: 0.59, MUFA:SFA ratio: 0.96) (70). In addition to these studies, a significant decrease in urinary 2,3-Dinor-TxB<sub>2</sub> was observed with feeding a high n-3 diet (2% energy from n-3) rich in salmon relative to a control diet (71). However there were no differences in urinary TXB<sub>2</sub>, 6-Oxo-prostaglandin F<sub>1 $\alpha$</sub> , 2,3-Dinor-6-oxo-

prostaglandin  $F_{1\alpha}$  or  $PGE_2$  between the two diets (71). A 2-wk fish diet significantly increased platelet and plasma phospholipid 20:5n-3 and 22:6n-3 and plasma phospholipid 22:5n-3 and decreased urinary 11-dehydro-TxB<sub>2</sub> (72).

Studies of fish and fish oil supplements have mostly shown that eicosanoid excretion can be altered by fish diets or fish oil supplementation of the diet. These studies were mostly done in men and the effects of a fish diet, high in n-3, on circulating sex hormones is unknown. The cell and animal studies outlined above indicate that a change in sex hormone metabolism may be observed by altering eicosanoid metabolism (particularly that of  $PGE_2$ ).

### **C. Human Studies: Omega-3 and Breast Cancer Risk**

#### **1. Epidemiological Studies**

Evidence from epidemiological studies of a role of n-3 FA on breast cancer risk is conflicting (See Table 1-4). Several prospective cohort and case-control studies utilized FFQ to gather data on intake of different types of dietary fat (73-78) and fish intake (74, 76). Goodstine et al (75) and Lof et al (77) found no associations between total fat and fat subtypes in their entire study populations, although risk was reduced in a subgroup of women over 50 years with high PUFA and MUFA intake (77). In the Malmo Diet and Cancer Cohort, total fat, MUFA, PUFA, and particularly n-6 PUFA were positively associated with breast cancer risk (73). Gago-Dominguez et al reported n-3 FA intake from shellfish was inversely associated with breast cancer risk in the Singapore Chinese Health Study (74) while no association was found between total fish intake and breast cancer risk in the European Prospective Investigation into Cancer and Nutrition (EPIC) study (76). In a relatively recent systematic review, MacLean et al reviewed prospective cohort studies that investigated the associations between n-3 FA and risk for 11 types of cancer (79). Breast cancer risk was addressed in eight of the studies reviewed: 1 showed increased risk of breast cancer with high fish intake; 1 showed decreased risk for women who had high intake of dried fish; 2 showed no association between fish intake and breast cancer risk; 2 indicated no association between total n-3 intake and breast cancer risk (although 1 of these showed decreased

risk for women in the highest quartile of marine FA intake); 1 study reported no association between EPA and DHA and breast cancer but a reduced risk for women in the highest quintile of ALA intake; and one reported an increased risk of breast cancer with high EPA + DHA intake. No clear pattern emerges regarding breast cancer risk and n-3 fatty acids in prospective cohort studies.

In addition to FFQ, dietary fat intake can be measured by analysis of biological samples (See Table 1-4). Analysis of biological samples as biomarkers of fat intake is attractive because the patient's memory and food details are no longer sources of error. Because n-3 and n-6 fatty acids cannot be synthesized *de novo* in humans, tissue concentrations of these fatty acids reflect dietary intake (80, 81). Red blood cell FA (82, 83), serum FA (84), and human adipose tissue (85-87) have been used as biomarkers. In some of these studies, breast cancer risk is reduced with increasing concentration of n-3 FA (83, 87) while others report no association (82, 84-86). Pala et al report a protective effect of n-6 FA (82). Saadatian-Eliahi and colleagues analyzed 3 prospective cohort studies and 7 case-control studies in a meta-analysis of fatty acid biomarkers and breast cancer risk (88). A decrease in risk was observed for total n-3, EPA and DHA in postmenopausal women in prospective cohort studies, but these results were not observed in case-control studies.

As noted by Terry et al in their 2003 review (89), most of the studies that investigated the marine and long-chain n-3 FA and breast cancer risk question were case-control studies (19 case-control studies vs. 7 prospective cohort studies), noted for recall bias addressed below. Terry et al reviewed both studies that used FFQ and tissue FA concentrations to assess dietary exposure to long-chain n-3 FA, and they concluded that there was little evidence of an association between breast cancer risk and fish or long-chain n-3 FA and recommended that future studies assess specific marine FA. Terry et al published an updated review (90) in 2004 that included an additional 7 prospective cohort studies and 6 case-control studies. The additional evidence did not clarify an association and they further concluded that the current epidemiological evidence does not support a recommendation regarding fish intake and prevention of breast cancer.



## **2. Limitations of Dietary Assessment Methods**

Most published studies investigating the effects of total fat and n-3 FA have relied on relatively inexpensive FFQ as dietary assessment methods, which are subject to recall bias and dietary measurement error (91). Recall bias may occur in case-control studies because the presence of the disease may affect the dietary reporting of cases but not controls (29). Prospective cohort studies are regarded with more confidence in this regard because diet is reported before the onset of disease; therefore the error associated with the dietary instrument is the same for women who develop the disease and for those who do not (29).

Dietary measurement error related to the FFQ may provide an explanation for null findings of cohort studies. A study by Bingham et al measured dietary intake by both FFQ and a 7-day food diary in a cohort of 13,070 women aged 45 – 74 years (92). Saturated fat was not significantly associated with breast cancer when diet was assessed by FFQ while a significant association was observed when the diet was assessed by 7-day food record. The authors stressed that measurement error of the FFQ may be attenuating the associations observed between dietary fat and breast cancer risk in cohort studies. Hietman and Frederiksen contend that even the 7-day food record is not adequate to assess diet-disease relationships because of the error in reporting in general, particularly in under-reporting by overweight and obese participants (93). Unfortunately, a biomarker for total fat intake has yet to be developed and validated for use in epidemiological investigations, so there is no alternative for these dietary assessment methods for measuring dietary fat intake at this time.

Studies measuring fish and n-3 fatty acid intake are also limited by the questionnaires used to measure dietary intake. Many studies used data from FFQ that did not distinguish between fatty and lean fish (89), rendering these studies insensitive in quantifying n-3 intake. More recent FFQ, such as the instruments used in the EPIC study (76) have addressed this problem, however there is still considerable heterogeneity in FFQ questions related to fish intake among research investigations and even among sites of a multi-site study.

### **3. Dietary Intervention Studies: Omega-3 and Tissue Fatty Acids**

Consumption of dietary sources of n-3 FA, such as fatty fish, flax seed and walnuts, or ingestion of fish oil supplements are ways to increase tissue concentrations of n-3 FA. A study by Bagga et al (50) instructed women with advanced cancer to follow a low fat diet (15% of energy from fat) and consume fish oil capsules supplying 3g of n-3/day for 3 months. Gluteal and breast adipose tissue and plasma samples were taken at baseline and after 3 months of the intervention. Plasma total n-6 PUFA, LA, and AA were significantly reduced while total n-3 PUFA, EPA, DHA, EPA:AA ratio and the n-3:n-6 PUFA ratio were significantly increased after the intervention. In breast and gluteal adipose tissue, total n-3, EPA and DHA concentration and EPA:AA and n-3:n-6 PUFA ratios increased significantly after the intervention. Breast adipose tissue more readily incorporated the n-3 FA than gluteal adipose tissue (94). In addition, one study showed that plasma levels of n-3 FA could be increased by consumption of a low fat diet utilizing whole foods without supplemental n-3 (94). Raatz et al conducted a cross-over design trial and found that feeding humans a low fat diet vs. a high fat diet with equal proportions of SFA, MUFA, and PUFA significantly increased concentrations and proportions of plasma total n-3, EPA, DHA, and AA and reduced total n-6 and LA phospholipid FA (PLFA) (94). The authors concluded that the low fat diet treatment was similar in effect to supplementation with n-3 FA due to decreased total n-6 FA and thus decreased competition for the enzymes of desaturation and elongation when the volume of n-6 was reduced. Increasing the proportion of n-3 FA in cell membrane phospholipids and in turn the breast microenvironment may mediate protective effects of n-3 FA on breast cancer.

#### **Summary of Human Studies Relating Dietary Fat and Breast Cancer Risk**

Ecological studies showed a positive association between dietary fat disappearance and breast cancer risk; however the epidemiological studies on total fat and breast cancer risk that followed did not strongly support an association. The single large-scale human intervention trial that aimed to reduce dietary fat intake in order to

reduce breast cancer risk in healthy women, the WHI, showed a nearly significant association between lower fat diet and breast cancer risk. However, the WHI intervention group did not meet the fat reduction goal, rendering the results difficult to interpret. Yet another similar low-fat dietary intervention in breast cancer survivors (WINS) increased relapse-free survival. It is possible to increase tissue n-3 fatty acids with diet and fish oil supplements, but epidemiological investigations that measured type of fat and fish intake by FFQ in relation to breast cancer risk yielded mostly null results. Studies that measured red blood cell phospholipids, serum FA, or human adipose as an index of dietary FA intake showed mixed results as well. While the hypothesis of whether dietary fat affects breast cancer risk has been debated for over 60 years, there is still no clear conclusion.

#### **D. Animal Studies: Dietary Fat and Mammary Cancer**

Unlike data from human trials, animal studies demonstrate a tumorigenic effect of high total fat diets on mammary tumors regardless of whether tumors were spontaneous, chemically induced or surgically implanted. (See Table 1-5). In the 1950's Silverstone and Tannenbaum fed spontaneous mammary tumor model mice diets ranging from 2-26% fat (by weight) and found that the incidence and rate of spontaneous mammary tumor formation increased in a dose-dependent manner (95). In contrast, a high fat diet (32.5% energy from fat) designed to induce obesity did not affect spontaneous mammary tumor incidence relative to an 11% energy from fat diet in mouse mammary tumor virus-transforming growth factor  $\alpha$  (MMTV-TGF $\alpha$ ) transgenic mice (96). However, tumor latency was reduced in the obesity-prone group of mice, indicating that the high fat diet enhanced tumor promotion.

As in human studies, both type of fat and amount of fat is of interest in animal studies. High n-6 FA in a high corn oil diet (20% corn oil by weight) increased chemically-induced mammary tumor incidence in rats (97). Yee et al demonstrated that a 25% of energy from fish oil diet reduced incidence and number of mammary tumors compared to a 25% of energy from corn oil diet in MMTV-Her-2/neu transgenic mice (98). High n-3 FA diets reduced the size of implanted mammary tumors (99, 100) and

reduced the amount of circulating (100) and tumor (99) PGE<sub>2</sub> in rats. Diets high in EPA and DHA reduced lung metastasis from implanted MDA-MB-435 cells in nude mice (101). Tumor incidence was decreased and the latency period of tumors was increased in rats fed diets high in menhaden fish oil (high in DHA and EPA) (102, 103). A menhaden oil (20% by weight) diet also reduced the number of malignant tumors and mammary eicosanoids relative to a 20% (by weight) corn oil diet in rats (104). A study designed to assess the effects of n-3 FA during early life fed 5-day old rats high fat (39% of energy from fat) or low fat (16% of energy from fat) n-6 (corn oil) or n-3 (menhaden oil) diets until age 25 days, then chemically induced tumors at age 50 days (105). Compared to the low fat n-6 diet, the low fat n-3 diet decreased mammary tumor incidence, promoted mammary terminal end bud differentiation, increased apoptosis, and reduced proliferation and COX-2 activity while high n-3 diets increased tumor incidence and proliferation and decreased apoptosis. This study indicates that low levels of n-3 feeding during early life are protective while high levels may be harmful.

A number of meta-analyses have been conducted on the vast animal literature on the topic of dietary fat and breast cancer risk. Freedman et al conducted a meta-analysis on the effects of caloric intake, body weight, and diets high in fat from a single source on breast carcinogenesis in rats and mice (106). The authors conclude that a high fat diet has an independent, greater tumor promoting effect relative to weight gain and caloric intake (106). The authors reason that extra calories from fat would have a greater tumor enhancing effect than if the animals ate an equivalent amount of lower-fat food (106). A later meta-analysis indicated the tumor-promoting effect of the diets was due predominantly to n-6 FA and to a lesser degree, SFA (107).

Wynder et al (108) maintain that there is a threshold amount between 20-30% of energy from fat above which tumor promotion is initiated (108). Standard rodent chow consists of roughly 10% of energy from fat (108), so it's important to note that the levels of fat in many of these experiments are far greater than in standard lab rodent diet. Applicability of these results to human disease is uncertain.

#### **IV. Postmenopausal Sex Hormones**

Hallmarks of the menopause include termination of the menstrual cycle brought about by exhaustion of ovarian hormone production and the cessation of reproduction in women (109). Few follicles remain in the ovaries at menopause and ovarian estradiol (E2) production drops dramatically. This drop is accompanied by a drop in levels of inhibins and an elevation of follicle stimulating hormone (FSH) levels as E2 feedback to the hypothalamus diminishes. The ovarian-derived E2 deficit following menopause has mixed effects on body tissues. Estrogens are protective against heart disease and osteoporosis (110), but relatively high levels of postmenopausal estrogens are associated with breast cancer (111). Symptoms including hot flashes, frequent urination and vaginal dryness accompany the reduction in circulating E2 with menopause (112).

As ovarian hormone production declines post menopause, the majority of estrogen production shifts to the peripheral tissues, such as adipose, bone and brain, where androgens are metabolized to estrogens (53, 58). The reaction by which the androgens testosterone (T) and androstenedione (A) are converted to the estrogens E2 and estrone (E1), respectively, is mediated by the aromatase enzyme, also known as cytochrome P450 19 (CYP19) (See Figure 1-2) (58, 113). Aromatase is the rate-limiting enzyme in the conversion of androgens to estrogens (114). Aromatase is expressed in a tissue-specific manner and is mediated by several promoter sites (58). Aromatase expression is regulated by cyclic AMP via FSH stimulation in the ovary and by PGE<sub>2</sub> in tumors (58). In peripheral tissues, estrogen production is no longer endocrine in nature; the tissues produce estrogens locally and they act in a paracrine or autocrine fashion (58). Plasma levels reflect hormones that have spilled over into the bloodstream (58).

##### **A. Sex Hormones and Breast Cancer in Postmenopausal Women**

The causes of breast cancer are myriad and related to endogenous factors, environmental factors, and their effects on genes. Two factors are notable in the causes of cancer: agents causing DNA lesions and agents quickening cell division (115). Genetic mutations form when DNA adducts are not rectified before cell division. Once

a DNA lesion has formed, exogenous or endogenous growth factors, such as estrogens, stimulate the damaged cell to replicate and the mutation becomes permanent. If a mutation is in an area of DNA critical to cell cycle control, DNA repair, or metabolic activation of carcinogens, the mutation may lead to cancer development.

Endogenous sex hormones affect the growth and differentiation of breast tissue throughout the life cycle and are found in high levels in hormone receptor positive breast tumors (53). The prevailing hypothesis regarding estrogen's role in breast cancer is that estrogens promote cancer by binding estrogen receptors and inducing expression of genes leading to cell growth and division (116). Enhanced cell division may lead to the accumulation of genetic mutations in the areas of DNA that code for apoptotic proteins, DNA repair enzymes, and cell cycle control proteins leading to cancerous transformation of the cell (116). Also, estrogens may be metabolized into genotoxic metabolites that bind DNA and induce cancer (116, 117). Blood and urinary sex hormone levels have been associated with breast cancer in a great number of case control studies and are utilized as biomarkers of breast cancer risk. Plasma sex hormone levels are convenient surrogates for measuring hormone activity at the tissue level but may not indicate true tissue levels (57, 58).

### **1. Plasma Hormones**

There is convincing evidence that elevated endogenous estrogens and androgens are associated with increased breast cancer risk in postmenopausal women. Circulating hormone levels and their relation to breast cancer risk was measured in a number of case-control studies (See Table 1-6). In postmenopausal women, elevated plasma E2 (118-126), E1 (119, 122-125), estrone-sulfate (E1-S) (119, 122-125), A (119, 122-125), T (118-126), dehydroepiandrosterone (DHEA) (125, 127, 128) and dehydroepiandrosterone-sulfate (DHEAS) (122-125, 127, 128) were associated with increased breast cancer risk, while elevated sex-hormone binding globulin (SHBG) level was associated with decreased risk (118-126). Breast cancer risk was not increased with elevated DHEAS concentration in one study (129). In postmenopausal women, concentrations of E2, E1, T and E1-S increased as BMI increased, while SHBG

decreased as BMI decreased (130). Dehydroepiandrosterone, DHEAS and A decreased with aging (130).

However, case-control studies are criticized because the higher concentration of circulating estrogen may be due to the presence of breast cancer (131). For this reason, prospective cohort studies are deemed superior because all women in the cohort were without a cancer diagnosis at the onset of the study. The authors of a quantitative review of 29 case-control and prospective cohort studies (conducted from 1966 – 1996) found that in the six prospective studies reviewed, concentrations of estradiol in cases were 15% greater than in women who did not develop breast cancer (131). Case-control studies that were reviewed had a similar pattern.

Additional evidence that sex hormones drive breast cancer is that the ablation of systemic estrogen action with the anti-estrogen, Tamoxifen (58, 132), and the removal of both ovaries greatly reduces risk (133). Also women, who have far greater life-time exposure to circulating estrogens than men, have a greatly increased risk of breast cancer.

## **2. Breast Tissue Sex Hormones**

Breast tissue analysis allows for measurement of hormone levels directly in the tissue (See Table 1-7). Breast tumor tissue, tissue adjacent to the tumor, and normal glandular tissue were analyzed for content of sex hormones and sex hormone metabolizing enzymes (134, 135). Tumor tissue contained significantly more E1-S, E2 and sulfatase and aromatase enzymes than normal tissue (134, 135). Breast tumor tissue contained significantly higher levels of sex hormones than plasma, suggesting that tumors sequester estrogens (134).

## **3. Urinary Sex Hormone Metabolites**

Metabolites of plasma estrogens may also affect carcinogenesis. Circulating estrogens are metabolized in the liver via several CYP450 enzymes which may lead to inert or readily excretable polar products or to activated compounds with increased bioactivity or carcinogenicity (114, 136). Some of the estrogen metabolizing enzymes

may be selectively expressed in peripheral tissues, including the breast (114). Effects of estrogen metabolites may or may not be mediated by estrogen receptors (114, 116).

Phase 1 metabolism of estrogens generally occurs via hydroxylations at the position of carbon 2, 4, or 16 by NADPH-dependent CYP450 enzymes (See Figure 2) (137, 138). The products of these hydroxylations at the 2 and 4 positions are 2-hydroxyestradiol (2OH-E2), 2-hydroxyestrone (2OH-E1), 4-hydroxyestradiol (4OH-E2), and 4-hydroxyestrone (4OH-E1) and are referred to as catechol estrogens (117). Hydroxylation at C16 produces 16 $\alpha$ -hydroxyestradiol (estriol) and 16 $\alpha$ -hydroxyestrone (16 $\alpha$ OH-E1) (137, 138). The 2-OH catechol metabolites of estradiol and estrone, metabolized by CYP1A2 and CYP3A4, are the major products formed (136, 136, 138, 139). The 2- and 4-OH catechol estrogens are further metabolized by catechol-O-methyltransferase (COMT) to a less oxidizable form with the addition of a methyl group to the 2- or 4- OH group respectively, yielding 2-methoxy E2 (2-ME2), 2-methoxy-E1 (2-ME1), 4-methoxy E2 (4-ME2), and 4-methoxy-E1 (4-ME1).

E2, E1, plus the phase I estrogen metabolites can be sulfated by estrogen sulfotransferase 1A1 which greatly increases the half-lives of the hormones (114, 137). Addition of a sulfide, glucuronide, or methyl moiety leads to excretion of the hormone or metabolite in the urine or feces (137).

The different metabolites of E2 and E1 have a variety of biological activities and some pathways of metabolism are associated with increased breast cancer risk. Some metabolites bind the estrogen receptor (ER) and elicit estrogenic activity while other activities are mediated by non-ER pathways (114). Other metabolites form reactive intermediates that form DNA adducts. In general, the 2-hydroxy and 2-methoxy metabolites are considered to be inert or have anti-cancer effects relative to estradiol, while the 4-hydroxy estrogens and 16 $\alpha$ OH-E1 are estrogenic and carcinogenic.

#### **a. Two-hydroxy Estrogens**

Hydroxylation of estrogens at the 2 position is quantitatively greater than hydroxylation at the 4 or 16 positions (114). The 2-hydroxy estrogen metabolites are



considered to be non-estrogenic due to their low-affinity for estrogen receptors (114, 138). Two-hydroxy-E2 and 2OH-E1 lack tumorigenic activity partially due to their high rate of clearance and rate of O-methylation via COMT to 2-ME2 and 2-ME1 (114). However, 2OH-E2 has been shown to damage DNA when COMT is inhibited (140). In contrast, others have shown that 2OH-E2 inhibits growth and proliferation in cell culture when COMT is inhibited (141).

#### **b. Two-methoxy Estrogens**

2OH-E2 is metabolized via COMT to 2-ME2, which has cancer preventive effects. 2-ME2 has antiproliferative, antiangiogenic, and antimetastatic properties due to its effects on microtubule stabilization, and thus cell migration and mitosis (138, 142, 143). In MCF-7 cells, 2-ME2 inhibited proliferation, DNA synthesis, and mitosis, possibly due to increased phosphorylation of S-phase mitotic proteins (144). 2-ME2 has demonstrated disruptive effects on spindle formation in tumor cell lines including MCF-7 and HeLa cells leading to cytotoxicity (145). 2-ME2 inhibited the angiogenesis of cornea (146) and melanoma (143) in mice. Analogs of 2-methoxy estrogens have been synthesized for the purpose of cancer treatment (142).

#### **c. Four-hydroxy Estrogens**

The small amounts of 4OH-E2 and 4OH-E1 metabolites generated can form quinone intermediates that react with DNA to form depurinating adducts (116, 147). The purine moiety dissociates from the DNA and point mutations may result if the apurinic site is not detected by DNA-repair enzymes. If lesions occurring at critical points in DNA are not corrected before cell division, the resulting mutation could initiate breast cancer (147). The reactive 4-hydroxy intermediates can also damage cellular proteins and lipids (114). Estrogen quinone DNA conjugates are indeed formed in MCF-7 breast cancer cells (116) and humans (148). Quinone intermediates may be inactivated by reduction to 4OH-hydroxy catechols or by the addition of glutathione by glutathione-S-transferase; therefore cellular levels of these enzymes are important for

cancer prevention (147). Methylation of catechol estrogens by COMT also limits the formation of estrogen quinones (117, 148). Both 4-hydroxy estrogens and 2-hydroxy estrogens form quinone-DNA adducts, however 2,3-quinones form stable adducts, while 3,4-quinones produce apurinic sites (117). In humans, 4-hydroxylation of estrogens was two to four times greater in microsomal preparations from breast tumors relative to normal breast tissue (149). Four-hydroxy estrogen quinones were greater in breast cancer tissue indicating impaired clearance of the reactive intermediates (147). The ratio of urinary quinone-estrogen DNA adducts to their parent catechol estrogen or conjugated catechol estrogen was significantly greater in breast cancer cases and women at high risk of breast cancer, indicating that the ratio could be used to predict breast cancer presence (148).

#### **d. Sixteen-hydroxy Estrogens**

16 $\alpha$ OH-E1 is a potent mitogen (141), tumor initiator (150), and tumor promoter in vitro (150). Sixteen- $\alpha$ OH-E1 can form a covalent bond with the ER $\alpha$  and the receptor /16 $\alpha$ OH-E1 complex localizes to the nucleus, indicating that the complex affects gene transcription (114, 151). Sixteen- $\alpha$ OH-E1 has demonstrated qualities of a cancer initiator in mouse epithelial cells where 16 $\alpha$ OH-E1 treatment transformed cells similarly to that of known carcinogen 7,12-dimethylbenz[ $\alpha$ ]anthracene (DMBA) (150). Initiation was evidenced by increases in cell proliferation, in DNA synthesis/repair, and in formation of soft agar colonies (150). Increased 16 $\alpha$ OH-E1 metabolism accompanies ras-oncogene cell transformation in mouse mammary epithelial cells (152). In human breast tissue, 16 $\alpha$ OH-E1 concentration was eight times higher in cancerous mammary terminal duct lobular units than in adjacent mammary fat tissue and four times higher than in non-cancerous terminal duct lobular units (153) indicating that estrogen metabolism is shifted towards the 16 $\alpha$ OH-E1 product in breast cancer. Circulating levels of 16 $\alpha$ OH-E1 were higher in breast cancer cases than controls (154, 155).

#### **e. Ratio of Two-hydroxy Estrogens to Sixteen-hydroxy Estrogens**

The ratio of 2OH-E1 to 16 $\alpha$ OH-E1 has been used as an index of breast cancer risk because 2OH-E1 is considered to be estrogenically inert and 16 $\alpha$ OH-E1 is highly estrogenic and carcinogenic. Two-OH-E1:16 $\alpha$ OH-E1 ratio has been assessed in both the blood and the urine relative to breast cancer risk in case-control and prospective cohort studies (See Table 1-8). The results of these investigations are conflicting. In postmenopausal women, urinary 2OH-E1:16 $\alpha$ OH-E1 ratio was significantly lower in breast cancer cases than controls in some studies (156-158) and this translated to decreased breast cancer risk for women with high 2OH-E1:16 $\alpha$ OH-E1 ratio (156, 157). In contrast, breast cancer risk was increased in women who were taking hormone replacement therapy who had relatively high urinary 2OH-E1 concentrations (159). No significant association between urinary 2OH-E1:16 $\alpha$ OH-E1 ratio and breast cancer risk was found by several others (160-164). Additionally there was no significant association between 2OH-E1:16 $\alpha$ OH-E1 ratio and breast cancer risk in the two studies that measured 2OH-E1 and 16 $\alpha$ OH-E1 in the blood (165, 166) although breast cancer risk was increased in women who had high concentrations of 2OH-E1 and 2OH-E1:16 $\alpha$ OH-E1 ratio and who also had estrogen receptor negative/progesterone receptor negative tumors (166).

Many of the studies were likely underpowered to detect an association given the high variability of urinary sex hormone measurements. In one study, high 2OH-E1:16 $\alpha$ OH-E1 ratio was associated with increased breast cancer risk in cases who gave urine samples after cancer treatment whereas associations were not detected in cases who gave samples prior to treatment for breast cancer or in the entire cohort (164). Other authors also mentioned that they saw large variability in the 2OH-E1:16 $\alpha$ OH-E1 ratio in samples collected before treatment and those collected 2 months after treatment (158). Thus the timing of sample collection from cases is likely to complicate many of these case-control studies.

#### **f. Urinary Estrogen-DNA Adducts**

One case-control study investigated the presence of urinary DNA-estrogen adducts in women with breast cancer, women at high risk for breast cancer, and control women (148). This is a great advancement because the DNA-adducts in the urine indicate the amount of DNA damage that has occurred due to reactive estrogen metabolite intermediates. The authors measured 40 urinary estrogens, androgens, estrogen metabolites and estrogen metabolite-DNA adducts and compared mean amounts of each compound among groups. They then compared estrogen metabolite-DNA adduct:parent estrogen metabolite ratios for each DNA adduct (148). Testosterone, A, E1, E2, 16 $\alpha$ OH-E2, 4MeOE2, and 4OH-E2-1-N3Adenine were significantly higher in high risk women than controls and 2OH-E2, 4OH-E2, 16 $\alpha$ OH-E2, and 4OH-E2-1-N7-Guanine were significantly greater in cases than controls. Ratios of estrogen metabolite-DNA adducts:parent estrogen metabolite were also higher in high risk women and cases than in control women, indicating that more severe genetic damage occurred in the cases and high risk women. Concentrations of estrogen metabolite-DNA adducts may be used in the future as an indicator of breast cancer risk.

#### **4. International Comparisons**

Differences in sex hormone metabolism may explain disparate breast cancer rates among nations. Adlercreutz et al (167) compared the estrogen metabolism of two groups of premenopausal women: Finnish women (high risk for breast cancer) and Oriental women who recently immigrated to Hawaii (low risk for breast cancer) and found that plasma E1-S and E2 and urinary E1, E2, and catechol estrogens were significantly higher in the Finnish women than the Oriental women. Another cross-sectional study compared healthy postmenopausal women from America to women from Singapore, which has a much lower incidence of breast cancer than the United States (168). Urinary 2OH-E1 concentration was significantly greater in the Singapore women, but concentrations of 16 $\alpha$ OH-E1 and the 2OH-E1:16 $\alpha$ OH-E1 ratio did not differ between groups. Urinary E1, E2, and E3 were significantly greater in the American women.

## **V. Dietary Fat, Sex Hormones, and Breast Cancer Risk**

Since sex hormones are related to breast cancer risk, it may be via alteration of sex hormone levels that dietary fat may affect breast cancer risk.

### **A. Epidemiological Studies**

There are a number of observational epidemiological studies investigating the influence of dietary fat on sex hormones (See Table 1-9). Early studies in small numbers of subjects (up to 13 per group) investigated the differences in hormone metabolism between groups with different diets as well as disparate breast cancer risks, such as Oriental women vs. American women and vegetarians vs. omnivores. When the diets of vegetarian postmenopausal women, at relatively reduced risk for breast cancer, were compared to those of omnivores, total energy did not differ between groups while the vegetarians consumed 36% as much animal fat as the omnivores consumed (169). Blood, urinary, and fecal hormones were measured in these women, and urinary excretion of E1, E2, and E3 did not differ while fecal excretion of these estrogens was significantly greater in vegetarians (169). This result indicates that the enterohepatic circulation of estrogens differs between the two groups. In another study, vegetarian and omnivorous women were studied with the addition of a group of breast cancer survivors (170). The omnivores reported consumption of significantly more total energy and total fat than either the breast cancer survivors or the vegetarians (170). Concurrently, the omnivores had significantly increased A, T, free T, and SHBG relative to the vegetarian group while the breast cancer survivors had higher T and A than vegetarians and reduced free T and SHBG compared to the other two groups (170). Armstrong et al compared a group of postmenopausal Seventh-Day Adventist vegetarians, who followed prudent lifestyle practices including abstaining from alcohol and cigarettes in addition to other dietary practices (171), to a group of omnivorous women and found that the Seventh-Day Adventists consumed less fat than the omnivores (172). Omnivores had increased plasma prolactin and SHBG and increased excretion of urinary E3 than the Seventh-Day Adventists (172). Taken together, these studies indicate that vegetarians tend to report a lower consumption of dietary fat and

tend to have lower concentrations of plasma androgens and SHBG. The results for fecal and urinary excretion of estrogens were mixed.

The diets and sex hormone concentrations of Oriental women who had recently immigrated to America were compared to Caucasian women living in the United States (173) and Caucasian women living in Finland (167). In both studies the dietary fat intake of the Caucasian women was significantly greater than that of the Oriental women. Urinary E1, E2, and E3 were relatively increased in both Caucasian groups relative to the Oriental women in each study. Plasma E2, E1 + E2, T and A were significantly greater in the Caucasian women from the United States (173) and plasma E1-S and E2 were significantly higher in the women from Finland (167) relative to their Oriental counterparts. Again, the women with the evidenced decreased breast cancer risk and concomitant lower dietary fat intake had lower plasma and urinary hormone concentrations.

However studies from larger cohorts have predominately failed to find associations between dietary fat or types of dietary fat and sex hormone concentrations. Two cross-sectional studies in American women failed to find associations between reported total fat intake and fat subtypes and any of the hormones measured (174, 175). Estradiol levels were inversely associated with total dietary fat intake in the NHS cohort, contrary to the prevailing hypothesis that high dietary fat intake raises estradiol levels (176). Cross-sectional studies of Oriental women from Singapore (177) and Japan (178) yielded conflicting results: no associations were found between dietary fat intake and sex hormone levels in one study (177), while serum E1 and DHEAS were positively associated with percent energy from fat in another study (178).

## **B. Dietary Intervention Trials**

### **1. Blood Hormones**

On the other hand, controlled intervention trials have shown changes in circulating sex hormone concentrations in postmenopausal women with dietary fat reduction (See Table 1-10). Prolactin levels decreased in free-living women instructed to reduce percent of energy from fat for 4 weeks (179). Circulating E2 levels were

significantly reduced in women enrolled in a residential wellness program in which participants living at the Pritkin Longevity Center (Santa Monica, CA) had free access to very low fat (<10% calories from fat), high fiber foods for three weeks (180). In another study at the Pritkin Longevity Center involving two groups of 11 women (11 on hormone replacement therapy (HRT), 11 not on HRT), SHBG increased significantly in both groups while insulin, body mass index (BMI), and total cholesterol decreased significantly after a three week low fat (<10% energy from fat) diet and exercise intervention (181). Estradiol was reduced in free-living women taking part in the Women's Health Trial, a preliminary version of the low fat intervention portion of the WHI (182). In the Diet and Androgens (DIANA) Randomized Trial, SHBG was significantly increased and T was significantly decreased in postmenopausal women randomized to a diet intervention aimed at reducing intake of fats from animal sources and refined carbohydrates and increasing intake of soy foods and unsaturated FA compared to controls (183). There are multiple dietary factors involved in these studies, particularly the DIANA intervention, so the hormone changes cannot be attributed solely to changes in dietary fat intake. In contrast, no hormone changes were seen after 8 weeks of a low fat (11% fat) diet, Step 1 Diet (30% fat) plus soy foods, or a Step 1 diet (30% fat) (184). Notably, participants in the above interventions (179, 180, 182, 183) lost a significant amount of weight during the studies, which alone may affect hormone levels and confound results. The above studies indicate that circulating sex hormone levels may be affected by changes in dietary fat intake. None of the studies isolated the effects of n-3 fatty acids on circulating sex hormone metabolism.

## **2. Urinary Hormones**

A few dietary intervention studies involving urinary sex hormones and dietary fat reduction or n-3 fatty acids have been carried out (See Table 1-11). Three studies investigated low fat dietary interventions in relation to estrogen excretion. In an early study, Longcope et al examined blood and urinary metabolism of radio-labeled E2 in premenopausal women (185). They found that following a low-fat diet (25% of energy from fat, 18% of energy from protein, and 57% carbohydrates) for two months, estrogen

metabolism was shifted significantly towards the 2-hydroxylation pathway and away from the 16-hydroxylation pathway compared to estrogen metabolism following a high fat diet (40% of energy from fat, 17% of energy from protein, and 43% of energy from carbohydrate) (185). Dorgan et al carried out a controlled, cross-over feeding study in men involving two diets, a low fat/high fiber diet (20% energy from fat with a PUFA:saturated fat ratio of 1.3 and ~61g fiber/day) and a high fat/low fiber diet (40% of energy from fat, PUFA:saturated fat ratio of 0.5, and ~ 27g fiber per day), for 10 weeks each (186). Plasma SHBG-bound T and urinary excretion of T were significantly greater with the high fat/low fiber diet, but urinary excretion of E1, E2, 2OH-E1, and 2OH-E2 were significantly higher with the low fat/high fiber diet (186). Carruba et al randomized healthy postmenopausal women to follow either their regular diets (control) or a traditional Mediterranean diet and attend a weekly meal preparation class on Mediterranean cooking (intervention). A panel of urinary estrogens (see Table 1-11) was measured before and after 6 months of the intervention or control diet. The intervention group reduced their intake of a number of nutrients, including total energy, total protein, animal protein, total fat, animal fat, sat fat, cholesterol, carbohydrates and sugar and the intervention group had significant reductions in total estrogens, 2OH-E2, 16keto-E2, and 17epiestriol. The Longcope et al (185) intervention was in premenopausal women and the Dorgan et al (186) intervention was in men, so they do not relate directly to estrogen metabolism in postmenopausal women. The Carruba (187) was in women, but it is difficult to isolate the effects of lowering the fat content of the diet when so many other dietary changes were made with the adoption of the Mediterranean diet.

Two studies investigated the intake of n-3 fatty acids and urinary estrogen metabolism (See Table 1-11). Osborne et al reported preliminary results from a pilot study that involved an n-3 FA supplement in a short article that gave no information about the study subjects or the dose/type of n-3 fatty acid supplement used (188). The authors reported decreased estrogen hydroxylation via the 16-hydroxylation pathway with the n-3 fatty acid supplement. Unfortunately, neither the final results of this study nor a mention of the larger-scale intervention the authors referred to in that article can



be found in the literature. Wu et al tested the effects of a DHA-rich algae oil supplement (2.14g/day DHA) vs. a placebo corn oil capsule on urinary 2OH-E1, 16 $\alpha$ OH-E1, 2OH-E1:16 $\alpha$ OH-E1 ratio, F2-isoprostanes and plasma lipids in postmenopausal women (189). Following 6 weeks of the supplement, there was no change in estrogen metabolism or F2-isoprostane even though plasma LDL DHA and EPA were significantly increased by the supplement. More studies on n-3 fatty acids and urinary estrogen metabolism are warranted due to the conflicting results of these two studies and the lack of information on any estrogen metabolites other than 2OH-E1, 16 $\alpha$ OH-E1 and the 2OH-E1:16 $\alpha$ OH-E1 ratio.

## **VI. Insulin-like Growth Factor (IGF) Pathway**

### **A. IGF Pathway Background**

The insulin-like growth factor (IGF) pathway is comprised of three hormones (IGF-I, IGF-II, and insulin), several IGF binding proteins (IGFBP 1-6), three receptors [IGF-I receptor (IGF-IR), IGF-II receptor (IGF-IIR) and insulin receptor (IR)], and their downstream signaling cascades. IGF-I is a peptide hormone predominantly secreted by the liver in response to pituitary-derived growth hormone (GH) (190). IGF-I is secreted to a lesser degree by peripheral tissues, including the breast, and acts in an autocrine and paracrine fashion in these tissues (191). In the blood, approximately 90% of IGF-I is complexed with IGFBP-3 and acid labile subunit in a 1:1:1 ratio which increases the half-life of IGF-I (192). IGF-I levels are greatly reduced with protein and energy restriction (193) and in cancer cachexia (194).

Circulating IGF-I and IGFBP-3 are markers of biological activity of the cellular-level IGF pathway (195). IGF-I binds the IGF-IR, which is a homodimer, cell-membrane embedded tyrosine kinase. IGF-IR is expressed in all tissues except the liver and white blood cells (190). IGF-I stimulates a number of cellular pathways by activating IGF-IR which affects the overall functioning of the cell (196). The intracellular part of the IGF pathway involves the adapter proteins insulin-receptor substrate (IRS) -1 and -2 through which the phosphatidylinositol 3' kinase (PI3K) /Akt pathway and the mitogen-activated protein kinase (MAPK) pathways are activated

(190). The mitogenic and anti-apoptotic properties of IGF-I are mediated through IRS-1 and the MAPK and P13-kinase pathways, while IRS-2 mediates IGF-I's metastatic mobility effects (190). The signaling proteins in these pathways transduce the IGF signal via phosphorylation reactions to a variety of intracellular proteins, including transcription factors (such as the estrogen receptor) and cell cycle control proteins (196, 197).

## **B. IGF Pathway and Breast Cancer**

### **1. IGF-I and IGF-IR and Breast Cancer**

IGF-I is involved in both the normal and abnormal growth of tissues (190, 191, 198, 199). During development, IGF-I mediates the indirect actions of GH and induces growth and differentiation in most tissues including the bones, muscle, nerves and cartilage. In the breast, IGF-I mediates the development of terminal end buds and ducts and the establishment of lactation (199). Following puberty, GH/IGF-I sustains these tissues.

Because IGF-I mediates cell growth, cell cycle progression and programmed cell death it is implicated in carcinogenesis (199). It is likely that sustained enhancement of cell turnover is required for tumorogenesis to occur (200). IGF-I may affect tumorogenesis by increasing the rate of cell division, which reduces the likelihood of repair of genetic damage that occurred during the lifetime of the cell and may result tumor growth (195). This feature, combined with IGF-I's anti-apoptotic effect allows cells harboring transforming mutations to survive and evolve into tumor cells (195).

Responsiveness of the breast tissue to the mitogenic properties of IGF-I is dependent upon the presence of IGF-IR. IGF-IR was present in most of the breast carcinoma specimens examined in one study (201) and IGF-IR was present at a significantly higher concentration in primary breast tumors than in controls in another study (202).

The IGF pathway is a target for treatment of cancers of the colon, breast, prostate, and lung (203). Therapeutic efforts to reduce signaling through the IGF

pathway include blocking IGF-I binding to the IGF-IR with antibodies, blocking intracellular kinase signaling with small molecules, reducing circulating IGF-I by administering excess IGFBP or soluble IGF-IR, administering IGF-IR antagonists, using somatostatin analogues to reduce GH/IGF-I levels, and inhibiting IGF-IR gene expression via small interference RNA (198, 204, 205). Pharmaceuticals targeting the IGF pathway are currently being tested in clinical trials for the treatment of cancer (198, 204). In dietary intervention trials for cancer prevention, nutritional and lifestyle interventions aim to reduce circulating IGF and raise circulating IGFBP-3.

The IGF-IR is similar in structure and sequence to the IR (205) to the degree that IGF-IR and IR heterodimers (IGF-IR/IR) can form and bind IGF-I. Hybrid IGF-IR/IR that bind IGF-I have been characterized (206). Therefore, the IR must be blocked as well as the IGF-IR in order to fully disable IGF-I signaling (207). However, blocking both receptors with pharmaceuticals is not without risk because Type II diabetes may result (204). Therefore, dietary interventions aimed at reducing circulating IGF-I levels are desirable.

#### **a. Animal and Cell Studies**

The importance of circulating GH, IGF-I and the IGF-IR in cell growth and tumorigenesis has been well documented in animal and cell studies (205). Cells lacking the IGF-IR resist transformation by a number of oncogenic factors. Cultured mouse embryo cells lacking the IGF-IR gene resisted transformation by simian virus 40 large tumor antigen (208) and H-ras (209) while wild-type cells were transformed. Transfection of these IGF-IR null cells with an IGF-IR expressing plasmid restored responsiveness of the cells to growth factors (209).

Similarly, animal models deficient in GH/IGF-I appear to be resistant to tumorigenesis resulting from exposure to known carcinogens. Spontaneous Dwarf rats (SDR) produce nonfunctional GH and therefore have very low levels of IGF-I (210). SDR developed 4% of the number of mammary tumors generated in Sprague-Dawley rats exposed to the same level of N-methyl-N-nitrosourea (210). SDR exposed to

DMBA followed by saline daily developed no tumors while rats that were exposed to 100 $\mu$ g or 400 $\mu$ g growth hormone daily following DMBA exposure developed 2 – 3.5 tumors/animal (211). Mammary tumors were also reduced in mice transfected with a GH antagonist compared to non-transgenic littermates when treated with DMBA (212).

The lit mutation in mice, in which GH-releasing hormone receptor is mutated and nonfunctional, serves as a model for GH/IGF-I deficiency (IGF-I reduced by 90% relative to controls) and allows for disruption of endocrine GH/IGF-I without interfering with other pituitary functions (213). Growth of tumor xenographs into lit/lit mice depends partly on endogenous tissues such as mammary stromal cells and angiogenic elements (213). In lit/lit mice implanted with MCF-7 cells, tumor growth was significantly reduced relative to controls (214). Transgenic mice with high levels of GH/IGF-I have increased tumorigenesis (215, 216) and GH supplementation has been shown to increase mammary hyperplasia in rhesus monkeys (217). Wu et al utilized the liver-specific IGF-I gene-deleted (LID) mouse to differentiate the effects of liver-derived IGF-I from the paracrine/autocrine effects of IGF-I (218). LID mice exposed to DMBA had significantly less mammary tumors per mouse and fewer percent of mice had tumors compared to controls (218).

Additionally, IGF-I is mitogenic in a number of cell lines (219-222). Antibodies against IGF-IR restricted growth and DNA synthesis of MCF-7 and MDA-231 cells in vitro (222) and restricted growth of MDA-231 cells implanted into nude mice (223). The above animal and cell studies indicate that the GH/IGF-I axis is instrumental in the initiation and progression of mammary tumorigenesis.

## **2. IGF and Estrogen Signaling Pathway Cross-Talk**

There is notable convergence of IGF-I and estrogen signaling pathways in the breast and other tissues (191, 224, 225). Estradiol potentiates the mitogenic effects of IGF-I, which in turn activates the ER $\alpha$  (196). Estrogens that bind the ER $\alpha$ , which is a transcription factor, regulate local expression of growth factors including IGF-I (226), growth factor receptors such as IGF-IR and epidermal growth factor receptor (227), intracellular signaling proteins such as IRS-1 and IRS-2 (227), cell-cycle regulators

such as cyclin D (227), and binding proteins including IGFBP-3 (228). Autocrine/paracrine IGF-I then binds its membrane-bound IGF-IR and initiates mitogenic signaling cascades mediated by kinases (MAPK) that activate/potentiate a number of signaling intermediates and transcription factors including ER $\alpha$  (191, 229). Therefore, IGF-I and ER $\alpha$  are thought to act synergistically to increase breast cancer growth and metastasis (191, 227, 229, 230).

#### **a. Animal and Cell Studies**

Estrogen and IGF-I cross-talk has been established in cell and in animal studies. Estradiol treatment reduced IGFBP-3 and IGFBP-3 mRNA and increased DNA synthesis in ER $\alpha$  positive MCF-7 cells (228). The net effect of this study is increased cell growth and increased bioavailability of IGF-I. Estradiol increased IGF-I signaling by increasing the concentration of IRS-I in MCF-7 cells, which activated downstream signaling pathways such as the MAPK and PI3-kinase/Akt pathways (196).

Ruan et al determined that estrogen was required for the full mitogenic effect of IGF-I on mammary development in hypophysectomized and oophorectomized rats (224). Ruan et al also found that estrogen magnifies GH-induced IGF-I gene transcription in the rat mammary (224). ER $\alpha$  was activated by IGF-I treatment although ER $\alpha$  mRNA was reduced in MCF-7 cells (231). Lee et al explored the effects of estrogens on IGF-IR and IGF pathway signaling proteins in cell culture and in breast cancer survivors (230). Expression of IGF-IR, IRS-1 and IRS-2 and signaling through the IGF-IR were enhanced by estradiol treatment in MCF-7 cells (230). Xenografts of MCF-7 cells in nude mice had similar effects of increased IRS-1 expression (230). Women with ER positive tumors and increased IRS-1 had significantly reduced disease-free survival (230). Estrogen was required for induction of proliferation resulting from IGF-I administration in mice (225). In ER $\alpha$  knockout mice and wild type mice, IGF-I administration stimulated the Akt and MAPK pathways via phosphorylation of the IGF-IR, IRS-1 and PI3 kinase. However, a mitogenic response was not observed in the ER $\alpha$  knockout mice indicating the ER $\alpha$  mediates IGF-I nuclear effects (225). IGF-I may

also be sufficient to activate the ER $\alpha$  when estrogens are absent, which further evidences cross-talk between the two pathways (191, 225). In the rat uterus IGF-I exposure in the absence of estradiol induced gene expression via ER $\alpha$  and increased phosphorylation of ER $\alpha$  (232). IGFBP-3, IGF-I and acid-labile subunit levels were significantly decreased by oral contraceptive exposure in postmenopausal women (233).

### **3. Insulin and Breast Cancer**

Insulin has anabolic effects in the liver, adipose tissue, and muscle and mediates glucose utilization in those tissues. In addition to stimulating synthesis of glycogen, lipids and proteins, insulin also affects apoptosis, mitogenesis, and proliferation (234). Similarly to IGF-I signal transduction, insulin binding its receptor (a cell membrane-embedded receptor tyrosine kinase) elicits autophosphorylation of intracellular tyrosine residues followed by phosphorylation of the IRS-1 and signal transduction to the MAPK and PI3K/Akt pathways which activate proteins involved in proliferation, cell cycle control and apoptosis along with mediators of carbohydrate and fat metabolic pathways (235).

Early studies in rat mammary carcinoma explants demonstrated increased proliferation in the presence of insulin (236). Additionally, the insulin receptor is upregulated in many breast tumors, and may play an important role in breast tumor growth. Insulin resistance, given sufficient  $\beta$ -cell function, is accompanied by a compensatory hyperinsulinemia in which glucose levels are returned to normal by increased circulating insulin (235). Hyperinsulinemia in the presence of increased insulin receptors on tumor cells may further increase proliferation (235). Insulin receptors were present at six times the concentration as in benign human breast tissue specimens in a study by Papa et al (237). As a result, more tyrosine kinase signaling would be initiated and this could activate a number of oncogenes and lead to growth and proliferation of tumor cells (237). Insulin receptors are present in breast cancer cell lines as well (238). Insulin also upregulates the expression of GH receptors in the liver (192), ultimately affecting the amount of IGF-I produced in the liver. Insulin may also

indirectly increase breast tumorigenesis by increasing bioavailable estrogen via increasing aromatase activity and reducing SHBG levels (239, 240).

**a. Human Studies**

**i. IGF-I and Breast Cancer**

The association of IGF-I and its binding proteins and breast cancer risk has been investigated in a number of epidemiological studies (See Table 1-12). In an early paper, Peyrat et al (202) determined that IGF-I levels were higher in breast cancer cases than controls. Vadgama also found that IGF-I levels were higher in cases than controls, and additionally they found that predicted survival of cases with low levels of IGF-I (<120ng/mL) is significantly greater than cases with high IGF-I levels (>120ng/mL) (241). IGF-I concentrations tend to decrease with age (242), and premenopausal women have higher IGF-I concentrations than postmenopausal women (241). Men tend to have higher concentrations of IGF-I than women (242). In a cross-sectional study, IGF-I levels correlated inversely with IGFBP-1 levels although there were no correlations between circulating sex hormone concentrations or urinary hormone metabolite concentrations and IGF-I (243) although a cross-sectional study in the EPIC cohort found that IGF-I was positively associated with E1, E2, free E2, T, free T, A and DHEAS and inversely associated with SHBG in postmenopausal women (244).

To date, IGF-I has mostly been associated with increased breast cancer risk in premenopausal women. Association of IGF-I and IGFBP-3 and development of several cancer types was examined in a meta-analysis of case-control studies by Renehan et al (203). Five of the 26 studies measured breast cancer risk relative to IGF-I and IGFBP-3 concentrations in postmenopausal women, but none of the associations were statistically significant in postmenopausal women (245-249), although IGF-I was associated with breast cancer risk in women with BMI over 26 in the study by Muti et al (248). Another meta-analysis by Shi et al (250) examined 18 studies of breast cancer related to IGF-I and IGFBP-3 concentrations conducted prior to July 2003 and seconded the findings of Renehan that breast cancer risk was not associated with IGF-I concentration in

postmenopausal women. More recent studies found no association between IGF-I and breast cancer risk in postmenopausal women (251-253) although a study in the EPIC cohort, which is much larger than those of the other studies, found that IGF-I and IGFBP-3 were positively associated with breast cancer risk in women diagnosed with breast cancer at age 50 above while no association was found for women under 50 (254).

## **ii. Insulin Resistance and Breast Cancer Risk**

Results of epidemiological studies of insulin resistance-related endpoints (glucose, insulin, C-peptide) are mixed (See Table 1-13). Measurement of fasting insulin concentration is a convenient measure of hyperinsulinemia with levels below 15 mU/L (104 pmol/L) deemed normal, 15 -20 mU/L (104 – 139 pmol/L) considered borderline high and above 20 mU/L (139 pmol/L) regarded as high (255). Elevated fasting insulin was not associated with breast cancer risk in women in a small (n = 45 cases) study nested within the Rancho Bernardo study (256) while elevated fasting insulin was associated with increased breast cancer risk in a larger nested case-control study within the WHI (n = 835 cases) (253). In both of these studies, most of the women had insulin levels within the normal range, so it may be difficult for them to detect an association. Relatively increased insulin concentrations were associated with breast cancer risk in women with BMI over 26 in a study nested within the ORDET cohort (248). Women had normal to borderline elevated fasting insulin levels in this study (248). One aspect that complicates an association of breast cancer risk with insulin concentration is that women with advanced diabetes and failing  $\beta$ -cells may have low levels of insulin (257). Increased recurrence and mortality was associated with increased fasting insulin concentrations in a cohort of breast cancer survivors without diabetes, however the highest quartile of insulin level was > 51.9 pmol/L, well within the normal range (258). The authors report that 95% of the insulin concentrations for the cohort were below 106 pmol/L, also within the normal range (258).



The human proinsulin connecting peptide, or C-peptide, is a chain of amino acids formed during the cleavage of proinsulin to insulin (259). C-peptide is measured as an indicator of insulin secretion because it is secreted in equimolar concentration from the pancreas  $\beta$ -cells and it has a longer half-life in the circulation than insulin (260). Neither Jernstrom(256) nor Tonilo (246)observed significant associations between C-peptide concentration and breast cancer risk in their nested case-control studies. However Bruning et al (261) found C-peptide levels to be higher in cases than controls and Yang et al (262) found a positive association between breast cancer risk and C-peptide level in their case-control study that analyzed blood drawn from cases before any cancer treatment was undergone . The pattern of results was similar in a systematic review by Xue et al (257) in which 17 studies that measured C-Peptide were reviewed. Results were mixed with half of the studies that measured fasting C-peptide showing significantly positive associations between fasting C-peptide and breast cancer risk while the other half showed no association (257). Non-fasting C-peptide studies showed mostly non-significantly positive associations for breast cancer risk (257).

In a meta-analysis of markers of hyperinsulinemia (measured insulin /C-peptide) and different types of cancer, Pisani (263) assessed case-control and prospective cohort studies of insulin levels and breast cancer. Cohort studies showed no association of insulin concentration and breast cancer risk while case-control studies indicated a positive association of insulin level and breast cancer risk (263). When the cohort studies and case-controlled studies were combined for analysis, there remained a positive association between insulin concentration and breast cancer risk, but the evidence for an association came entirely from the case-controls studies, which is problematic due to presence of the disease in cases (263).

Type II diabetes results when target cells become resistant to insulin or when insulin levels drop due to failing  $\beta$ -cells and blood glucose levels rise chronically. Type II diabetes is clinically diagnosed when hemoglobin A1C concentration, an indicator of long-term blood glucose level, is  $\geq 6.5\%$ , fasting plasma glucose is  $\geq 126$  mg/dL (7.0 mmol/L), plasma glucose is  $\geq 200$  mg/dL (11.1 mmol/L) 2 hours following a 75g oral

glucose load (oral glucose tolerance test, OGTT) and/or if a random plasma glucose level is  $\geq 200$  mg/dl in a symptomatic patient (264). Neoplasms exhibit increased glucose uptake, expression of glycolytic enzymes, and flux through the glycolytic pathway (265). The increased blood glucose concentration of diabetic hyperglycemia could be utilized by cancerous cells for proliferation. Evidence of an association of relatively high glucose levels and breast cancer risk are mixed. Glucose and hemoglobin A1C were higher in cases than controls, although insulin was higher in controls, in a study by Lopez-Saez et al (266). Krajcik et al (247) observed an increased risk in postmenopausal women if their blood glucose was in the range of Type II diabetes. However, glucose concentrations were not associated with breast cancer risk in a recent analysis of participants of the WHI studies (267).

Evidence suggests that women with Type II diabetes are at increased risk of breast cancer development and women with breast cancer are at increased risk of developing Type II diabetes. The systematic review of case-control and prospective cohort studies by Xue et al indicated that women with invasive breast cancer, and particularly postmenopausal cases, were more likely to be diagnosed with diabetes than control women (257) and likewise women with a recent diagnosis of diabetes were more likely to have a history of breast cancer than women without diabetes (268). A higher early mortality rate is observed in women with breast cancer who have a comorbid condition, including Type II diabetes (269, 270).

Insulin and glucose levels also vary with body mass index and body fat distribution (waist to hip ratio). In a study by Gamayunova et al (271), fasting glucose was significantly greater in postmenopausal breast cancer patients with BMI over 30. Also, fasting glucose and glucose and insulin 2 hours after an oral glucose load (OGTT) were significantly greater in postmenopausal breast cancer patients with waist-hip ratio over 0.85. Most studies mentioned above adjusted for body mass index.

### **C. IGF Pathway and Aging**

Aging is accompanied by reductions in lean body tissue and bone mineral density and increased adiposity along with a higher risk vascular profile associated with

increased mortality and morbidity resulting from cardiovascular events. GH/IGF-I deficiency has similar symptoms and therefore the symptoms accompanying the decrease in GH/IGF-I with aging are referred to as the somatopause (272). GH concentrations decrease markedly following menopause (272). With an increasingly elderly population, the burden on health care services increases; therefore strategies to blunt the symptoms of advanced age are welcomed. Unlike with cancer prevention and treatment, in which reductions in circulating IGF-I and IGF signaling is the goal, raising IGF-I concentrations is desirable in reversing/preventing the effects of aging. Thus a nutritional intervention that increases IGF-I concentrations in elderly individuals would be desirable.

GH replacement in individuals with GH deficiency results in improvements in the associated impaired biochemical/physical parameters by increasing bone mineral density, lean body tissue, and skin thickness and decreasing adiposity (273, 274). Whether these changes result in reduced fatigue or related impairments and increased quality of life is of great concern, and little data is available relating to these functional issues (273). A recent meta-analysis reviewed the results of trials involving GH replacement and its effects on functional exercise-related endpoints in individuals with GH deficiency (275). According to their analysis, exercise capacity was significantly increased with GH replacement (275), which lends support in terms of GH improving quality of life.

Using GH treatment to combat the effects of aging in normal individuals is more controversial. Definition of who would benefit from GH therapy and cut-offs for IGF-I concentrations deemed inadequate and thus appropriate for therapy and target IGF-I levels resulting from treatment need to be determined (272). Common side effects of GH treatment include carpal tunnel syndrome, edema and increased weight due to fluid retention, which may be alleviated by reduction of GH dose (274). Whether GH therapy results in extension of independent living is a primary objective that has yet to be determined (272). Long-term studies need to be done in order to determine the appropriateness and cost-effectiveness of GH therapy in healthy elderly adults. Authors of a systematic review of use of GH in healthy elderly individuals concluded that the

small improvements in body composition afforded by GH therapy in healthy adults do not offset the associated adverse side-effects (276).

#### **D. IGF Pathway and Cardiovascular Disease**

IGF-I is also associated with cardiovascular disease risk. Low IGF-I has been associated with increased risk of myocardial infarction (277), stroke (278), and increased propensity of atherosclerotic plaque accumulation (279). Additionally, IGF-I directly affects the heart muscle and may play a role in prevention of heart disease and recovery of myocardial cells following ischemia (280). Lower IGF-I concentrations were associated with poor outcome (death or congestive heart failure) in patients following myocardial infarction (281). Concentrations of IGF-I below 140 ng/mL correlated with increased risk of congestive heart failure in elderly individuals in the Framingham Heart Study (282). Results of a meta-analysis of clinical trials of GH treatment in chronic heart failure showed improvements in physical and functional (including exercise duration and oxygen uptake) cardiovascular parameters (283). Long-term effects of GH treatment need to be determined in a large-scale randomized controlled trial (283). Long-term GH therapy does not seem to increase cancer incidence (284). Again, a nutritional intervention to raise IGF-I concentrations would be desirable for the prevention of cardiovascular disease.

#### **E. IGF Pathway and Total Dietary Fat Intake**

##### **1. IGF-I and IGFBP's and Total Fat Intake**

Most of the evidence for altering IGF-I concentration with diet comes from studies of protein and/or energy deprivation (285). Fasting and malnutrition result in GH resistance, possibly due to decreased circulating insulin, a regulator of GH receptors (285). Protein deprivation may result in post GH receptor signal attenuation and down-regulation of IGF-I synthesis (286). Increased dietary protein intake was associated with increased circulating IGF-I in several epidemiological studies (287-289).

The data relating total dietary fat intake and IGF-I concentrations are limited, but a few studies indicate that altering dietary fat may impact circulating IGF-I

concentrations. Dietary fat intake was positively associated with serum IGF-I and IGFBP-3 in a group of healthy men and women (290) and inversely associated with IGFBP-3 in premenopausal women (178). Total fat intake (288) and saturated fat intake (242, 288) were associated with decreased IGFBP-3 concentrations in a couple of studies. However, there were no associations detected between total fat or fat subtypes (SFA, MUFA, PUFA) and IGF-I and IGFBP-3 in participants of the EPIC study (287) and no associations were found between total fat and SFA and IGF-I and IGFBP-3 in the Multi-Ethnic Cohort (291).

A few dietary intervention studies have investigated the effects of dietary fat on circulating IGF-I and IGFBP's (See Table 1-14). Concentrations of IGFBP-1 and IGFBP-2 were significantly increased, but IGF-I level did not change significantly in postmenopausal women following the dietary intervention of the DIANA randomized trial, which include alteration of a number of dietary factors including limiting animal fat and refined carbohydrates and increasing intakes of fish and olive oil (292). Dietary fat decreased by 7% in the intervention group of the DIANA trial (292). A low fat diet (< 15% energy from fat) and exercise program significantly reduced insulin and IGF-I and significantly increased IGFBP-1 after two weeks of the intervention (293) however it is unknown whether the changes in IGF-I and IGFBP-1 would be sustained long-term. Analysis of fasting insulin, glucose, IGF-I and IGFBP-3 in men and women in the Polyp Prevention Trial yielded no significant differences between endpoints between the intervention group that received low fat (20% of energy from fat), high-fiber and high fruit and vegetable dietary intervention counseling compared to the control group that received no counseling, although fasting glucose was reduced in the intervention group in subjects with a BMI of 25 or lower (294). The authors of that study point out that the insulin levels of the participants were in the lower normal range, allowing little space for improvement with the intervention (294). Levels of IGF-I, IGFBP-1 and IGFBP-3 did not change significantly in premenopausal women who participated in a low fat, high fiber dietary intervention compared to a minimally counseled control group of women after 12 months (295). None of these interventions isolated the effects of n-3 FA on IGF-I or IGFBP's.

## **2. Insulin Resistance and Total Fat Intake**

There is a large amount of evidence that indicates dietary composition and total fat intake affect insulin sensitivity. High-fat diets impair insulin sensitivity in animal models, and type of fat in the diet has differential effects on insulin sensitivity (296). Diets high in palmitic acid (SFA) and low in oleic acid (MUFA) in humans tend to promote insulin resistance (297).

Total fat intake was positively associated with fasting insulin concentrations (298, 299) and negatively associated with insulin sensitivity (300) in cross-sectional studies in non-diabetic persons. However, dietary intervention studies in healthy persons of total dietary fat intake and insulin sensitivity-related endpoints have given mixed results (See Table 1-15). Low fat/high carbohydrate diets improved insulin sensitivity (301-303) and fasting insulin (181, 301, 304) concentrations in several intervention studies relative to a high fat diet (302, 303) or habitual diet (181, 301, 304) in healthy persons. However, insulin concentrations decreased significantly with a more moderate fat intake (30% of energy) relative to a very low fat diet (11% energy from fat) in another study of healthy individuals (184). A study employing liquid diets with extremely low carbohydrate (83% energy from fat, 2% energy from carbohydrate, 15% energy from protein) compared to an extremely low fat diet (0% energy from fat, 85% energy from carbohydrate, 15% energy from protein) and an intermediate fat/carbohydrate diet (41% energy from fat, 44% energy from carbohydrate, 15% energy from protein) resulted in reduced fasting insulin and glucose and increased fasting fat oxidation with the high fat diet (305). Endogenous glucose production and fat oxidation were also increased during a hyperinsulinemic euglycemic clamp technique with the high fat liquid diet (305). However, that study has questionable significance since the nutrient compositions of the liquid diets are not likely to be consumed by free-living humans. No change in insulin or glucose parameters was observed in a study that compared substituting low fat foods for high fat foods, restricting high fat foods, or a combination of substituting or restricting high fat foods (306).

Riccardi et al contend that fat composition appears to have more of an effect on insulin sensitivity than total amount of fat in humans (307). Some studies have investigated the effects of changing dietary fat composition on insulin-sensitivity parameters. In a highly-regarded multi-center intervention study with ample sample size (n = 162), the KANWU study (308), subjects were counseled to follow a high SFA diet or a high MUFA diet with equal percentages of energy from total fat for three months. Following the intervention, weight did not change, but insulin sensitivity declined in the high SFA arm while insulin sensitivity was unchanged and fasting insulin decreased in the high MUFA arm (308). Another finding of the KANWU study was that insulin sensitivity was also impaired by the MUFA intervention in subjects in which dietary fat intake exceeded 37% (308). In subjects whose fat intake was < 37%, insulin sensitivity was increased by 8.8% with the MUFA diet (308). In another study, type of dietary fat (SFA vs. MUFA vs. trans FA) had no effect on insulin sensitivity or secretion with an isocaloric weight-maintaining cross-over intervention (309). Earlier studies in smaller numbers of subjects (n = 8 - 20) in which percentage of energy from fat was held constant and type of fat was varied and esteemed methodology such as the hyperinsulinemic euglycemic clamp, oral glucose tolerance test or frequently sampled intravenous glucose tolerance test was used yielded consistently negative effects on insulin sensitivity in healthy adults (310).

Overweight/obesity (particularly abdominal obesity) and sedentary lifestyle are repeatedly associated with development of insulin resistance and Type II diabetes (311). Consumption of an energy-dense/high fat diet is clearly related to overweight/obesity (307); however it is unclear to what extent diet composition affects weight loss independent of calorie restriction. Weight-reduction strategies have generally been to reduce fat intake in order to reduce total calorie consumption. A Cochrane meta-analysis of weight loss interventions in overweight and obese individuals concluded that low fat diets are no better than reduced-calorie diets for weight loss (312). In a recent meta-analysis of dietary interventions in patients with Type II diabetes, low fat/high carbohydrate diets tended to increase fasting insulin and triglycerides (TG) and decrease HDL cholesterol relative to high fat/low carbohydrate diets (313). Hemoglobin A1c,

TG and fasting glucose tended to decline with carbohydrate-restricted diets in patients with Type II diabetes in a meta-analysis by Kirk et al (314). Furthermore, there is recent evidence that carbohydrate restriction is effective in improving parameters of the metabolic syndrome, which is defined by a cluster of symptoms attributed to underlying insulin resistance: obesity, dyslipidemia (low HDL and high TG), hyperglycemia and hyperinsulinemia. A recent study by Volek et al (315) compared a mildly-ketotic carbohydrate-restricted diet (average 12% energy from carbohydrate, 28% energy from protein, 59% energy from fat) to a low fat diet (56% energy from carbohydrate, 24% energy from fat, and 20% energy from protein) in individuals with atherosclerotic dyslipidemia. Interestingly, both diets had similar reductions in energy intake (~1500 kcal per day average intake) however the carbohydrate-restricted group experienced almost twice the degree of weight loss relative to the low fat group (10.1 vs. 5.2 kg) amounting to a decrease of 10% of baseline weight in the carbohydrate-restricted group (315). Total body fat and abdominal fat was concomitantly reduced significantly greater with the carbohydrate-restricted group than the low fat group (315). Fasting glucose, insulin, insulin sensitivity, and postprandial TG following a fat challenge were all significantly reduced with carbohydrate restriction relative to low fat diet (315). Another carbohydrate-restriction intervention study by Meckling et al (316) demonstrated reduced circulating insulin following carbohydrate restriction to 15% of energy for 10 weeks. Carbohydrate restriction resulting in compensatory high protein intake is not recommended for individuals with Type II diabetes due to the risk of diabetic nephropathy, but for individuals with the metabolic syndrome rigorous carbohydrate restriction appears to be beneficial.

## **F. IGF Pathway and Omega-3 Fatty Acids**

### **1. IGF-I and Omega-3 Fatty Acids**

Very limited information on the effects of n-3 FA on the molecular level IGF signaling pathway is available in the literature. However, the cellular signaling effects of LA and ALA treatment of HCT116 colon cancer cells were investigated in one study by Seti et al (317). Although IGF-I gene expression levels did not appear to be affected



by LA and ALA treatment of the cells, IGF-I-dependent and ligand independent phosphorylation of the IGF-IR in HCT116 cells was significantly increased by LA and ALA treatment (317). Furthermore, downstream signaling kinases Akt and MAPK were phosphorylated to a significantly greater degree and more cells progressed to the S phase of the cell cycle with LA and ALA treatment than with bovine serum albumin control (317). Linoleic acid and ALA also reduced apoptosis as evidenced by reduced Poly-ADP ribose polymerase cleavage (317). Taken together, these results indicate that treatment of colon cancer cells with LA and ALA results in increased IGF-IR-mediated cell signaling resulting in increased proliferation and decreased apoptosis.

Few studies regarding n-3 FA and circulating IGF-I concentrations in humans are available in the literature. Probst-Hensch et al (242) used a validated FFQ (318) to evaluate dietary factors in relation to circulating IGF-I and IGFBP-3 concentrations in a subset of Chinese men and women (> 50 years old) who took part in the Singapore Chinese Health Study. In their analysis, n-3 FA intake was associated with increased IGFBP-3 while SFA intake was associated with decreased IGFBP-3 (242). Intake of n-3 from fish was associated with non-significantly increased ( $P = 0.07$ ) IGF-I concentrations and was significantly associated with increased IGFBP-3 concentrations in another study of pre- and postmenopausal women (288). However, intake of fish and shellfish was not associated with IGF-I or IGFBP-3 concentrations in a subset of women in the EPIC cohort (289). Other studies that measured the association of total fat and fat subtypes and IGF pathway factors did not differentiate PUFA into n-3 and n-6 FA or report fish intake (287, 291, 319). No dietary intervention studies of fish intake and circulating IGF-I and IGFBP's were found in the literature, although one study added fish oil supplements to a controlled 40% fat diet and found that IGF-I and GH concentrations were significantly decreased following the fish oil intake for 10 weeks (320). However, when 200 mg Vitamin E was added to the fish oil intervention for 8 weeks longer, IGF-I concentrations returned to baseline levels (320). This result suggests that the oxidation status of the fish oil PUFA is related to its effects on IGF-I concentration.

## **2. Insulin Resistance and Omega-3 Fatty Acids**

There is evidence of a reduction in insulin resistance with n-3 FA feeding in animal models. As mentioned above, high fat feeding in animal models induces insulin resistance, and this effect is thought to be partially attributed to accumulation of triglyceride in insulin-sensitive tissues such as muscle and liver. In a study by Simoncikova et al (321), incorporation of 10% of energy from fish oil in a high fat diet (70% of energy as fat) in male rats compared to high fat diet (70% of energy as fat) attenuated the insulin resistance, increases in TG, FFA and glycerol and increased accumulation of tissue TG induced by high fat diet. Lesser amount of n-3 were also successful in inhibiting the development of insulin resistance. Replacement of 6% of energy from safflower oil with 6% of energy from fish oil in a high fat diet prevented the development of insulin resistance in rats (322). High sucrose diet can also induce insulin resistance in animal models. A fish oil-rich diet was effective in preventing the hypertriglyceridemic and hyperinsulinemic effects of a high sucrose diet in rats, but could not reverse these changes once they had been established (323).

Altered composition of membrane phospholipids and membrane fluidity may be one mechanism by which n-3 FA affect insulin sensitivity (297, 324). Increased unsaturation in the lipid membrane may affect conformation of receptor binding sites and subsequent ligand binding and may affect permeability of the membrane to ion flux, requiring increased energy input to maintain ion gradients across the membrane (325). A great percentage of n-3 FA in muscle phospholipids in rats was correlated with greater insulin sensitivity in muscle (326). In normal humans, greater percentages of PUFA in muscle phospholipids (327) and particularly greater percentages of PUFA in membrane phosphatidylcholine is associated with increased insulin sensitivity (328).

The effects of fish oil supplementation on insulin sensitivity-related endpoints in the diets of humans are less striking (See Table 1-16). In healthy men, fish oil supplementation of 15g/day as part of a controlled diet (40% of energy from fat) significantly increased glucose concentrations relative to the controlled diet plus placebo oil capsules (320). Fish oil supplements (4g/d EPA + DHA) decreased blood pressure but did not affect insulin sensitivity and increased glycosylated hemoglobin in

hypertensive individuals (329). To better understand the effect of fish oil supplementation on glucose homeostasis, Delarue et al (330) conducted a study of the metabolism of glucose and fructose loads in a small number ( $n = 5$ ) of healthy individuals. Supplementation with fish oil (1.1g/day EPA + 0.7g/day DHA) resulted in a small (5%) but significant increase in fasting glucose and a greater (40%) decrease in fasting insulin concentrations as well as reduced oxidation of fructose and glucose and increased fatty acid oxidation following the glucose and fructose challenges (330). As mentioned above, the KANWU study was an intervention in which participants were instructed to follow a high SFA diet or a high MUFA diet (308). Half the participant in either randomization received a daily fish oil supplement (2.4g/day EPA +DHA) but the addition of fish oil did not alter any of the insulin-related endpoints (308). As expected, TG were reduced and LDL was increased in the individuals who received fish oil (308). There was also no change in insulin sensitivity-related endpoints in an intervention study in which participants received study-specific fats and oils and were instructed to follow either a low or high n-6 FA diet for 6 weeks followed by the addition of a fish oil supplement (2.5g/day EPA + DHA) for 6 weeks (331). In another study in healthy individuals where fats and oils were provided, changing the ratio of n-6 to n-3 FA had no effect on glucose, insulin, or insulin sensitivity although diets high in EPA and DHA reduced TG and small, dense LDL (332). These studies indicate that supplementation with n-3 fish oil does not result in meaningful changes in insulin sensitivity in healthy, non-diabetic individuals. No studies were found that investigated the effects of fish diets on insulin sensitivity-related endpoints.

A vast number of studies on fish oil supplementation have been conducted in individuals with Type II diabetes, which repeatedly show effects of reducing TG concentrations and increasing LDL concentrations (333, 334). However n-3 FA supplementation does not appear to affect insulin sensitivity once the glucose intolerance of Type II diabetes has set in (335). The results of animal and human studies may differ due to the amount of n-3 FA administered. In animal studies, some experiments used fish oil percentages of up to 20% by weight (~6 g/day), which would be equal to about 24g/day of fish oil per day in humans, an unrealistic amount that

exceeds the amount given in studies in Type II diabetics (335). In addition, most of the studies in diabetic patients have been too short in duration to allow for incorporation of n-3 FA into cellular membranes and subsequently affect insulin signaling (336).

In summary, n-3 FA supplementation with fish oil is effective in reducing TG concentrations and treating dyslipidemia in non-diabetic and diabetic individuals; however there are no established effects of n-3 FA on insulin sensitivity or glucose control.

## **VII. Dissertation Rationale**

The purpose of the research described in this dissertation was to determine the effects of altering type and amount of dietary fat on breast cancer risk markers in healthy postmenopausal women. Chapter 1 of this dissertation provides a review of the literature supporting this hypothesis.

Chapters 2-4 detail the outcome of the clinical trial that was performed to determine the effects of low fat diet and n-3 fatty acids on markers of breast cancer risk. In total, a diet low in fat with or without a high level of n-3 FA was expected to reduce endpoints associated with increased breast cancer risk. The greatest effects were hypothesized to occur with a low fat diet plus n-3 fatty acids. Three diets were employed in a cross-over design clinical trial in healthy, postmenopausal women. The three diets were a high fat diet (40% of energy from fat) a low fat diet (20% of energy from fat) and a low fat, high n-3 FA diet (23% of energy from fat including 3% of energy from n-3 FA). Many endpoints were evaluated at the conclusion of the trial including plasma PLFA [LA (18:2n6), ALA (18:3n3), AA (20:4n6), EPA (20:5n3) DHA (22:6n3), total n-3, total n-6, and the n-6:n-3 ratio]; urinary eicosanoids (prostaglandin E metabolite, 11-dehydrothromboxane B<sub>2</sub>); plasma sex hormones (E2, E1, E1-S, FSH, DHEA, DHEAS, T, A, SHBG, Free E2, Free T); urinary estrogens and metabolites [E1, E2, estriol (E3), 2OH-E1, 2OH-E2, 2-ME1, 2-ME2, 4-ME1, 4-ME2, 16 $\alpha$ OH-E1, 2:16 $\alpha$ OH-E1 ratio, 4OH-E1, 4OH-E2]; and IGF pathway endpoints [insulin, glucose, IGF-I, IGFBP-3, homeostatic model assessment of insulin resistance (HOMA-IR), IGF-I:IGFBP-3 ratio].

Chapter 2 comprises the results of the clinical feeding trial on plasma PLFA, urinary eicosanoids, and plasma sex hormones. Both a diet low in fat and a diet low in fat and high in n-3 FA were hypothesized to increase plasma PLFA and reduce urinary eicosanoids and thereby reduce plasma estrogens relative to a high fat diet.

Chapter 3 details the effects of the three test diets on urinary estrogen endpoints. Urinary estrogens and metabolites were expected to change in a pattern associated with decreased breast cancer risk with both a low fat diet and low fat diet with n-3 FA: urinary total estrogens, 4-hydroxy estrogens and 16 $\alpha$ OH-E1 were expected to decrease and the ratio of 2OH-E1 to 16 $\alpha$ OH-E1 was expected to increase.

Chapter 4 presents the effects of the three test diets on IGF-I related endpoints. Endpoints associated with the IGF pathway were predominantly exploratory; there is limited evidence that IGF-I and IGFBP-3 could be altered with reduction of dietary fat while maintaining equivalent and adequate protein and caloric intake.

Chapter 5 provides a brief summary of the dissertation research. Appendix 1 contains the menus for the dietary feeding trial and Appendix 3 contains forms and questionnaires used for subject recruitment, assessment and education.

**Table 1-1: Total dietary fat and breast cancer incidence prospective cohort studies.**

Reference	Endpoints	Results
Holmes (30)	From FFQ: total fat, total energy, SFA, animal fat, vegetable fat, PUFA, MUFA, trans fat, long-chain n-3 PUFA, ALA, cholesterol; breast cancer incidence.	Four FFQ administered to the NHS prospective cohort every 4 years from 1980 – 1990 were assessed. 1) No association between breast cancer risk and any of the fat types.
Velie (33)	From FFQ: total fat, SFA, unsaturated fat, LA, oleic acid, protein, carbohydrate (all as % of energy); total energy; breast cancer incidence.	FFQ prospectively self-administered to mammography screening cohort. 1) No significant associations in entire cohort. 2) In a subset of women with no history of BBD, total fat, largely due to oleic acid, was positively associated with breast cancer risk.
Byrne (32)	From FFQ: total fat, SFA, unsaturated fat, oleic acid, LA, trans FA, energy intake; breast cancer incidence.	FFQ from the NHS prospective cohort were assessed. 1) No increase in rate of breast cancer with increased dietary fat or types of fat in women with no history of BBD was observed.
Sieri (35)	From FFQ: total fat, animal fat, vegetable fat, SFA, MUFA, PUFA, LA, ALA, total protein, animal protein, vegetable protein, total carbohydrates, starch, sugar, fiber, alcohol, total energy; breast cancer incidence.	FFQ administered prospectively to postmenopausal women in the ORDET cohort. 1) Significant positive relationship between total fat and breast cancer risk; 2) Inverse relationship for carbohydrate and breast cancer risk.
Kim (31)	From FFQ: total fat, SFA, animal fat, vegetable fat, PUFA, MUFA, trans fat, long chain n-3 PUFA, ALA, cholesterol; breast cancer incidence.	FFQ from 20 years follow-up (1980 – 2000) of the NHS were assessed. 1) No association between breast cancer risk and any of the fat types were observed.
Thiebaut (20)	From FFQ: total fat, SFA, MUFA, PUFA; breast cancer incidence.	Assessed FFQ from the NIH - American Association of (AARP) Persons Diet and Health Study. 1) Positive association of total and all fat subtypes and breast cancer risk.

**Table 1-2: Total dietary fat and breast cancer incidence/recurrence controlled dietary intervention studies.**

Reference	Intervention	Outcome Measures	Results
Prentice (37)	WHI randomized dietary modification clinical trial. Group 1: Dietary modification to reduce total fat to 20% of calories, consume 5 servings of fruit and vegetables/day, and 6 servings of grains/day. Group 2 = control group (no dietary changes).	Breast cancer incidence	1) Dietary fat intake was significantly lower in the dietary modification group, but breast cancer incidence was significantly different between groups. 2) The intervention group did not meet their goal of 20% calories from fat. 3) Increase of 1 serving/day of fruit/vegetables in dietary modification group. 4) In a subset of compliant subjects, risk was reduced for women with a higher fat intake at baseline.
Chlebowski (39)	WINS randomized trial. Group 1: Dietary intervention (reduce fat intake to 15% of calories). Group 2 = control group (no dietary changes).	Disease-free survival in women previously diagnosed with breast cancer	WINS randomized trial. 1) Dietary fat intake was significantly lower in the dietary intervention group. 2) Intervention group lost significantly more weight. 3) Relapse events were significantly lower in the intervention group. 4) Intervention most effective for ER-/PR- tumors.
Pierce (42)	WHEL randomized trial. Group 1: Dietary Intervention group received telephone counseling plus cooking classes and newsletters with aim to reduce dietary fat to 15-20% of calories, increase vegetable/fruit servings to 8/3. Group 2: Received newsletters and optional cooking classes tailored to the "5 A Day" campaign.	Recurrent and new primary breast cancer and all-cause mortality in women previously diagnosed with breast cancer	1) Intervention group increased fruit, fiber, and vegetable intake significantly. 2) No significant differences between intervention and control groups in disease recurrence or death.

**Table 1-3: Dietary intervention studies involving n-3 fatty acids and eicosanoid concentrations.**

Reference	Subjects/ Design	Intervention	Outcome Measures	Results
Fischer (63)	Single arm. Healthy men (n=8) ages 22-42.	20mL 2x/week cod liver oil (containing ~ 4g EPA) to diet for 25 days.	PLFA, TxB <sub>3</sub> formed in vitro with collagen stimulation, platelet aggregation.	1) Phospholipid EPA and DHA increased significantly. 2) TxB <sub>3</sub> produced in platelets. 3) Platelet aggregation decreased post-intervention.
Fischer (64)	Three arms. Healthy men, n=18.	3 groups: 1) 40 mL cod liver oil (~ 4g EPA) daily for 24 days. 2) 750g mackerel daily for 3 days. 3) control.	From urine: 2,3-dinor-6-keto-PGF <sub>1α</sub> and Δ17-2,3-dinor-6-keto PGF <sub>1α</sub> (major metabolites of PGI <sub>2</sub> and PGI <sub>3</sub> ); plasma fatty acids.	1) Plasma EPA and DHA were significantly increased and LA was significantly decreased by the cod liver oil. 2) Plasma EPA significantly increased and LA significantly decreased after mackerel feeding. 3) PGI <sub>3</sub> metabolites increased from non-detectable levels after both interventions. 4) PGI <sub>2</sub> increased with mackerel feeding.
von Schacky (65)	Single arm. Healthy med, n=6, ages 26-36.	10-40mL cod liver oil (9.4% EPA and 13.8% DHA) for 5 months: 4 weeks: 10mL/day; 4 weeks: 20mL/day; 4 weeks: 40mL/day; 8 weeks: 20mL/day.	From blood free fatty acids, plasma and platelet phospholipid fatty acids, TxA <sub>3</sub> , TxA <sub>2</sub> , TxB <sub>2</sub> , TxB <sub>3</sub> , platelet aggregation upon collagen stimulation, triglycerides (TG), cholesterol, HDL. From urine: 2,3-dinor-6-keto-PGF <sub>1α</sub> and Δ17-2,3-dinor-6-keto PGF <sub>1α</sub> (major metabolites of PGI <sub>2</sub> and PGI <sub>3</sub> ).	1) EPA and DHA increased significantly and according to a dose-response in free fatty acids and plasma phospholipids. LA and AA changed insignificantly. 2) AA increased in plasma phospholipids at high doses of oil. 3) EPA and DHA in platelet fatty acids increased slowly relative to plasma PLFA. 3) TG decreased significantly and according to a dose-response. 4) Cholesterol was unchanged. 5) Platelet concentration and aggregation decreased significantly. 5) Δ17-2,3-dinor-6-keto PGF <sub>1α</sub> increased from non-detectable amount to 50 ng/mg creatinine at peak of oil intake. 6) 2,3-dinor-6-keto-PGF <sub>1α</sub> did not change (~ 100 ng/mg creatinine). 7) TxB <sub>2</sub> dropped significantly. 8) TxB <sub>3</sub> was undetectable due to lack of a standard.



**Table 1-3 continued: Dietary intervention studies involving n-3 fatty acids and eicosanoid concentrations.**

Reference	Subjects/ Design	Intervention	Outcome Measures	Results
Ferretti (66)	Case study: woman (56 years old).	Woman was taking 10-50g/day MaxEPA (1.8 – 9.0g/day EPA); 1.3 – 6.5g/day DHA) for 4 years in addition to her normal diet.	Urinary derivatives of PGE <sub>3</sub> .	1) Urinary PGE <sub>3</sub> metabolites were identified in the subject's urine. 2) After the subject quit taking the supplement for 16 weeks, no PGE <sub>3</sub> metabolites were detected.
Ferretti (70)	Cross-over design. Healthy men (n=24) 24-54 years old.	10 weeks/diet, two diets: 1) regular (R) diet (41% energy from fat; P/S 0.59; M/S 0.96; 13.9 g/day fiber; 600 mg/day cholesterol); 2) experimental (E) diet (19% energy from fat; P/S 1.31; M/S 1.48; 35.5 g/day fiber; 280 mg/day cholesterol).	Urinary 7 $\alpha$ -hydroxy-5,11-dioxo-tetranorpropane-1,16-dioic acid (a metabolite of PGE <sub>1</sub> + PGE <sub>2</sub> ).	1) Urinary output of 7 $\alpha$ -hydroxy-5,11-dioxo-tetranorpropane-1,16-dioic acid decreased by 14.2% after E diet feeding.
Ferretti (67)	Cross-over design. Healthy men (n=10) 31-65 years old.	Two diets (both 16% protein, 54% carbohydrate, 30% fat): 1) salmon diet (S) contained 450 g/day salmon n-6:n-3 ratio of 3.6 (fat composition was 7.5% n-6, 2% n-3) and 2) reference (R) diet with n-6:n-3 ratio of 19.5 (fat composition was 7.5% n-6, 0.3% n-3). Diets supplemented with $\alpha$ -Tocopherol to 200% RDA. 20 days on R diet then either S or R diet for 40 days, then switch diets for 40 days.	Urinary 11 $\alpha$ -hydroxy-9,15-dioxo-2,3,4,5,20-pentanor-19-carboxy-prostanoic acid (an index of PGE <sub>1</sub> + PGE <sub>2</sub> turnover).	1) Effects of changing the n-6:n-3 FA ratio: urinary 11 $\alpha$ -hydroxy-9,15-dioxo-2,3,4,5,20-pentanor-19-carboxy-prostanoic acid significantly reduced by 40 days on S diet.
Ferretti (68)	Cross-over design. Healthy men (n=40) ages 24-57 years old.	Three sequential experimental periods, each involving consumption of a reference diet (40% energy from fat [P/S 0.8:1] ) plus one of three supplement regimens: 1) 15g/day placebo oil capsules (15g oil with distribution of FA similar to reference diet) (10 weeks); 2) 15g/day fish oil capsules (7.5 g/day n-3) (10 weeks); 3) 15g/day fish oil capsules plus extra 200mg/day $\alpha$ -Tocopherol (8 weeks).	Urinary 11 $\alpha$ -hydroxy-9,15-dioxo-2,3,4,5,20-pentanor-19-carboxy-prostanoic acid (an index of PGE <sub>1</sub> + PGE <sub>2</sub> turnover).	1) Excretion of urinary 11 $\alpha$ -hydroxy-9,15-dioxo-2,3,4,5,20-pentanor-19-carboxy-prostanoic acid was significantly lower at the end of period 2 vs. the end of period 1. 2) There was no difference between the values at the end of period 2 and period 3. Thus, vitamin E made no difference in excretion.

**Table 1-3 continued: Dietary intervention studies involving n-3 fatty acids and eicosanoid concentrations.**

Reference	Subjects/ Design	Intervention	Outcome Measures	Results
Ferretti (69)	Cross-over design. Healthy men (n=35) ages 24-57.	Two sequential experimental periods, each involving consumption of a reference diet (40% energy from fat [P/S 0.8:1] ) plus the following supplement regimens: 1) 15g/day placebo oil capsules (15g oil with distribution of FA similar to reference diet) (10 weeks); 2) 15g/day fish oil capsules (7.5 g/day n-3) (10 weeks).	Urinary 11-dehydro-TxB <sub>2</sub> (metabolite of TxA <sub>2</sub> ).	1) Urinary 11-dehydro-TxB <sub>2</sub> was reduced by 36% by the fish oil capsule intervention.
Prakash (71)	Cross-over design. Healthy men (n=9) ages 31 – 65 years.	Two diets: 1) reference diet (R) and 2) a salmon diet (S) containing 450g salmon/day (6g n-3 FA/day; 2% energy). Both diets had the composition of 19% energy from protein, 56% energy from carbohydrate, and 25% energy from fat.	Urinary TxB <sub>2</sub> , 2,3-dinor-TxB <sub>2</sub> , 6-oxo-PGF <sub>1α</sub> , 2,3-dinor-6-oxo-PGF <sub>1α</sub> , and PGE <sub>2</sub> ; platelet production of TxB <sub>2</sub> .	1) 2,3-dinor-TxB <sub>2</sub> was significantly lower after the S diet indicating reduced TxA <sub>2</sub> production. 2) All other measures did not differ between diets.
Mann (72)	Cross-over design. Men (n=14) and women (n=15) ages 22 – 52.	Three, 3-week diet periods separated by 3-week washout periods in the following order: 1) 1 week of vegetarian meals followed by 2 weeks of provided white meat; 2) 1 week of vegetarian meals followed by 2 weeks of provided red meat; 3) 1 week of vegetarian meals followed by 2 weeks of provided fish. All diets had 15% energy from fat.	Serum PLFA, platelet aggregation, ex vivo platelet TxB <sub>2</sub> production, urinary 2,3-dinor-6-keto-PGF <sub>1α</sub> (from 20:4n-6), Δ17-2,3-dinor-6-keto-PGF <sub>1α</sub> (from 20:5n-3) and 11-dehydro-TxB <sub>2</sub> .	1) No difference in platelet aggregation among diets. 2) Ex vivo TxB <sub>2</sub> production increased after the vegetarian periods, but decreased after the fish period. 3) 2,3-dinor-6-keto-PGF <sub>1α</sub> decreased after the vegetarian diet of each treatment period. No further lowering of output by either the fish or white meat diets. 4) Δ17-2,3-dinor-6-keto-PGF <sub>1α</sub> was only detected after fish diet. 5) 11-dehydro-TxB <sub>2</sub> was significantly reduced by the fish diet.

**Table 1-4: Omega-3 fatty acids and risk of breast cancer case-control and prospective cohort studies.**

Reference	Endpoints	Results
Wirfalt (73)	From FFQ and 7-day menu book: Total fat, SFA, MUFA, PUFA, SFA:PUFA ratio, n-3, n-6, n-3:n-6 ratio; breast cancer risk	Case-control study nested within the Malmo Diet and Cancer cohort. 1) Total FA, MUFA and PUFA positively associated with breast cancer risk, especially n-6 FA.
Gago-Dominguez (74)	From FFQ: total fat, SFA, MUFA, PUFA, n-3, n-3 marine, n-3 other foods, n-6, fish/shellfish intake; breast cancer risk.	Singapore Chinese Health Study cohort. 1) n-3 from fish/shellfish significantly inversely related to risk. 2) No association between n-6 and risk.
Goodstine (75)	From FFQ: total energy, total carbohydrate, total fat, total SFA, total MUFA, total PUFA, EPA, DHA, n-3, and long-chain n-3:n-6 ratio; breast cancer risk.	Case-control study. 1) No trends related to type of fat and risk in the whole population. 2) In premenopausal women, there was a non-significant decrease in breast cancer in the highest vs. lowest quartile of dietary fat intake.
Engeset (76)	From FFQ: fish intake at baseline and followed for 6.4 yr; breast cancer incidence.	EPIC prospective cohort. 1) No significant association of total fish intake and breast cancer risk. 2) Both lean and fatty fish measured; FFQ varied among centers; both recall and FFQ data used, which varied greatly between the two types of tools.
Lof (77)	From FFQ: total fat, MUFA, PUFA, SFA; breast cancer incidence.	Prospective cohort. 1) Total fat, MUFA, PUFA, and SFA not associated with risk in entire cohort. 2) In a subset of women in the highest quintile of MUFA and PUFA intake, risk was reduced in those over 50.
Cui (78)	From FFQ: association of two dietary patterns ("vegetable-soy" and "meat-sweet"); risk of breast cancer.	1) Risk increased in post-menopausal women with the "meat-sweet" pattern, similar to the Western diet.

**Table 1-5: Dietary fat and mammary tumor incidence animal studies.**

Reference	Intervention	Results
Silverstone (95)	Experiment 1 (n=260): Female C3H mice fed equicaloric diets of five fat percentages (by weight): 1.6, 5.7, 12, 26 and 24 +2% cholesterol. Experiment 2 (n=300): semi-purified feed plus fat at five percentages (by weight): 2, 4.1, 8, 16, and 23.7%.	1) Incidence of spontaneous tumors and rate of tumor appearance correlated directly with percent of dietary fat.
Cleary (96)	MMTV-TGF $\alpha$ transgenic mice (n=76) fed two diets ad libitum: 1) low fat chow (11% energy from fat); or 2) high fat diet (32.5% energy from fat). (fat from corn oil)	1) Spontaneous tumor incidence of groups did not differ. 2) Tumor latency shortest for the obesity-prone group.
Yee (98)	Four groups: corn oil (25% of energy) or fish oil (25% of energy) with or without rosiglitazone drug.	1) Rosiglitazone had no effect on spontaneous tumor incidence. 2) Fish oil diet significantly reduced tumor number and incidence.
Karmali (99)	Four groups of F344 rats fed chow (5% energy from fat) plus 1) 17mg, 2) 33mg, 3) 67mg of each DHA and EPA per day or 4) control. R2320AC tumor was implanted at one week after starting the diet.	1) Size of implanted mammary carcinomas was significantly reduced in DHA and EPA supplemented diet groups compared to control. 2) Tumor PGE <sub>2</sub> and PGF <sub>1</sub> were significantly decreased in supplemented groups.
Kort (100)	Four diets fed to BN/Bi female rats (n=100): 1) high SFA cocoa butter; 2) high n-6 PUFA; 3) high n-3 PUFA; 4) low SFA, low n-6 PUFA diets. Mammary adenocarcinoma implanted at 12 weeks age; removed when 20mm. Two weeks later, animals killed and lung metastasis counted.	1) PGE <sub>2</sub> and TXB <sub>2</sub> were significantly lower in high n-3 PUFA group. 2) Number of lung metastasis was not different, but tumor growth was significantly reduced in high n-3 PUFA group.
Rose (101)	Athymic nude mice (n=248) were fed one of four high fat (20% by weight) diets after injection of MDA-MB-435 cells into mammary fat pad: 8% from LA diet, or substituted 8, 4, or 2% of fat from EPA or DHA diet.	1) Both the EPA and DHA diets significantly reduced lung metastasis. Dosing prior to excision of primary tumor was most effective.
Gammal (97)	Sprague-Dawley rats (n=66) were fed three diets ad libitum after weaning. Diet 1: high (20% by weight) corn oil diet; diet 2: high (20% by weight) coconut oil diet; or diet 3: low fat (0.5% by weight) corn oil diet.	1) High corn oil diet significantly increased mammary formation of DMBA –induced tumors relative to low fat diet or coconut oil diet.

**Table 1-5 continued: Dietary fat and mammary tumor incidence animal studies.**

Reference	Intervention	Results
Jurkowski (102)	Two experiments: 1) BUF rats fed diets of 0.5, 3, or 20% (by weight) menhaden oil; 2) diets of 0.5 or 20% (by weight) corn oil, 0.5 or 20% (by weight) menhaden oil or control (Formulab chow).	1) 20% menhaden oil diet significantly reduced N-methyl-N-nitrosurea (NMU)-induced tumor incidence. Tumor latency period was also increased. 2) 20% corn oil increased incidence and tumor reduced latency period. 3) Membrane FA analysis on the menhaden oil-treated rats' liver microsomes revealed an inverse association between EPA in the lipids and tumor incidence.
Braden (103)	Sprague-Dawley rats (n=220) were fed in two experiments: 1) DMBA administration at 50 days, then fed diets of 3, 10, or 20% (by weight) sunflower oil or 3% (by weight) sunflower oil mixed with either 7% or 10% (by weight) coconut oil. 2) DMBA dose at 50 days, then 1 week later fed diets: 2, 10, or 20% (by weight) corn oil or menhaden oil.	1) Experiment 1: 10% and 20% sunflower groups had significantly more tumors than the other groups. 2) Experiment 2: significantly fewer carcinomas in rats fed medium and high levels of menhaden oil. Menhaden oil also increased the latent period.
Abou-El-Ela (104)	Sprague-Dawley rats (n=108) fed three diets: 20% (by weight) primrose oil, menhaden oil, or corn oil.	1) Rats fed primrose oil or menhaden oil diets had significantly fewer DMBA-induced malignant tumors than the corn oil group. 2) Eicosanoid synthesis in all the high fat groups was significantly greater than in control rat mammary. 3) PGE <sub>1</sub> and LTB <sub>4</sub> significantly greater with primrose oil. 4) PGE <sub>1</sub> /PGE <sub>2</sub> highest in corn oil group. 5) Mammary synthesis of eicosanoids was significantly lower in menhaden oil group than primrose oil or corn oil.
Olivo (105)	5-day old Sprague-Dawley rats (n=80) were fed either a low fat (16% of energy from fat) n-6 (corn oil) diet, high fat (39% of energy from fat) n-6 diet, low fat (16% of energy from fat) n-3 (menhaden oil) diet, or a high fat (39% of energy from fat) n-3 diet.	1) High fat n-3 diet rats had increased DMBA-induced tumor incidence while low fat n-3 diet rats had decreased incidence. 2) Terminal end bud differentiation was induced by low fat n-3 diet. 3) Low fat n-3 diet reduced COX-2 activity. 4) Low fat n-3 diet increased apoptosis and reduced proliferation. High fat n-3 diet did the opposite.

**Table 1-5 continued: Dietary fat and mammary tumor incidence animal studies.**

Reference	Intervention	Results
Badawi (337)	Sprague-Dawley rats (n=20) were fed one of four diets for three weeks: Low fat menhaden oil diet (7% fat by weight); High fat menhaden oil diet (21% fat by weight); low fat safflower diet (7% fat by weight); and high fat safflower oil diet (21% fat by weight).	1) Amount of COX-1 mRNA was significantly less in the menhaden oil diets than the safflower oil diets. 2) COX activity was significantly less in the high fat menhaden oil diet than both the low fat menhaden oil diet and the high fat safflower oil diet. 3) PGE <sub>2</sub> was significantly less with high fat menhaden oil feeding than with both the low fat menhaden oil diet and the high fat safflower oil diet. 4) p21ras protein was significantly higher in the high fat safflower diet compared to the other three diets.

**Table 1-6: Case-control and cross sectional studies of breast cancer and circulating sex hormones.**

Reference	Design/Subjects	Outcome Measures	Results
Gordon (127)	Case-control study. n=30 cases; n=59 matched controls; postmenopausal samples from serum blood bank	DHEA and DHEAS	1) DHEA significantly increased among cases.
Barrett-Connor (129)	Case-control study within Rancho Bernardo Cohort. Three groups of cases: 1) n=21 diagnosed > 1 year following draw; 2) n=20 diagnosed before draw or < 1 year after draw; 3) n=10 with unknown date of onset. Controls n= 534. Postmenopausal women.	DHEAS	1) No significant associations between any of the case groups and controls. 2) Relative risk of tertiles of DHEAS not significantly different.
Toniolo (119)	Nested case-control study within cohort of NYC women. Cases n=130; controls n=260; postmenopausal women.	E1, total E2, free E2, free E2 %, Albumin-bound E2 and %, SHBG-bound E2 and %, FSH	1) Free E2 positively associated with risk. 2) % of E2 bound to SHBG inversely associated with risk.
Berrino (121)	Nested case-control within ORDET study. Cases n=24; matched controls n=88; postmenopausal women.	DHEAS, E2, total T, free T, SHBG	1) Free T, total T, and E2 significantly higher in cases than controls; 2) Risk of breast cancer significantly positively associated with T level.
Dorgan (118)	Nested case-control study with samples from Columbia, MO serum bank. Cases n=71; matched controls n=142; postmenopausal women.	E2, free E2, E1, E1-S, T, A, DHEAS, SHBG	1) Total and free E2 significantly associated with breast cancer risk; ratio of E1-S:E1 inversely related to risk; T significantly positively associated with risk.
Dorgan (128)	Nested case-control with samples from Columbia, MO serum bank. Cases n=71; matched controls n=142; postmenopausal women.	DHEA, DHEAS, ADIOL	1) DHEA and ADIOL significantly associated with breast cancer risk; 2) elevated risk with DHEAS in highest quartile.

**Table 1-6 continued: Case control and cross sectional studies of breast cancer risk and circulating sex hormones.**

Reference	Design/Subjects	Outcome Measures	Results
Zeleniuch-Jacquotte (120)	Nested case-control study within cohort of NYC women. Cases n=85; controls n=85; postmenopausal women.	T, DHEAS, total E2, % E2 bound by SHBG, % free E2	1) T associated w/ breast cancer risk until adjusted for E2. 2) E2 significantly associated with breast cancer risk and independent of T adjustment.
Hankinson (123)	Nested case-control study within the NHS. Cases n=156; matched controls n=312; postmenopausal women.	Total E2, free E2, bioavailable E2, E1, E1-S, Andro, T, DHEA, DHEAS	1) Risk of breast cancer positively associated with E2, E1, E1-S, DHEAS.
Cauley (122)	Nested case-control study within the Study of Osteoporotic Fractures. Cases n=97; controls n=244; postmenopausal women.	Total E2, non SHBG-bound E2, free E2, E1, E1-S, A, total T, free T, DHEAS, SHBG	1) Relative risk for breast cancer was significantly greater with high free E2 level and with high free T levels.
Missmer (338)	Nested case-control study within the NHS. Cases n=322; matched controls n=643.	Total E2, free E2, E1, E1-S, progesterone, A, T, free T, SHBG DHEA, DHEAS.	1) Significant positive association between each hormone and breast cancer risk except progesterone and SHBG. 2) Greatest positive associations seen in ER+/PR+ cases.
Kabuto (126)	Prospective cohort study of Hiroshima bomb survivors. Cases n=72; controls n=150.	Total E2, bioavailable E2, SHBG, DHEAS, prolactin.	1) Odds ratio significant for bioavailable E2 when both pre and postmenopausal women assessed together but not significant as separate groups.
Kaaks (124)	Nested case-control study within the EPIC study. Cases n=677; matched controls n=1309.	DHEA-S, A, T, E2, E1, SHBG, free T and free E2 calculated using SHBG.	1) All hormones and fractions positively associated with breast cancer risk; 2) SHBG negatively associated with risk.



**Table 1-7: Cross-sectional studies of breast cancer risk and breast tissue sex hormone levels.**

Reference	Subjects	Outcome Measures	Results
Pasqualini (135)	Cases: n=34 (11 premenopausal women and 23 postmenopausal women).	Breast tissue and plasma levels of E1, E2, E1-S; sulfatase activity and aromatase activity in breast tissue.	1) Level of E1-S in breast tissue was significantly greater in post vs. premenopausal women; 2) Tissue levels of all hormones are several-fold higher than plasma levels suggesting that tumors sequester estrogens; 3) E1-S sulfatase activity in both groups was significantly higher than aromatase.
Chetrite (134)	Cases = 14 peri/postmenopausal women	Tumor tissue, area adjacent to the tumor, and a distant normal glandular area were sampled. Tissue levels of E1, E2, E1-S, E2-S, sulfatase and aromatase activity were assayed.	1) E1-S and E2 were significantly higher in tumor tissue than normal tissue. 2) Sulfatase activity was higher than aromatase activity in all tissues. 3) Sulfatase level was higher in the tumor and adjacent tissue than in normal tissue. 4) Aromatase levels were higher in tumor tissue than in normal tissue.

**Table 1-8: Case-control and prospective cohort studies of 2OH-E1:16 $\alpha$ OH-E1 ratio and breast cancer risk.**

Reference	Design/Subjects	Outcome Measures	Results
Ursin (160)	Case-control study. Postmenopausal women: cases (n=25) and controls (n=23).	Urinary (first morning urine) E1, E2, E3, 2OH-E1, 16 $\alpha$ OH-E1, 2OH-E1:16 $\alpha$ OH-E1 ratio.	1) Urinary E1, E2, and E3, were higher in cases than controls, but not significantly different. 2) 2OH-E1:16 $\alpha$ OH-E1 ratio was lower in cases, but not significantly different than controls.
Kabat (156)	Case-control study. Pre- and postmenopausal women: cases (n=42) and controls (n=64).	Urinary (spot urine sample) 2OH-E1, 16 $\alpha$ OH-E1, 2OH-E1:16 $\alpha$ OH-E1 ratio, breast cancer risk.	1) 2OH-E1:16 $\alpha$ OH-E1 ratio was significantly lower in postmenopausal cases than controls. 2) Odds ratio of lowest and intermediate tertiles of 2OH-E1:16 $\alpha$ OH-E1 ratio were significantly increased relative to the highest tertile (indicating increased risk with low 2OH-E1:16 $\alpha$ OH-E1 ratio).
Zheng (158)	Case-control study: 20 case, 20 controls. Does not indicate ages.	Urinary (overnight urine sample) 2OH-E1:16 $\alpha$ OH-E1 ratio.	1) 2OH-E1:16 $\alpha$ OH-E1 ratio was significantly reduced in cases relative to controls.
Meilahn (161)	Prospective cohort study. Postmenopausal women: 42 cases and 139 controls; pre-menopausal women: 60 cases and 184 controls.	Urinary (spot urine sample; stored for up to 19 years) 2OH-E1, 16 $\alpha$ OH-E1, 2OH-E1:16 $\alpha$ OH-E1 ratio, breast cancer risk.	1) Risk reduced by 30% in highest tertile of 2OH-E1:16 $\alpha$ OH-E1 ratio for postmenopausal women, but the comparison was not significantly different.
Ursin (163)	Case-control study in postmenopausal women (cases=66; controls=76).	Urinary (first morning urine) E1, E2, E3, 2OH-E1, 16 $\alpha$ OH-E1, 2OH-E1:16 $\alpha$ OH-E1 ratio, breast cancer risk.	1) Urinary E1, E2, 2OH-E1, 16 $\alpha$ OH-E1, and 2OH-E1:16 $\alpha$ OH-E1 ratio were higher in cases than controls, but not significantly. 2) 2OH-E1:16 $\alpha$ OH-E1 ratio was not significantly different in cases than controls.
Muti (162)	Prospective cohort study in pre- and postmenopausal women: 144 cases (71 postmenopausal) and 576 controls (4 per case).	Urinary (overnight urine) 2OH-E1, 16 $\alpha$ OH-E1, 2OH-E1:16 $\alpha$ OH-E1 ratio, breast cancer risk.	1) Neither pre- or postmenopausal women were at significantly increased risk with low 2OH-E1:16 $\alpha$ OH-E1 ratio.
Fowke (164)	Case-control study in pre- and postmenopausal women (cases=110) and (controls=110).	Urinary 2OH-E1, 16 $\alpha$ OH-E1, 2OH-E1:16 $\alpha$ OH-E1 ratio, breast cancer risk. Urine collected prior to cancer treatment in 78 cases and after treatment in 32 cases.	1) Women with reduced 2OH-E1:16 $\alpha$ OH-E1 ratio were more likely (nonsignificantly) to be diagnosed with breast cancer. 2) Significantly increased risk for breast cancer was observed with high 2OH-E1:16 $\alpha$ OH-E1 ratio if urine sample was collected after treatment.

**Table 1-8 continued: Case-control and prospective cohort studies of 2OH-E1:16 $\alpha$ OH-E1 ratio and breast cancer risk.**

Reference	Design/Subjects	Outcome Measures	Results
Cauley (165)	Prospective cohort study in women 65 and older: cases=272 and controls =291.	Serum 2OH-E1, 16 $\alpha$ OH-E1, 2OH-E1:16 $\alpha$ OH-E1 ratio, breast cancer risk.	1) No difference in 2OH-E1:16 $\alpha$ OH-E1 ratio between case and controls. 2) Women with high 2OH-E1:16 $\alpha$ OH-E1 ratio were at nonsignificantly increased risk of breast cancer.
Wellejus (159)	Prospective cohort study in postmenopausal women: cases=426, controls=426.	Urinary (spot urine) 2OH-E1, 16 $\alpha$ OH-E1, 2OH-E1:16 $\alpha$ OH-E1 ratio, breast cancer risk.	1) Risk of estrogen-receptor positive breast cancer was increased in hormone replacement therapy users with high 2OH-E1. 2) No increased risk in non-hormone replacement therapy users.
Eliassen (166)	Prospective cohort study in postmenopausal women: cases=340 and controls=677.	Serum 2OH-E1, 16 $\alpha$ OH-E1, 2OH-E1:16 $\alpha$ OH-E1 ratio, breast cancer risk.	1) No association overall between risk of breast cancer and concentration of 2OH-E1, 16 $\alpha$ OH-E1, or 2OH-E1:16 $\alpha$ OH-E1 ratio. 2) For women with estrogen receptor negative/progesterone receptor negative tumors, 2OH-E1 and 2OH-E1:16 $\alpha$ OH-E1 ratio were positively associated with breast cancer risk.
Im (339)	Case-control study in pre- and postmenopausal women: 77 high-risk women, 30 cases, 30 controls.	Urinary (spot urine) 2OH-E1, 16 $\alpha$ OH-E1, 2OH-E1:16 $\alpha$ OH-E1 ratio, breast cancer risk.	1) High risk women and cases had similar 2OH-E1:16 $\alpha$ OH-E1 ratio. Controls had significantly higher 2OH-E1:16 $\alpha$ OH-E1 ratio. 2) Reduced 2OH-E1:16 $\alpha$ OH-E1 ratio significantly associated with breast cancer risk in cases and high risk women relative to controls.
Ho (157)	Pre- and postmenopausal women: cases=65 and controls=36.	Urinary 2OH-E1, 16 $\alpha$ OH-E1, 2OH-E1:16 $\alpha$ OH-E1 ratio, breast cancer risk	1) 2OH-E1 and 2OH-E1:16 $\alpha$ OH-E1 ratio significantly reduced in cases. 2) 16 $\alpha$ OH-E1 significantly increased in cases. 3) Women with highest 2OH-E1:16 $\alpha$ OH-E1 ratio were at significantly reduced risk of breast cancer.

**Table 1-9: Dietary intake and blood and urinary sex hormones observational studies.**

Reference	Subjects/Design	Outcome Measures	Results
Goldin (169)	Pre- (ages 20 -30; n=7-9) and postmenopausal (n=7-9) vegetarian and omnivorous women (4 groups). Dietary intake was assessed by diet history at 3, 3-month intervals.	Fecal (72-hour sample) E1, E2, E3, urinary (72-hour sample) conjugated E1, E2, E3; plasma unconjugated E1, E2, T, A.	1) Total energy did not differ among groups. 2) Vegetarians consumed 33% the amount of animal protein and 36% the amount of animal fat as omnivores (did not indicate statistical significance). 3) Fecal excretion of E1, E2, E3 was significantly greater in vegetarians than omnivores and significantly greater in pre- vs. postmenopausal women. 4) T and A levels were significantly higher in premenopausal vegetarians than premenopausal omnivores, but postmenopausal levels were higher in omnivores than vegetarians. 5) Urinary estrogens did not differ among groups.
Armstrong (172)	Postmenopausal vegetarian Seventh-Day Adventists (ages 50-79; n=46) and omnivores (n=47). Qualitative dietary data assessment.	Urinary (12-hour sample) E1, E2, E3, E3 ratio (E3:[E1 + E2 + E3])and total estrogens; plasma FSH, prolactin, cortisol, SHBG T, cholesterol, TG, HDL.	1) Dietary assessment indicated that Seventh-Day Adventists ate less fat than omnivores. 2) Plasma prolactin and SHBG were significantly greater in omnivores than vegetarians. 3) Urinary E3excretion was significantly greater in omnivores than vegetarians.
Adlercreutz (170)	Postmenopausal women: vegetarians (n=9), omnivores (n=10), breast (n=8) cancer survivors. Women gave samples and dietary info 4 times in one year to control for seasonal variation.	Plasma T, A, Free T, % Free T, DHEAS, SHBG.	1) Omnivores consumed significantly more energy and total fat than both the vegetarian and breast cancer survivors groups. 2) A, T, Free T, and SHBG significantly greater in omnivores than vegetarians. 2) Protein and total fat intake were positively correlated with A, T, and Free T. 3) Breast cancer survivors had significantly higher levels of A and T compared to vegetarians and higher levels of Free T and lower SHBG than the other two groups.

**Table 1-9 continued: Dietary intake and blood and urinary sex hormones observational studies.**

Reference	Subjects/Design	Outcome Measures	Results
Goldin (173)	Participants: premenopausal Caucasians (n=10); premenopausal Oriental immigrants (n=12); postmenopausal Oriental immigrants (n=11); postmenopausal Caucasians (n=11).	Plasma E1, E2, E1 + E2, T and A; urinary (24-hour) E1, E2, E3, total estrogens; fecal (24-hour) E1, E2, E3, total estrogens; dietary intake from 3-day food records (2/person).	1) Total energy, total fat, and saturated fat were significantly greater in both pre- and postmenopausal Caucasians than the Oriental women of each age group. 2) Premenopausal Oriental women excreted significantly more E1, E2, E3, total estrogens in the feces than their Caucasian counterparts. 3) Pre- and postmenopausal Caucasian women excreted more E1, E2, E3, total estrogens in the urine than the Oriental women. 4) Plasma E1, E2, E1 + E2, T and A were significantly greater in premenopausal Caucasian women and plasma E2, E1 + E2, T and A higher in postmenopausal Caucasian women than the Oriental women in their age-group. 5) Total fat and saturated fat positively correlated with plasma E1 and E2 in premenopausal women.
Adlercreutz (167)	Premenopausal women: Oriental immigrants to Hawaii (n=13) and Finnish women (n=12).	Plasma E1-S, E1, E2, SHBG; fecal E1, E2, E3; urinary E1, E2, E3, 2OH-E1, 4OH-E1, 2OH-E2, 17-epiestriol, 16-epiestriol, 2MeO-E1, 16 $\alpha$ OH-E1, 15 $\alpha$ OH-E1, 16 $\beta$ OH-E1, 16-ketoestradiol, Total estrogens.	1) Dietary fat intake was significantly higher in Finnish women (73% higher). 2) Finnish women had significantly increased plasma E1-S and E2. 3) Urinary E1, E2 and catchol estrogens were elevated in Finnish women. 4) 16 $\alpha$ OH-E1 was elevated in Oriental women relative to Finnish women. 5) Oriental women had elevated fecal estrogen excretion relative to Finnish women.
Fowke (341)	Cross-sectional Study. Healthy postmenopausal women (n=37). Two urine collections 2 weeks apart with 6, 24-hour diet recalls in-between.	Urinary (24-hour) 2OH-E1, 16 $\alpha$ OH-E1, 2OH-E1:16 $\alpha$ OH-E1 ratio.	1) Concluded that 75% of the variation in 2OH-E1:16 $\alpha$ OH-E1 ratio measurements in postmenopausal women is due to intra-individual variation. 2) Low fat (and low saturated fat) high fiber diet was significantly and positively associated with 2OH-E1:16 $\alpha$ OH-E1 ratio.

**Table 1-9 continued: Dietary intake and blood and urinary sex hormones observational studies.**

Reference	Subjects/Design	Outcome Measures	Results
London (174)	Perimenopausal women (n=325) ages 50-60 (American).	Reported dietary intake from a FFQ; plasma E1, E2, percent free E2, SHBG, cortisol, FSH, LH.	1) No associations were detected between any of the hormones and total dietary fat, saturated fat, or LA.
Newcomb (175)	Postmenopausal women (n=253) (American).	Diet from FFQ; serum E1, DHEAS, SHBG, total T, free T.	1) Total fat and saturated fat were not correlated with any hormone measure.
Holmes (176)	Cross-sectional study within the NHS. Cohort subset (n=381) (American).	Reported fat intake from 2 FFQ (1986 and 1990) and plasma of E2, bioavailable E2 and % E2, free E2, E1, E1-S, SHBG, A, T, DHEA, DHEAS.	1) Percentage energy from fat inversely associated with E2 levels.
Wu (177)	Cross-sectional study. Postmenopausal Chinese women (n=144) in Singapore.	Dietary intake from a FFQ; plasma E1, E2, A.	1) No associations were detected between any of the hormones and total fat, sat fat, MUFA, or PUFA.
Nagata (178)	Cross-sectional study. Healthy postmenopausal Japanese women (n=324).	Dietary intake from FFQ; serum E1, E2, T, DHEAS.	1) Serum E1 positively associated with percent energy from fat. 2) DHEAS positively associated with percent energy from total fat, sat fat, MUFA, PUFA.

**Table 1-10: Dietary fat and blood sex hormones dietary intervention studies.**

Reference	Subjects/Design	Intervention	Outcome Measures	Results
Prentice (182)	Single-arm intervention in 73 healthy postmenopausal women who were part of the Women's Health Trial.	10-22 weeks of free-living low fat diet.	E1, E1-S, E2, Free E2, bioavailable E2 weakly bound E2, SHBG-bound E2, SHBG, cholesterol.	1) Significant reduction in E2. 2) Significant reduction in plasma cholesterol and weight loss. 3) Average reduction in dietary fat from 68.5 to 29.5g/day.
Bennett (342)	Parallel arm intervention, n=39 premenopausal women.	Intervention 1): subject's standard diet + encouragement to eat meat; 2) vegetarian group; 3) vegetarian group + encouraged to eat fish 3x/wk. Duration: 3 months.	Dietary intake at baseline and end by 7, 24-hour food records; total prolactin, total progesterone, total T, SHBG, total E2, non-protein-bound E2.	1) E2 significantly decreased in vegetarian group. 2) SHBG decreased significantly with decreased fat consumption. 3) Free E2 increased significantly with increased fat consumption.
Heber (180)	Single arm intervention in 13 postmenopausal women located at Pritkin Longevity Center.	3-4 weeks of cafeteria food <10% calories from fat.	Height, weight, serum cholesterol, TG, HDL, LDL, E2.	1) Significant weight loss. 2) Significant decreases in serum cholesterol, LDL, HDL and E2.
Crighton (179)	Single arm intervention n=37 (19 post and 18 premenopausal women).	4 weeks of free living diet aimed to reduce fat calories to 20% of total.	7-day food records for intake; sex hormones: E2, prolactin, SHBG, free E2.	1) Fat intake decreased significantly; 2) Nonsignificant increase in SHBG and decrease in prolactin in postmenopausal group.
Tymchuk (181)	Single arm study in 22 postmenopausal women; 11 on HRT, 11 not on HRT. Located at Pritkin Center.	Low fat (<10%), high fiber (65-70 g/day) diet plus exercise intervention. Three weeks in duration.	Anthropomorphic measures, total cholesterol, LDL, HDL, TG, glucose, insulin, SHBG.	1) SHBG increased significantly in both groups. 2) Insulin decreased significantly in both groups. 3) BMI and cholesterol significantly reduced.

**Table 1-10 continued: Dietary fat and blood sex hormones human intervention studies.**

Reference	Subjects/ Design	Intervention	Outcome Measures	Results
Berrino (183)	Parallel arm study; n=104 postmenopausal women with high T levels (DIANA trial).	Control vs intervention of diet counseling and 2x weekly group meal prep over 4.5 months. Meals were Mediterranean or macrobiotic in nature, and women received a loaf of flax bread per week and other items	Serum T, E2, SHBG, anthropomorphic measures; diet analyzed by FFQ and 24h food diaries	1) Significant increase in SHBG in active group. 2) T reduced significantly in active group. 3) Non-significant reduction in E2 in active group. 4) Active group lost significantly more weight. 5) Fat intake reduced about 7% in active group.
Wu (184)	Parallel arm study of 57 postmenopausal women.	Intervention 1: very low fat diet (VLFD; 11% energy from fat; n=20); Intervention 2: Step 1 diet plus soy food,(SFD; 30% energy from fat; n=20); Control: Step 1 diet (CD; 30% energy from fat; n=17). 8 week controlled diet; meals were prepared at a GCRC.	Total cholesterol, TG, HDL, LDL, total E2, free E2, E1, E2/E1, T, free T, A, SHBG, IGF-1, IGFBP-3, insulin, leptin	1) No difference in hormone levels between either of the intervention groups and the controls at 8 weeks. 2) Insulin decreased significantly in SFD group and CD group. 3) Leptin decreased significantly in VLFD and SFD groups. 4) IGF-1 level increased significantly in the VLFD group.



**Table 1-11: Dietary fat and urinary sex hormones dietary intervention studies.**

Reference	Subjects/ Design	Intervention	Outcome Measures	Results
Longcope (185)	Single arm. Premenopausal women (n=6).	High fat diet (40% of energy from fat; 75g protein, 105 g carbohydrates) for 4 weeks, followed by low fat diet (25% of energy from fat, 80g protein, 257g carbohydrates) for two months. Meals prepared at USDA metabolic kitchen. On day 5-7 of menstrual cycle, each woman was given [6,7- <sup>3</sup> H]E2 orally and [4- <sup>14</sup> C]E2 by iv.	Blood samples at 8 hours (14 time points) after administration of radiolabeled E2 were analyzed for [ <sup>3</sup> H]/[ <sup>14</sup> C] E1, [ <sup>3</sup> H]/[ <sup>14</sup> C] E2, [ <sup>3</sup> H]/[ <sup>14</sup> C] E2 glucuronide, [ <sup>3</sup> H]/[ <sup>14</sup> C] E1-S, [ <sup>3</sup> H]/[ <sup>14</sup> C] E1 glucuronide.  Urine was collected for 96 hours after administration of radio-labeled E2 and assayed for radiolabeled estrogen glucuronides.	1) Urinary 16 $\alpha$ -hydroxylation of estrogens was reduced significantly relative to the catechol estrogen pathway with the low fat diet.
Osborne (188)	No information on subjects given.	Placebo, levothyroxine, or n-3 fatty acids. No dose information mentioned.	Urinary 16 $\alpha$ OH-E1.	1) Reduction in 16 $\alpha$ -hydroxylation of estrogens with n-3 fatty acid supplement.
Dorgan (186)	Controlled, cross-over feeding study in men (n=43)	Either a low fat/high fiber diet (20% fat, PUFA:sat fat 12) or a high fat/low fiber diet (40% fat, PUFA: sat fat ratio of 0.5) for 10 weeks each; separated by a 2-week wash-out period.	Plasma SHBG, T (free, total, albumin-bound, SHBG-bound) DHEA, DHEAS, A, E1, E2 (free, total, albumin-bound and SHBG-bound; and urinary T, E1, E2, E3, 2OH-E1, 2OH-E2.	1) SHBG-bound T significantly greater with high fat diet. 2) Urinary T excretion significantly greater with high fat diet. 3) Urinary E1, E2, 2OH-E1, and 2OH-E2 significantly increased with LF diet.

**Table 1-11 continued: Dietary fat and urinary sex hormones dietary intervention studies.**

Reference	Subjects/ Design	Intervention	Outcome Measures	Results
Carruba (187)	Healthy postmenopausal women (n=115, selected based on serum T level $\geq 0.14$ mg/mL).	Intervention group consumed a traditional Mediterranean diet, attended a weekly cooking course. The Mediterranean diet was stressed to include whole grains, beans, fish, nuts and seeds and exclude animal fats and refined sugars. Control women consumed their normal diets. Study lasted 6 months.	Dietary intake by FFQ at beginning and end of study; urinary (12-hour) E1, E2, E3, 2OH-E1, 4OH-E1, 2OH-E2, 4OH-E2, 17-epiestriol, 16-epiestriol, 2MeO-E1, 4MeO-E1, 2MeO-E2, 4MeO-E2, 16 $\alpha$ OH-E1, 16-ketoestradiol, Total estrogens.	1) Intervention group consumed significantly less total energy, total protein, animal protein, total fat, animal fat, sat fat, cholesterol, carbohydrates and sugar. 2) Total estrogens, 2OH-E2, 16keto-E2, and 17epiestriol decreased significantly in the intervention group.
Wu (189)	Randomized/placebo-controlled trial. Postmenopausal vegetarian women (n=27).	2-week run-in period with 6g corn oil/day. Then subjects took either corn oil (6g/day) or 6g DHA-rich algae oil (2.14g DHA/day) for 6 weeks.	Urinary 2OH-E1, 16 $\alpha$ OH-E1, 2OH-E1:16 $\alpha$ OH-E1 ratio, F2-isoprostanes; plasma LDL fatty acids, TG, LDL-C, HDL-C, $\alpha$ -tocopherol, LDL T-bars.	1) Plasma LDL DHA and EPA increased significantly with the DHA supplement. 2) No difference between groups for urinary estrogens or F2-isoprostanes. 3) DHA supplement decreased plasma cholesterol.

**Table 1-12: Case-control and prospective cohort studies of IGF pathway and breast cancer related endpoints.**

Reference	Subjects	Outcome Measures	Results
Peyrat (202)	Case-control study. Cases n=47; controls n=134. Pre- and postmenopausal women.	Plasma IGF-I	1) IGF-I levels were significantly higher in breast cancer cases than controls.
Lonning (243)	Cross-sectional study. Cases n=32, postmenopausal.	Blood hormones and binding proteins: E2, E1, E1-S, DHEA, DHEA-S, A, T, SHBG, IGF-I, IGFBP-1. Urinary hormone metabolites: 2OH-E1, 2OH-E2, 4OH-E1, E1, E2, E3, 16 $\alpha$ OH-E1, 16 $\beta$ OH-E1, total urinary estrogens.	1) No association between androgen and estrogen levels and IGF-1. 2) No correlations between urinary estrogens and IGF-I or IGFBP-1. 3) IGFBP-1 correlated inversely with IGF-I.
Hankinson (245)	Nested case-control study within the NHS. Cases n=397; controls n=620. Pre- and postmenopausal.	IGF-I and IGFBP-3; breast cancer risk.	1) No association in postmenopausal women for IGF-1 and breast cancer risk although there was a positive association for premenopausal women.
Jernstrom (256)	Nested case-control study within the Rancho Bernardo Study. Cases n=45, controls n=393 aged 53-90 years.	BMI, IGF-I, proinsulin, C-peptide, fasting insulin, and breast cancer risk.	1) In women who gained weight since baseline or used HRT, breast cancer risk was increased. 2) IGF-I not associated with breast cancer risk.
Vadgama (241)	Retrospective cross-sectional study. Cases (n=130) were minority women ages 31-80; Controls (n= 42) were ages 22-74.	IGF-I and IGFBP-3, tumor characteristics, tamoxifen status.	1) Cases had significantly higher IGF-I and IGFBP-3 levels than controls. 2) Premenopausal cases had significantly higher IGF-I levels than postmenopausal cases. 3) IGF-I levels were significantly higher in premenopausal cases with recurrence than those without recurrence. 4) Survival of cases with low levels of IGF-I was significantly greater than those with high levels of IGF-I.

**Table 1-12 continued: Case-control and prospective cohort studies of IGF pathway and breast cancer related endpoints.**

Reference	Subjects	Outcome Measures	Results
Toniolo (246)	Nested case-control study within the NYU Women's Health Study. Cases n=287 (172 premenopausal and 115 postmenopausal); controls n=220.	IGF-I, IGFBP-3, C-peptide, and breast cancer risk.	1) In premenopausal women, IGF was directly associated with breast cancer risk. 2) No associations in postmenopausal women.
Muti (248)	Nested case-control study within the ORDET study. Cases n=144; controls n=576. Pre- and postmenopausal women.	Serum glucose, IGF-I, free IGF-I, insulin, IGFBP-1, IGFBP-2, IGFBP-3; breast cancer risk.	1) In premenopausal women, glucose, insulin, IGFBP-3 and IGF-1 were positively associated with risk. 2) When postmenopausal women were stratified by BMI, glucose, insulin, and IGF-I were positively associated with breast cancer risk in women with BMI over 26.
Yu (249)	Case-control study. Cases n=300; controls n=300. Pre- and postmenopausal women.	IGF-1, IGF-II and IGFBP-3; breast cancer risk.	1) IGF-1 and IGFBP-3 were higher in cases than controls. 2) Breast cancer risk increased as level of IGFBP-3 and IGF-1 increased. These associations were stronger for premenopausal women.
Probst-Hensch (242)	Nested cross-sectional study within the Singapore Chinese Health study. n=312 men and 326 postmenopausal women.	Information from a FFQ, IGF-I, IGFBP-3, IGF-I:IGFBP-3 ratio.	1) Levels of IGF-I and IGFBP-3 were significantly greater in men. 2) Levels of IGF-I and IGFBP-3 decreased with increasing age. 3) SFA decreased and n-3 fat and fiber increased relative to IGFBP-3.
Krajcik (247)	Nested case-control study within the Kaiser Permanente Medical Care Program. Cases n=126 (66 premenopausal, 60 postmenopausal) women; matched controls n=126.	IGF-I, insulin, glucose, IGFBP-1, IGFBP-2, IGFBP-3, breast cancer risk.	1) IGF-I associated with breast cancer risk in premenopausal women but not postmenopausal women. 2) IGFBP-3 higher in premenopausal cases than controls and was associated with increased risk. 3) IGFBP-3 was lower in postmenopausal cases than controls. 4) IGFBP-3 was inversely associated with risk in postmenopausal women.

**Table 1-12 continued: Case-control and prospective cohort studies of IGF pathway and breast cancer related endpoints.**

Reference	Subjects/Design	Outcome Measures	Results
DeLellis (343)	Nested case-control study within the Multi Ethnic Cohort. Cases n=400; controls n=400; equal numbers of postmenopausal women from 4 ethnic/racial groups	IGF-I, IGF-I genotype, breast cancer risk	1) Latino women had the lowest IGF-1 levels and the lowest breast cancer risk in the cohort. 2) IGF-I genotyping not associated with breast cancer risk.
Groenbaek (344)	Nested case-control study within Danish "Diet, Cancer, and Health" Cohort. Cases n=311; controls n=397; postmenopausal women.	IGF-I, IGF-II, IGFBP-2, IGFBP-3, breast cancer risk.	1) Positive association between IGFBP-3 and IGF-II and ER+ breast cancer risk.
Bezemer (244)	Cross-sectional study of the EPIC cohort. Healthy women: n=743 premenopausal women; n=1217 postmenopausal women.	BMI, C-peptide, IGF-I, IGFBP-3, T, free T, A, DHEAS, SHBG, E1, E2, free E2.	1) BMI and waist circumference (adiposity) correlated positively with E1, E2, free E2, insulin, and inversely with SHBG. 2) In postmenopausal women, IGF-I positively associated with E1, E2, free E2, T, free T, A and DHEAS and inversely associated with SHBG.
Schernhammer (251)	Nested case-control study within the NHS. Cases n=800; controls n=1129.	IGF-1, IGFBP-3 (ELISA), IGFBP-3 (RIA), IGFBP-1, free IGF-I, breast cancer risk.	1) None of the IGF's were associated with breast cancer risk in postmenopausal women. 2) IGF modestly associated with breast cancer risk in premenopausal women.
Schernhammer (252)	Nested case-control within the NHS II. Cases n=317; controls n=634; 75% premenopausal women.	IGF-I, IGFBP-3, IGFBP-1, GH., breast cancer risk.	1) No association of any of the outcome measures and breast cancer risk in the total group or in a subset of premenopausal women.

**Table 1-12 continued: Case-control and prospective cohort studies of IGF pathway and breast cancer related endpoints.**

Reference	Subjects/Design	Outcome Measures	Results
Rinaldi (254)	Nested case-control study within the EPIC study. Pre- and postmenopausal women: cases (n=1081) and controls (n=2098).	IGF-I and IGFBP-3.	1) Increased breast cancer risk in women over 50 with increased IGF-I and IGFBP-3. 2) No association in younger women.
Johansson (345)	Cross-sectional study of the EPIC cohort. n=226 postmenopausal women.	Mammographic density, E2, SHBG, FSH, prolactin, C-peptide, IGF-I, IGFBP-3.	1) E2 was positively correlated with breast density. 2) Women with BMI $\leq$ 25 had denser breasts, higher SHBG, FSH, and lower E2 levels than overweight women.
Verheus (346)	Prospective study within the EPIC cohort. n=684 premenopausal women.	Premenopausal IGF-1, pre- and postmenopausal breast density by mammogram and changes in breast density over menopause.	1) Women with higher IGF-I levels premenopause have denser breasts post menopause.
Lopez-Saez (266)	Case-control study. Cases n=204 (96 premenopausal and 108 postmenopausal women); controls n=250.	Glucose, insulin, glycosylated hemeoglobin (HbA1c), c-peptide, IGF-I, total cholesterol, TG, HDL-c, LDL-c, selenium, BMI.	1) In premenopausal women, HbA1c, insulin, C-peptide, IGF-I, total cholesterol, and selenium differed significantly between cases and controls. 2) In postmenopausal women, glucose, HbA1c, C-peptide, total cholesterol, LDL-c, and BMI greater in cases than controls. Insulin, selenium, and HDL-c greater in controls than cases.
Gunter (253)	Nested case-control study within the WHI. Postmenopausal cases (n=835) and controls (n=816).	Fasting serum insulin, glucose, total IGF-I, free IGF-I, IGFBP-3, E2.	1) No association of total or free IGF-I and breast cancer risk. 2) E2 associated with increased breast cancer risk.

**Table 1-13: Case-control and prospective cohort studies of insulin resistance-related endpoints and breast cancer risk.**

Reference	Subjects/Design	Outcome Measures	Results
Brunning (261)	Case-control study. Cases (n=223) and controls (n=441).	C-peptide, TG, SHBG, E2, glucose, fructosamine, anthropomorphic measures.	1) C-peptide levels were increased in cases relative to controls independent of BMI or waist-hip ratio.
Gamayunova (271)	Case-control study. Breast cancer patients (n=113), endometrial cancer patients (n=18), and controls with BBD (n=35); premenopausal and postmenopausal.	Fasting glucose and insulin, glucose and insulin 2 hr after oral glucose tolerance test.	1) Fasting glucose was significantly greater in postmenopausal breast cancer patients with BMI over 30. 2) Fasting and 2-hour glucose and 2-hour insulin were significantly greater in postmenopausal breast cancer patients with waist-hip ratio over 0.85. 3) Postmenopausal women with BBD had significantly higher 2-hour glucose than postmenopausal breast cancer patients.
Jernstrom (256)	Nested case-control study within the Rancho Bernardo Study. Cases n=45, controls n=393 aged 53-90 years.	BMI, IGF-I, proinsulin, C-peptide, fasting insulin, and breast cancer risk.	1) Proinsulin, fasting insulin, and c-peptide were not associated with breast cancer risk.
Toniolo (246)	Nested case-control study within the NYU Women's Health Study. Cases n=287 (172 premenopausal and 115 postmenopausal); controls n=220.	IGF-I, IGFBP-3, C-peptide, and breast cancer risk.	1) No association between C-peptide and breast cancer risk in postmenopausal women.
Yang (262)	Case-control study. 143 matched pairs as part of the Shanghai Breast Cancer study; postmenopausal and pre-menopausal.	C-peptide	Samples collected before cancer therapy. 1) C-peptide associated with increased risk of breast cancer in both pre- and postmenopausal women.
Goodwin (258)	Prospective study breast cancer survivors (n=512).	Fasting insulin, IGF-I, IGF-II, IGFBP-3, E2.	1) Relatively increased fasting insulin was positively associated with breast cancer recurrence and mortality in women without diabetes.

**Table 1-13 continued: Case-control and prospective cohort studies of insulin resistance-related endpoints and breast cancer risk.**

Reference	Subjects/Design	Outcome Measures	Results
Muti (248)	Nested case-control study within the ORDET study. Cases n=144; controls n=576. Pre- and postmenopausal.	Serum glucose, IGF-I, free IGF-I, insulin, IGFBP-1, IGFBP-2, IGFBP-3; breast cancer risk.	1) When postmenopausal women were stratified by BMI, glucose, insulin, and IGF-I were positively associated with breast cancer risk in women with BMI over 26.
Krajcik (247)	Nested case-control study within the Kaiser Permanente Medical Care Program. Cases n=126 (66 premenopausal, 60 postmenopausal) women; matched controls n=126.	IGF-I, insulin, glucose, IGFBP-1, IGFBP-2, IGFBP-3, breast cancer risk.	1) Postmenopausal women were at increased risk of breast cancer if they had glucose levels within the diabetic range.
Lopez-Saez (266)	Case-control study. Cases n=204 (96 premenopausal and 108 postmenopausal women); controls n=250.	Glucose, insulin, glycosylated hemeoglobin (HbA1c), c-peptide, IGF-I, total cholesterol, TG, HDL-c, LDL-c, selenium, BMI.	1) In postmenopausal women, glucose, HbA1c, C-peptide, total cholesterol, LDL-c, and BMI greater in cases than controls. Insulin, selenium, and HDL-c greater in controls than cases.
Gunter (253)	Nested case-control study within the WHI. Postmenopausal cases (n=835) and controls (n=816).	Fasting serum insulin, glucose, total IGF-I, free IGF-I, IGFBP-3, E2.	1) Insulin associated with increased risk of breast cancer among non-users of HRT.
Kabat (267)	Longitudinal study within the WHI: 190 cases in 5450 postmenopausal women.	Fasting insulin, glucose.	1) Glucose not associated with breast cancer risk. 2) Baseline insulin associated with increased risk of breast cancer.



**Table 1-14: Dietary fat and IGF-I and IGFBP dietary intervention studies.**

Reference	Subjects/ Design	Intervention	Outcome Measures	Results
Kaaks (292)	Parallel arm n = 99 postmeno- pausal women: 49 in intervention; 50 in control group.	Intervention: group meals/cooking 2x/wk for 18 wks in Mediterranean/ macrobiotic style. Intervention group (ad libitum) instructed to limit meat, eggs, dairy, refined carbohydrates, and eat soy 1x/day, use olive oil and consume seeds and fish. 1x/wk women received flax bread. Control group given public pamphlet on diet.	Serum T, E2, SHBG, IGF-I, IGFBP-1, IGFBP-2, IGFBP-3, GH, GHBP, insulin, C- peptide, glucose, anthropomo rphic measures; diet analyzed by FFQ and 24h food diaries.	1). Significant decrease in C-peptide, fasting glucose, AUC insulin, and increased IGFBP-1 and IGFBP-2 in intervention group. 2) GHBP increased significantly in intervention group. 3) Significant reduction in T, significant increase in SHBG, and non-significant reduction in E2 in intervention group. 4) Active group lost significantly more weight. Most hormonal changes were no longer statistically significant after adjustment for weight loss. 5) Fat intake reduced about 7% in active group. 6) No effect on IGF-I or IGFBP-3.
Barnard (293)	Single arm study of 38 overweight/ obese postmenopa usal women (28 on HRT; 10 not on HRT) at Pritkin Longevity Center.	Two-week diet and exercise intervention: prepared meals (10- 15% fat [SFA:PUFA 1.24], 15-20% protein, 2 serving of nonfat milk and 3.5 oz of fish or poultry, 30- 40 g of fiber. Food adlibitum except for fish/poultry. Exercise was 30-60 min 5-7x/week.	Insulin, E2, IGF-I, IGFBP-1. Serum effect on cell growth in cell culture experiments with MCF- 7, T-47D, and ZR-75- 1 cells.	1) Weight, BMI, E2 (in both HRT and non-HRT groups), insulin, and IGF-I were significantly reduced after intervention. 2) IGFBP-1 was significantly increased after intervention. 3) Serum- induced growth reduced and apoptosis increased significantly in all cell types.

**Table 1-14 continued: Dietary fat and IGF-I and IGFBP dietary intervention studies.**

Reference	Subjects/ Design	Intervention	Outcome Measures	Results
Flood (294)	Parallel arm: 375 subjects with recurrent colon polyp and 375 subjects without recurrent colon polyp (men and women aged >35 years) selected from the Polyp Prevention Trial.	Four-year low-fat (20% of energy) high-fiber dietary intervention high in fruits and vegetables (5-8 servings per day) plus dietary counseling. Blood drawn at baseline, year 1 and year 4. Non-intervention group received Dairy Council information but no dietary counseling.	Insulin, glucose, IGF-I and IGFBP-3.	1) Neither insulin nor glucose levels were significantly changed within or between groups. 2) No significant change in IGFBP-3 levels. 3) IGF-I level decreased significantly within each group but the difference between groups was not significantly different. 4) Among participants with BMI < 25, glucose was significantly decreased in the intervention group at 4 years.
Gann (295)	Parallel arm study in healthy premenopausal women (n=154).	Low-fat, high fiber intervention (< 20% energy from fat, >25g fiber/day) vs. normal diet for 12 menstrual cycles followed by either a soy supplement with or without isoflavones for 3 cycles.	IGF-I, IGFBP-1 and IGFBP-3.	1) No change in IGF-I, IGFBP-1 or IGFBP-3 following the intervention or compared to control after 12 cycles.

**Table 1-15: Total dietary fat and insulin sensitivity-related endpoints dietary intervention studies.**

Reference	Subjects/ Design	Intervention	Outcome Measures	Results
Fukagawa (301)	Single-arm study in 12 healthy men and women.	Habitual diet followed by 3-4 week intervention of high-carbohydrate, high-fiber diet (HC; 63% carbohydrate, 14% fat with 33g fiber /1000 kcal).	Glucose, cholesterol, TG, insulin, insulin sensitivity by hyperinsulinemic euglycemic clamp.	1) Fasting glucose, insulin, and cholesterol significantly lower after HC diet. 2) Glucose disposal with the clamp increased significantly with the HC diet.
Lovejoy(302)	Cross-over study in 31 women (20 African American and 11 Caucasian).	Isocaloric controlled diets for three weeks: high fat (50% fat, 35% carbohydrate, 15% protein) or low fat (20% fat, 55% carbohydrate, 15% protein).	Fasting insulin, fasting glucose, insulin sensitivity, leptin, total cholesterol, HDL, LDL, TG.	1) Insulin sensitivity decreased with high fat feeding and increased with low fat feeding.
Straznicky (303)	Cross-over study in 14 healthy men.	Two diets: low fat diet (LF; 25% fat, 54% carbohydrate, 21% protein); and high fat diet (HF; 45% fat, 36% carbohydrate, and 19% protein) for two weeks each, with a 2-week wash-out period between.	Blood pressure, total cholesterol, LDL, HDL, fasting glucose, glucose area under the curve, fasting insulin, insulin area under the curve.	1) TG concentrations declined significantly from baseline with both diets. 2) Total and LDL and HDL cholesterol declined significantly from baseline with LF. 3) Fasting glucose and glucose area under the curve were decreased significantly with LF compared to HF. 4) Insulin sensitivity was significantly increased with LF. 5) Blood pressure decreased significantly with LF.
Tymchuk (181)	Single arm study in 22 postmenopausal women; 11 on HRT, 11 not on HRT. Located at Pritkin Center.	Low fat (<10%), high fiber (65-70 g/day) diet plus exercise intervention. Three weeks in duration.	Anthropomorphic measures, total cholesterol, LDL, HDL, TG, glucose, insulin, SHBG.	1) SHBG increased significantly. 2) Insulin decreased significantly. 3) BMI and total cholesterol, LDL, HDL, and TG significantly decreased.

**Table 1-15 continued: Total dietary fat and insulin sensitivity-related endpoints dietary intervention studies.**

Reference	Subjects/ Design	Intervention	Outcome Measures	Results
Bisschop (305)	Cross-over study in 6 healthy men.	Three isocaloric liquid diets for 11 days each (all with 15% protein): low fat/high carbohydrate (LFHC; 0% fat and 85% carbohydrate), intermediate-fat/intermediate carbohydrate (IFIC; 41% fat, 44% carbohydrate), and high fat/low carbohydrate (HFLC; 83% fat, 2% carbohydrate).	Insulin sensitivity using hyperinsulinemic euglycemic clamp.	1) Fasting insulin and glucose were reduced with the HFLC diet. 2) HFLC diet reduced suppression of endogenous glucose production and glucose oxidation during the hyperinsulinemic clamp. 3) Fat oxidation was increased with HFLC diet relative to the other two diets at baseline and during clamp technique. 4) Fat oxidation was reduced during the clamp technique compared to baseline with the LFHC and IFIC diets.
Vessby (308)	Parallel arm study in 162 healthy men and women. The KANWU study.	Participants were instructed to follow either a high SFA diet (SF; 37% fat with 17% SFA, 14% MUFA, and 6% PUFA) or a high MUFA diet (MF; 37% fat, 8% SFA, 23% MUFA, 6% PUFA) for 3 months. Within treatments, they were also randomized to receive either fish oil (2.4g EPA + DHA) or placebo (olive oil) per day. Edible fats were provided.	Fatty acids, insulin, insulin sensitivity index, glucose, oral glucose tolerance, cholesterol, TG, LDL, HDL, ApoB, ApoA-I.	1) Weight did not change during the interventions. 2) Insulin sensitivity index decreased significantly with SF, but remained unchanged with MF. 2) Fasting insulin decreased significantly with MF. 3) Addition of fish oil did not change any of the insulin-related endpoints. 4) Total cholesterol and LDL increased significantly with SF. 5) Fish oil increased LDL. 6) TG decreased in subjects given fish oil.
Lovejoy (309)	Cross-over trial in 25 men and women.	Three controlled diets, each with composition of 57% carbohydrate, 28% fat and 15% protein and of 4 weeks in duration: 1) MUFA (M; 9% oleate); 2) SFA (S; 9% palmitic acid); 3) trans (T; 9% trans oleic acid). Two-week washout between diets.	Fasting glucose, insulin, insulin sensitivity index, glucose effectiveness, total cholesterol, LDL, HDL, TG.	1) Total cholesterol and HDL were significantly lower with the M diet than the S diet. 2) No difference in insulin parameters among diets.

**Table 1-15 continued: Total dietary fat and insulin sensitivity-related endpoints dietary intervention studies.**

Reference	Subjects/ Design	Intervention	Outcome Measures	Results
Hall (304)	Parallel arm in postmenopausal minority women (n=2208).	Feasibility trial for the WHI in ethnic populations. Women were randomized to a low fat dietary intervention (LF; goal of 20% of energy from fat and increased fruit and vegetable intake) or control group who received no dietary counseling for 6-18 months.	Anthropometrics, blood pressure, insulin, glucose.	1) Weight, BMI, and waist circumference decreased significantly with the LF compared to control. 2) Trends for decreased insulin and glucose with the LF.
Heald (306)	Four arm parallel study in women (n=80) who consumed a high fat diet at baseline.	Women were counseled on four interventions: 1) substitute low fat foods for high fat foods; 2) reduce high-fat foods; 3) both reduction and substitution of high fat foods; 4) no counseling for 3 months.	Anthropometrics, fasting TG, total cholesterol, LDL, C-reactive protein, IGF-I, IGFBP-1, fasting insulin and glucose.	1) Weight, body fat percent, TG, cholesterol and C-reactive protein decreased significantly with substitution method. 2) IGF-I increased with both the substitution and reduction methods. 3) No change in insulin or glucose observed.
Meckling (316)	Parallel arm study in overweight men (n=9) and women (n=24). Weight loss study.	10 weeks of one of two dietary interventions (free-living; counseling provided for each group): low carbohydrate (LC; goal of 50 – 70 g/day) vs. low fat group (LF).	Blood pressure, total cholesterol, LDL, HDL, TG, insulin, glucose.	1) Diastolic and systolic blood pressure decreased significantly in each group. 2) Total cholesterol, LDL, and HDL decreased significantly with the LF group. 3) TG decreased significantly in both groups. 4) Insulin decreased significantly in the LC group. 5) Significant weight loss, but equal between the two groups.
Wu (184)	Parallel arm study of 57 postmenopausal women.	Intervention 1: very low fat diet (VLFD; 11% energy from fat); Intervention 2: Step 1 diet plus soy food, (SFD; 30% energy from fa); Control: Step 1 diet (CD; 30% energy from fat). 8 week controlled diet; meals prepared at a GCRC.	Total cholesterol, TG, HDL, LDL, total E2, free E2, E1, E2/E1, T, free T, A, SHBG, IGF-1, IGFBP-3, insulin, leptin.	1) No difference in hormone levels between either of the intervention groups and the controls at 8 weeks. 2) Insulin decreased significantly in SFD group and CD group. 3) Leptin decreased significantly in VLFD and SFD groups. 4) IGF-1 level increased significantly in the VLFD group.

**Table 1-15 continued: Total dietary fat and insulin sensitivity-related endpoints dietary intervention studies.**

Reference	Subjects/ Design	Intervention	Outcome Measures	Results
Volek (315)	Parallel arm weight-loss study in 40 men and women with dyslipidemia.	Subjects were instructed to follow one of two reduced-calorie diets (~1500 kcal): carbohydrate-restricted diet (CRD; 12% carbohydrate, 59% fat, 28% protein) or a low fat diet (LFD; 56% carbohydrate, 24% fat, 20% protein) for 12 weeks.	Glucose, insulin, insulin resistance, leptin, ketones, fatty acids, TG.	1) Energy-reduction between diets was similar, but weight loss ~ twice that of LFD in CRD. 2) Total body and abdominal fat mass decreased significantly more with CRD than LFD. 3) Glucose and insulin reduced significantly more with CRD than LFD. Insulin resistance reduced significantly with CRD. 4) Ketones were significantly increased with CRD. 5) Post-prandial TG were significantly decreased following a fat challenge with CRD vs. LFD.

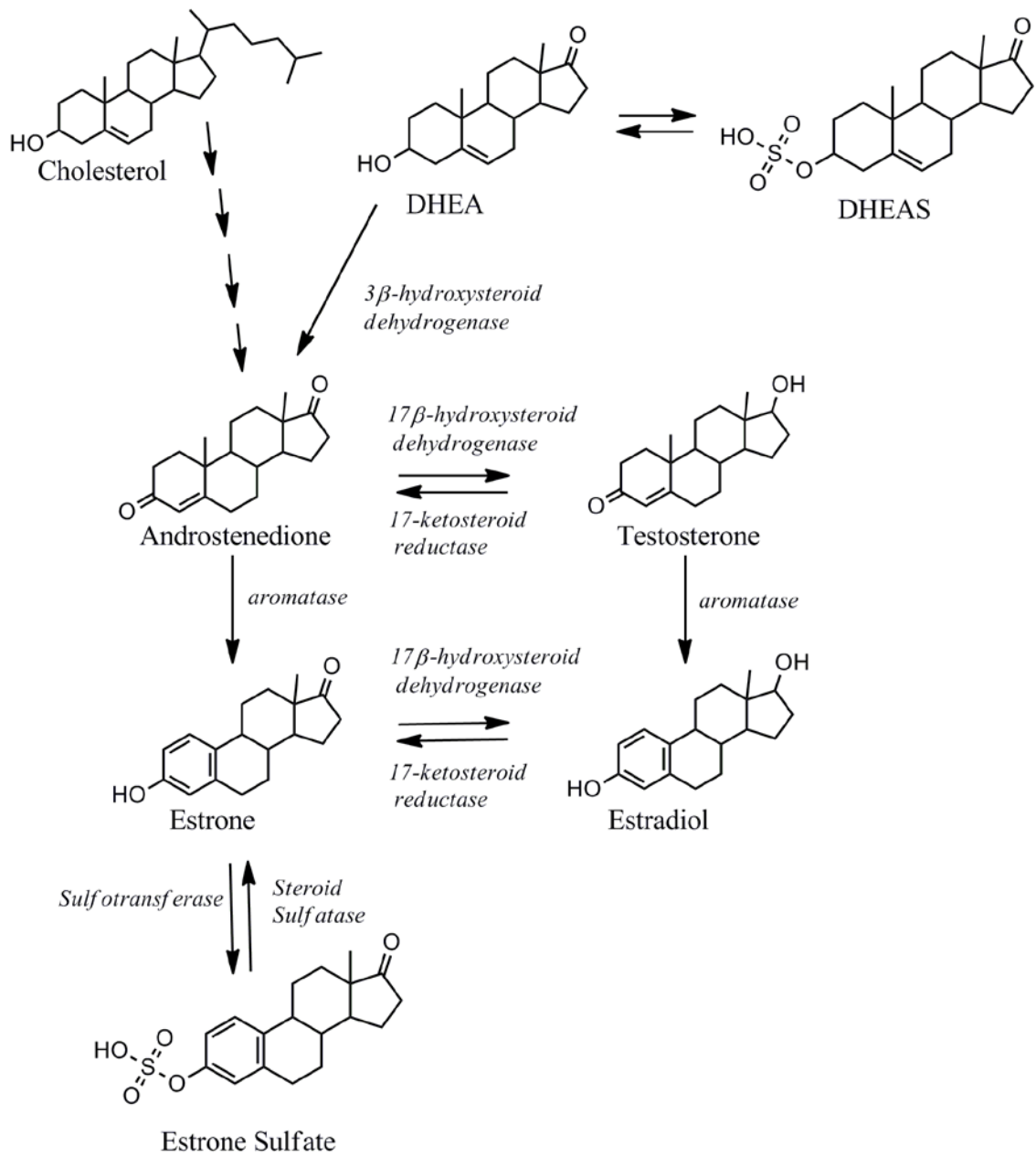
**Table 1-16: Omega-3 fatty acids and insulin sensitivity-related endpoints dietary intervention studies.**

Reference	Design	Intervention	Outcome measures	Results
Bhathena (320)	Single arm study in healthy men (n=40).	Diet of 40% fat fed for 28 weeks total with 15g placebo capsules the first 10 weeks followed by 10 weeks of 15g fish oil followed by addition of 200mg of Vitamin E to fish oil.	Fasting glucose, TG, total cholesterol, insulin, GH, IGF-I, glucagon, cortisol, DHEAS.	1) Fasting glucose significantly increased with fish oil. 2) TG and cholesterol significantly decreased with fish oil. 3) GH and IGF-I significantly decreased with fish oil and GH decreased further with addition of Vitamin E. IGF-I returned to baseline levels with addition of Vitamin E. 4) DHEAS declined significantly with addition of Vitamin E to fish oil.
Toft (329)	Parallel arm study in 78 men and women with hypertension .	Random assignment to supplement of DHA + EPA (4g/day) or corn oil (4g/day) for 16 weeks.	Oral glucose tolerance, insulin sensitivity and release and glucose disposal by hyperglycemic clamp, insulin sensitivity by hyperinsulinemic euglycemic clamp, blood pressure, C-peptide, glycosylated hemoglobin, TG, total cholesterol, LDL, HDL, VLDL, phospholipid EPA, phospholipid DHA, n-3:n-6 ratio.	1) Phospholipid EPA, DHA and n-3:n-6 ratio increased significantly with the fish oil group and EPA and n-3:n-6 ratio decreased significantly with the control group. 2) Blood pressure in the fish oil group reduced significantly compared to the control group. 3) Glycosylated hemoglobin increased significantly with the fish oil group. 4) No change in insulin or glucose parameters with either intervention.
Delarue (330)	Single arm study in healthy men (n=4) and a woman.	Two consecutive controlled diet periods: 1) High SFA diet (PUFA:SFA=0.21); 2) 6g oil in diet 1 was replaced with 6g fish oil (1.1g EPA and 0.7g DHA/day) for 3 weeks each separated by a 2.5-3 month wash-out period.	Fasting insulin and glucose, C-peptide, fatty acids, lipid, glucose and fructose oxidation.	1) Fasting glucose was significantly increased (+5%) and fasting insulin was significantly decreased (-40%) with the fish oil supplement. 2) Glucose and fructose oxidation were reduced and non-oxidative glucose disposal was increased by fish oil.

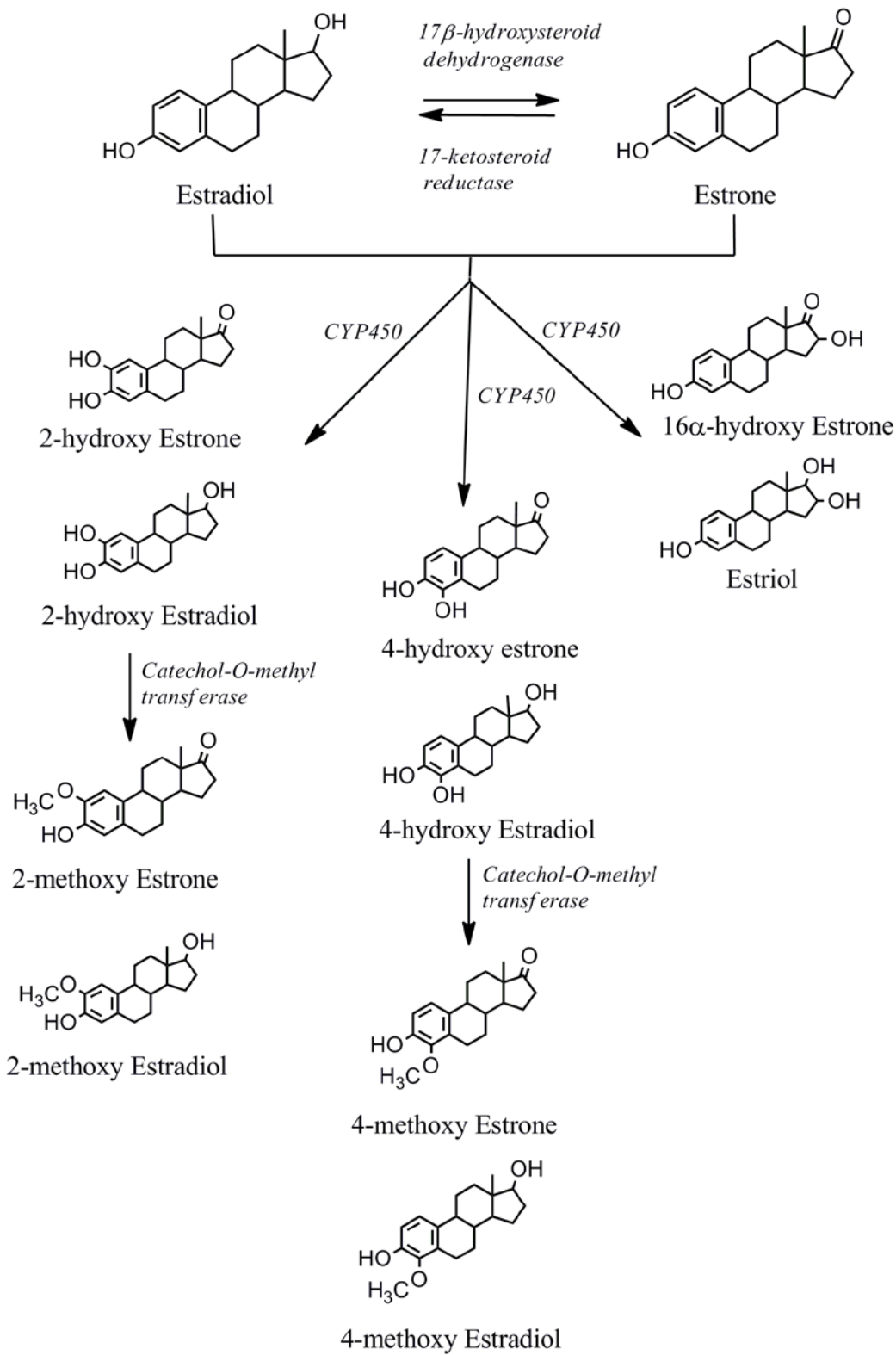
**Table 1-16 continued: Omega-3 fatty acids and insulin sensitivity-related endpoints dietary intervention studies.**

Reference	Design	Intervention	Outcome Measures	Results
Vessby (308)	Parallel arm study in 162 healthy men and women. The KANWU study.	Participants were instructed to follow either a high SFA diet (SF; 37% fat with 17% SFA, 14% MUFA, and 6% PUFA) or a high MUFA diet (MF; 37% fat, 8% SFA, 23% MUFA, 6% PUFA) for 3 months. Within treatments, they were also randomized to receive either fish oil (2.4g EPA + DHA) or placebo (olive oil) per day. Edible fats were provided.	Fatty acids, insulin, insulin sensitivity index, glucose, oral glucose tolerance, cholesterol, TG, LDL, HDL, ApoB, ApoA-I.	1) Weight did not change during the interventions. 2) Insulin sensitivity index decreased significantly with SF, but remained unchanged with MF. 2) Fasting insulin decreased significantly with MF. 3) Addition of fish oil did not change any of the insulin-related endpoints. 4) Total cholesterol and LDL increased significantly with SF. 5) Fish oil increased LDL. 6) TG decreased in subjects given fish oil.
Brady (331)	Parallel arm study in Indian Asian men (n=29),	Subjects consumed wither a high n-6:n-3 ratio diet (16) or a moderate n-6:n-3 ratio diet (9) for 6 weeks followed by 2.5 g EPA + DHA per day in both groups for 6 weeks.	Fasting and postprandial TG, fatty acids, total cholesterol, LDL, HDL, insulin sensitivity by homeostatic model of insulin resistance and frequently-sampled intravenous glucose tolerance test.	1) No difference in fasting or postprandial glucose or insulin or in insulin sensitivity after EPA + DHA. 2) Postprandial and fasting TG decreased significantly following EPA + DHA.
Griffin (332)	Parallel arm trial with 5 arms in 258 men and women (aged 45-70).	All diets had 6% PUFA with different n-6:n-3 ratio: 1) High LA diet; 2) moderate LA diet; 3) ALA + EPA +DHA diet; 4) EPA +DHA diet; 5) control diet for 6 months. Spreads and oils with desired fat composition provided.	Fasting glucose and insulin, insulin sensitivity by homeostatic model assessment of insulin resistance and the revised quantitative insulin sensitivity test, TG, HDL, LDL.	1) No change in glucose, insulin or insulin sensitivity with any of the diets or between diets. 2) Diets with long-chain PUFA reduced TG and small, dense LDL.





**Figure 1-1: Metabolism of sex hormones in postmenopausal women.**



**Figure 1-2: Sex hormone metabolites formed from oxidative metabolism.**

**Chapter 2:**  
**Effect of Dietary Fat and Omega-3 Fatty Acids on Urinary Eicosanoids and Sex  
Hormone Concentrations in Postmenopausal Women: a Randomized Controlled  
Feeding Trial**

Substantial evidence relates increased sex hormone concentrations with increased breast cancer risk. Varying omega-3 (n-3) fatty acid (FA) intake may lead to alterations in eicosanoid balance and changes in circulating sex hormones that reduce risk. To clarify effects of dietary fat and n-3 FA intake on breast cancer risk markers, circulating sex hormones and urinary eicosanoids were measured in response to controlled feeding of diets designed to increase plasma concentrations of n-3 FA, a controlled cross-over feeding trial in postmenopausal women was conducted using three diets: high fat (HF; 40% energy from fat), low fat (LF; 20% energy from fat) and low fat plus n-3 FA (LFn3; 20% of energy from fat plus 3% of energy from n-3 FA) in 8-week feeding periods. Plasma phospholipid fatty acid n-3 increased with the LFn3 relative to HF and LF ( $P < 0.0001$ ). Plasma estradiol increased by 51% with HF ( $P = 0.03$ ). Urinary prostaglandin E metabolite increased with HF relative to LF ( $P = 0.02$ ) and urinary 11-dehydro-thromboxane B<sub>2</sub> increased with HF ( $P = 0.01$ ). These results support a role of low fat diet, but are inconclusive with respect to n-3 FA, in the reduction of breast cancer risk factors.

## I. Introduction

A vast literature investigating the effects of dietary fat intake on breast cancer risk has accumulated. While international correlation studies (22, 24, 108), migrant studies (27), and animal experiments (106) have established a persuasive link between high dietary fat and breast cancer incidence and tumorigenesis, epidemiological cohort studies have historically shown weakly positive or null associations (34, 36, 347). However, recent evidence adds weight in support of a positive association of total fat and saturated, monounsaturated, and polyunsaturated fat intake and breast cancer incidence (20). In addition to amount of fat, long-chain, or marine, omega-3 (n-3) fatty acids (FA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to inhibit breast cell proliferation and invasiveness in vitro (348, 349) and mammary tumorigenesis in animal models (350, 351). Incidence of breast cancer is lower in populations with high fish intake (352); however, few epidemiological studies show significant reductions in breast cancer risk with relatively increased intake of fish and n-3 FA (89).

Reduction of tumorigenesis may be mediated by the inhibitory effects of n-3 FA on the synthesis of inflammatory eicosanoids derived from arachidonic acid (AA), an omega-6 (n-6) FA, and subsequent alteration of sex hormone metabolism. Measurement of blood hormone concentrations is a convenient surrogate for tissue exposure to hormones and substantial evidence relates relatively increased serum or plasma sex hormone concentrations with an increased risk of breast cancer in postmenopausal women (125). Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), an eicosanoid derived from AA, has been shown to stimulate the expression and activity of aromatase, the enzyme that converts androgens to estrogens (55, 353). Prostaglandin E<sub>3</sub>, derived from EPA, does not have this effect. Because PGE<sub>2</sub> is derived from n-6 FA, estrogen levels would be expected to decrease with decreased total n-6 FA intake (similar to the low fat dietary intervention in the present study) and increased consumption of n-3 FA (comparable to the low fat, high n-3 FA diet intervention of the current study) in proportion to n-6 FA (60). A major urinary metabolite of PGE<sub>2</sub> was reduced by salmon-containing diets (67) and fish oil supplements (68) in humans, although the

effects of a high n-3 FA diet on circulating sex hormones is unknown. Therefore we conducted a well-controlled feeding study to determine the effects of fat content (high vs. low fat diet) and n-3 FA on urinary eicosanoids and plasma sex hormones in postmenopausal women.

## **II. Materials and Methods**

### **A. Experimental Protocol**

A randomized, crossover design was used to compare the effects of diets containing varying levels of fat and n-3 FA on plasma sex hormone profiles and urinary eicosanoids in healthy postmenopausal women. All subjects were fed a controlled high fat (45% energy from fat; HF), a low fat (20% energy from fat; LF) and a controlled low fat, high n-3 FA diet (23% energy from fat; LFn3) in random order. Each diet lasted for a total of 56 days (8 weeks). After completion of the first experimental diet subjects returned to their habitual diet for a washout period of 42 to 84 days (6 – 12 weeks). All subjects then crossed over to the second treatment in the randomization. Upon completion of the second diet, the wash out was repeated and subjects returned for the third dietary treatment. Study meals were prepared in the metabolic kitchen of the General Clinical Research Center (GCRC) at the University of Minnesota. Subjects picked up bagged study meals (breakfast, lunch, dinner, and a snack) daily on weekdays. Weekend meals were packaged for 3-d pick-up. Compliance with the dietary intervention was monitored by use of a daily questionnaire that included questions about whether the subject ate all of her study meals and whether she consumed any foods from outside the study and amounts. Subjects weighed themselves at food pick-up times and recorded this weight in their study files.

Approval for the study was obtained from the University of Minnesota Committee for the Use of Human Subjects in Research and the U.S. Army Medical Research and Materiel Command's Human Subjects Research Review Board. Informed consent was obtained from all study participants. The entire study was performed at the GCRC of the University of Minnesota.

## **B. Subjects**

Study subjects were postmenopausal women recruited from the Minneapolis/St Paul, Minnesota metropolitan area using flyers and email announcements distributed within the University of Minnesota campus community, mailings of flyers to area churches and synagogues, presentations at local women's group meetings, and advertisements placed in local newspapers. The inclusion criteria were: age 45-70 y, postmenopausal status ( $\geq 1$  y since last menstrual period, screening follicle stimulating hormone (FSH) concentration of  $> 23$  IU/L or  $> 55$  y), BMI of 19-32, stable body weight for the prior 6 mo, willing to discontinue the use of aspirin, ibuprofen, and other non-steroidal anti-inflammatory drugs, and no use of hormone replacement therapy or fish oil supplements within 2 mo of beginning the study. Exclusion criteria were: current smoker, history of hormone-related cancer, use of prescription medications excluding high blood pressure medication, bilateral oophrectomy, and known disease processes (diabetes mellitus, inflammatory disease). Health status was determined by responses to a medical history questionnaire to eliminate subjects with current medical problems and participants who were taking any excluded medications.

Participants who failed to complete at least two of the three diets were omitted from the analysis. Thirty-two subjects were recruited and 24 were enrolled (see Figure 2-1). Six subjects dropped from the study and one was excluded from the analysis due to fluctuating FSH (perimenopausal status) following biological sample analysis. A total of 17 subjects (one of which completed 2 diets; missing LF) were included in the plasma sex hormone and fatty acid statistical analysis ( $n = 17$  for the HF and LFn3;  $n = 16$  in the LF) except for the following omissions: one subject was excluded from plasma Estradiol (E2), Estrone-sulfate (E1-S), and free E2 analysis in the LF because of outlier values that were outside of the postmenopausal range at baseline, thus  $n = 15$  in the LF and  $n = 17$  in the HF and LFn3 for these endpoints. Another subject was missing values for all diets for plasma FSH (laboratory error), thus  $n = 16$  in the HF and LFn3 and  $n = 15$  in the LF for FSH.

A total of 16 subjects are included in the statistical analysis of urinary eicosanoids because one subject was missing all urine data due to failure to correctly

collect 24-h urine specimens. One subject was missing HF treatment urine samples (missing samples) for all urinary endpoints, thus n = 15 for the HF and LF and n = 16 for the LFn3.

### **C. Dietary Intervention**

Isoenergetic HF, LF, LFn3 diets were designed to provide a varied content of total fat and fatty acid composition. Specifically, the LF (20% energy from fat, 15% energy from protein, and 65% energy from carbohydrate) and HF (40% energy from fat, 15% energy from protein, 45% energy from carbohydrate) contained a similar distribution of PUFA to monounsaturated fatty acids (MUFA) to SFA (1:1:1) with a very limited content of n-3 FA. The LFn3 (23% energy from fat, 15% energy from protein, and 62% energy from carbohydrate) was supplemented with 3% of energy from those vegetable and animal sources that are high in n-3 FA (e.g. salmon, flax seed oil, walnuts) amounting to approximately 5.8 g/day  $\alpha$ -linolenic acid (ALA) and 1.2 g/day EPA +DHA. Nutrient analysis software (Nutritionist V) that utilized the USDA standard reference database was used to determine the nutrient composition of the experimental diets (see Table 2-1). The three test diets were designed using commonly consumed foods (see sample menu items in Table 2-2). The three dietary interventions differed in amount of dietary fat and carbohydrate, but protein (15% energy), dietary fiber (10-12g/4184 kJ [10-12g/1000 kcal]), and cholesterol (100mg/4184 kJ [100mg/1000 kcal]) were held constant in the three diets (see Table 2-1).

A weight-maintaining energy level was calculated for each subject using the Harris-Benedict equation [ $655.1 + 9.56 \times \text{wt (kg)} + 1.85 \times \text{height (cm)} - 4.68 \times \text{age (y)}$ ] multiplied by an activity factor based on reported activity (1.4 – 1.7; mean 1.6). The energy level of the diets was adjusted in 840 kJ (200 kcal) increments if the subject gained or lost  $\geq 1.0$  kg.

Energy and n-3 FA content of any foods consumed in addition to the study diets were estimated from the daily compliance records and a mean total energy and n-3 FA deviation of all foods was estimated for all subjects.



#### **D. Plasma Collection**

Fasting blood samples were obtained from subjects in the morning two days in a row, in order to decrease recognized variation in sex hormone concentrations (354-356), at baseline and 8 weeks of each diet period for sex hormone analysis and one day at baseline and 8 weeks for fatty acid analysis. Samples were centrifuged to separate the plasma and aliquots were stored at -80°C until analysis.

#### **E. Plasma Sex Hormone Analysis**

Equal volumes of plasma from each of the two days at baseline and 8 weeks were pooled before analysis. E2, estrone (E1), E1-S, dehydroepiandrosterone (DHEA), dehydroepiandrosterone-sulfate (DHEAS), androstenedione (A), and testosterone (T) were analyzed by radioimmunoassay (<sup>125</sup>I labeled hormone) (Diagnostics Systems Laboratories, Austin, TX). Sex hormone binding globulin (SHBG) and FSH were analyzed by ELISA (IBL America, Minneapolis, MN). Each sample was run in triplicate, and all samples from a given subject were run in the same batch. Intra-assay CV's were 5.1% for E2, 5.5% for E1, 5.6% for E1-S, 4.6% for A, 3.5% for DHEA, 4.2% for DHEAS, 5.3% for T, 4.4% for FSH and 3.9% for SHBG. Free E2 index and Free T index were calculated as [E2 (nmol/L) / SHBG (nmol/L)] and [T (nmol/L) / SHBG (nmol/L)], respectively.

#### **F. Plasma Phospholipid Fatty Acid Analysis**

Gas chromatography (Lipid Technologies LLC, Austin, MN) was utilized to determine plasma PLFA percent composition of linoleic acid (LA; 18:2n6),  $\alpha$ -linolenic acid (ALA; 18:3n3), AA (20:4n6), EPA (20:5n3) DHA (22:6n3), total n-3, total n-6, and the n-6:n-3 ratio. Chloroform:methanol (2:1, by volume) was used to extract lipids from the plasma as previously described (357). Phospholipids were separated from neutral lipids by thin-layer chromatography using a Restek FFAP capillary column (30 meters, 0.25 mm internal diameter with a 0.25 $\mu$ m internal coating). Parameters of the GC were: injector temperature 300 °C, detector temperature 300 °C, split ratio 25:1, temperature program 190-240 °C with a final time of 5 minutes. Peaks were identified

by comparison of retention times to authentic fatty acid standards (NuChek Prep, Elysian, MN). Fatty acid methyl esters (FAME) of the aforementioned lipid classes were formed by transesterification with boron trifluoride (12%) in excess methanol (Supelco, Bellefonte, PA). All samples from a given subject were run in the same batch.

### **G. Urine Collection and Analysis**

Two consecutive 24-h urine collections (begun following the morning void of first day and including all voids during the day and the morning void of the following day) were collected by each subject at baseline and 8 weeks of each diet period. Samples were collected in opaque 3.5 L jugs containing 1 g ascorbic acid per liter. Urine samples were kept on ice or stored in a refrigerator during collection time. Urine collections were pooled and aliquots were stored at -80°C until analysis. Prostaglandin E metabolite (PGE-M), a derivative of 13,14-dihydro-15-keto PGA<sub>2</sub> and 13,14-dihydro-15-keto PGE<sub>2</sub> (degradation products of PGE<sub>2</sub> in vivo) and 11-dehydro thromboxane B<sub>2</sub> (TxB-M), stable product of thromboxane B<sub>2</sub> metabolism, were analyzed in triplicate using enzyme immunoassay kits (Caymen Chemical, Ann Arbor, MI). All samples from a given subject were run in the same batch. Intra-batch CV's were 6.1% for PGE-M and 5.1% for TxB-M. Data for urinary eicosanoids was analyzed as ng/mg creatinine and ng/d and the results of the statistical analysis did not differ, so data is expressed as ng/d.

### **H. Anthropometric Measures**

Body weight was measured for endpoint determination on an electronic scale (Scale-Tronix 5005 Stand-On Scale) with subjects wearing light clothing and no shoes; height was measured on the stadiometer attached to the scale at baseline and 8 weeks of each diet period.

## **I. Baseline Diet Analysis**

At the baseline screening visit, reported diet was analyzed using the National Institutes of Health Diet History Questionnaire (NIH, Bethesda, MD) (358).

## **J. Statistical Analysis**

For each of the primary outcomes, the final value on each diet was used as the response. Highly skewed responses were log transformed for analysis. Diets were compared using a general linear mixed model (SAS Proc Mixed, SAS Institute Inc., SAS® 9.2, Cary, NC: SAS Institute Inc., 2002-2008) with a random effect for participant in order to account for the correlation of repeated measurements within each participant. This model accommodates missing values and allows assessment of period and carryover effects. Adjusted means and SEM from the mixed model are reported; for responses analyzed on the log scale, geometric means and 95% CI are reported. Within diet differences were evaluated using paired t-tests. Statistical significance obtained at  $P < 0.05$ . For E2 and PGE-M, statistically significant differences were observed at baseline; therefore baseline E2 was included in the model for the 8 week analysis of E2 and baseline PGE-M was included in the model for the 8 week analysis of PGE-M.

## **III. Results**

The seventeen postmenopausal women had a mean age of 57 y with a mean BMI of  $28 \text{ kg/m}^2$  (Table 2-3). Based on the subjects' reported baseline dietary intakes of total dietary fat, the HF was closer to their habitual intake of dietary fat than the LF (Table 2-4). Mean body wt decreased significantly within all diets (-1.1 kg with the HF, -1.3 kg with the LF, and -0.7 kg with the LFn3) but mean weight was not significantly different among the diets at 8 weeks. Reported compliance to the treatment diets estimated from the daily questionnaire was  $< 1\%$  deviation in energy and  $< 0.5\%$  deviation in n-3 FA.

### **A. Plasma Phospholipid Fatty Acids**

Plasma PLFA were measured to assess compliance with the diets. Indistinguishable baseline proportions for all fatty acid classes (SFA, MUFA, PUFA, n-3, n-6) confirmed that subjects consumed their typical diets during the wash-out periods. Plasma percent composition of n-3 PLFA classes increased significantly from baseline with LFn3, indicating the subjects' adherence (Table 2-5). Results of the PLFA analysis were in the directions anticipated with significant increases in PLFA n-3 and significant decreases in PLFA n-6 with the LFn3. LFn3 doubled plasma PLFA 18:3n-3, 20:5n-3 and 22:6n-3. Plasma PLFA 18:2n-6 and the n-6:n-3 ratio increased with HF and decreased with LFn3. Plasma PLFA 20:4n-6 increased with the LF and decreased with the LFn3. Total PLFA n-6 decreased with LFn3 but increased with HF.

### **B. Urinary Eicosanoids**

There were baseline differences in mean urinary PGE-M ( $P = 0.05$ ) (Table 2-6); therefore the 8 week means of PGE-M were adjusted for baseline PGE-M. After adjustment for baseline PGE-M, mean PGE-M at 8 weeks was significantly higher with HF than LF ( $P = 0.02$ ). Mean urinary TxB-M was significantly increased with the HF ( $P = 0.01$ ).

### **C. Plasma Sex Hormones**

Significant differences in mean plasma E2 were observed at baseline among the three test diets with a high baseline in the LF group ( $P = 0.02$ ) (Table 2-7); therefore the 8 week means were adjusted for baseline E2. E2 was significantly increased by the HF ( $P = 0.03$ ) and there was a trend towards increased E2 with the HF relative to LF and LFn3 ( $P = 0.11$ ). There was a trend for increased free E2 index with HF, which supports the higher E2 on this diet. FSH was significantly reduced by the LF ( $P = 0.03$ ). The following trends were observed: reduced E1 at 8 weeks with the LFn3 relative to HF and LF ( $P = 0.14$ ); reduced sex hormone binding globulin with LF and LFn3 compared to HF ( $P = 0.06$ ); increased free T index with LF compared to HF and

LFn3 ( $P = 0.10$ ). No differences were observed in any of the other sex hormones (E1-S, A, DHEA, DHEAS, and T) for 8 week means.

#### **IV. Discussion**

We found that consumption of a high fat diet for eight weeks resulted in a significantly increased ratio of n-6 to n-3 plasma PLFA compared to two low fat diets, one of which was enriched in n-3 FA. As expected, the difference was particularly striking comparing HF with LFn3. These differences in fatty acid profiles were accompanied by increased PGE-M and TxB-M and differences in plasma sex hormone profiles with the HF resulting in higher levels of total E2 and a trend towards increased free E2. An unexpected result was that SHBG concentrations trended lower with the low fat diets.

To our knowledge, this is the first dietary intervention study using whole foods designed to isolate the effects of n-3 FA on sex hormone levels in postmenopausal women. Significant changes in n-3 FA and n-6 FA and a trend towards reduced E1 were observed with the LFn3. Increasing n-3 FA (fish) intake was part of the intention of the Diet and Androgens Randomized trial (183), however the trial had a number of additional objectives including reducing animal fat and low glycemic-index foods and increasing soy product intake, so it is impossible to identify the effects of increasing n-3 FA intake alone.

Previous low fat diet intervention studies yielded mixed results, with some studies reporting marked reductions in sex hormone concentrations while others show no changes. In a controlled feeding study by Wu et al (184), a very low fat diet (12% energy from fat), a soy food diet (30% energy from fat) or a control diet (30% energy from fat) was provided to postmenopausal women for 8 weeks. Wu et al did not observe any significantly different changes in E2, free E2, E1, T, free T, A or SHBG concentrations among the three diets. However, an estimate of -23% change in E2 concentration with low fat diet was calculated from a pooled analysis (359) of four dietary fat reduction interventions (179, 180, 182, 360) in postmenopausal women. Also, estradiol concentrations were reduced and SHBG concentrations were increased

significantly in a subset of postmenopausal women after participation for one year in the Women's Health Initiative Dietary Modification Trial low fat dietary intervention group relative to a subset of women in the comparison group (37). Fiber intake was greatly increased and significant weight loss accompanied the dietary fat reductions in many of these studies, which may confound the results. A small but statistically significant weight loss occurred in the present study as well, however final mean weight did not differ and the amount of dietary fiber was held constant across diets.

It is well established that long-term consumption of foods high in marine n-3 FA imparts relatively high levels of tissue long-chain n-3 FA as seen in coastal Japanese fishing village inhabitants (61) and in the Canadian Inuit population (62). Our results demonstrate that dietary n-3 FA are incorporated to a significant degree into plasma PLFA. We did not observe a reduction in TxB-M or PGE-M with the LFn3. Other studies have observed significant changes in eicosanoid metabolism upon feeding a fish-rich diet or n-3 FA supplements. A significant decrease in urinary 2,3-Dinor-thromboxane B<sub>2</sub> was observed with feeding a high n-3 FA diet (2% energy from n-3 FA) rich in salmon relative to a control diet (71). However there were no differences in urinary thromboxane B<sub>2</sub>, 6-Oxo-prostaglandin F<sub>1α</sub>, 2,3-Dinor-6-oxo-prostaglandin F<sub>1α</sub> or PGE<sub>2</sub> between the two diets (71). A urinary marker of PGE turnover was reduced with salmon feeding in a study by Ferretti et al (67). Urinary excretion of a metabolite of PGE<sub>1</sub> and PGE<sub>2</sub>, was reduced by 14% in men fed a controlled diet containing negligible n-3 FA plus 15 g/d fish oil supplements (50% n-3) for 10 weeks relative to the controlled diet (68). A 2-week fish diet significantly increased platelet and plasma phospholipid 20:5n-3 and 22:6n-3 and plasma phospholipid 22:5n-3 and decreased urinary TXB-M (72). Studies of fish and fish oil supplements have given mixed results on eicosanoid excretion, however in studies most similar to ours (67, 71), metabolites of both PGE<sub>2</sub> and TxB<sub>2</sub> were decreased with 40 days of feeding a fish diet or fish oil supplements.

Dietary intake is reflected in tissue fatty acid composition (361). In our study, subjects were fed a known amount of n-3 FA with the LFn3 diet and plasma PLFA levels of 18:3n-3, 20:5n-3, 22:6n-3, and total n-3 increased significantly while 18:2n-6,

20:4n-6, total n-6, and n-6:n-3 ratio decreased significantly. This is in agreement with a study by Bagga et al (50) in which the investigators instructed breast cancer survivors to follow a low fat diet (15% energy from fat, 15% energy from protein, 70% energy from carbohydrate) plus take 10 g/d fish oil capsules (3 g/d EPA + DHA). Plasma, gluteal adipose, and breast adipose tissue was obtained before and after the 3-mo intervention. Plasma n-3 FA and n-6 FA changed significantly following the intervention, in a pattern similar to our study. Gluteal adipose and breast adipose tissue n-3 FA and n-3/n-6 ratio were significantly increased after 3 mo, but total n-6 was not different. Low fat diet and fish oil supplements appear to increase the n-3 FA content of the breast microenvironment; however it is unknown whether these changes affect the risk of breast cancer. In a meta-analysis of fatty acid biomarker studies in which tissue (blood, red blood cell, adipose) fatty acids were measured, tissue n-3 FA were inversely associated with breast cancer risk in prospective cohort studies (88).

There are several strengths of the current dietary intervention study. One, unlike an association study, we are able to evaluate changes in biomarkers directly related to the dietary intervention. Secondly, the entire study was carried out at the GCRC of the University of Minnesota and a trained metabolic kitchen staff prepared all the meals for the study duration. Also, the high n-3 FA level of the LFn3 diet was achieved using whole foods in amounts that are feasible for a person on a self-selected diet to achieve. In addition, the proportions of SFA, MUFA and PUFA were 1:1:1 in both the HF and LF diets, therefore the effects of decreasing the amount of dietary fat to half from the HF to the LF could be examined without interference by changing the proportions of fatty acids.

A weakness of the current study is that the randomization schemas are not balanced. There were three diets (HF, LF, and LFn3) and six treatment order schemas possible. Because of the pattern of dropped subjects and exclusion following sample analysis, we ended up with four subjects in one schema, three subjects in three of the schemas, and two subjects in two of the schemas. However, all subjects except one completed all of the dietary treatments. Statistical analysis considered treatment order. Also, the sample size may be too small to detect the differences in sex hormone

concentrations. In addition, biochemical markers of compliance for the HF and LF diet interventions were not included. Compliance with the LFn3 diet was estimated by incorporation of n-3 FA into PLFA and it was clear from the PLFA measurements that the subjects consumed the LFn3 diet. As a compliance measure we instructed the subjects to fill out a daily food consumption questionnaire, which we assume the subjects filled out honestly. An additional weakness is that two of the sources of n-3 FA (flax oil and walnuts) in the LFn3 diet supplied only ALA, not long-chain EPA and DHA. Because conversion of ALA to long-chain n-3 FA is low in adults, we may not have provided enough EPA and DHA to see the expected results.

One may argue that altering blood hormone or fatty acid levels late in life may confer little protection from breast cancer when the ideal time of exposure may be years or decades earlier. However even a small decrease in circulating estrogens may impart beneficial effects long-term and women at high risk of breast cancer may benefit from such dietary interventions.

## **V. Conclusion**

Breast cancer risk factors appear to be increased by the HF. Relative to the HF, the LF and LFn3 appear to confer decreased risk, although the results are less conclusive regarding the LFn3. Long term consumption of a diet similar to LF and LFn3 may have an impact on breast cancer risk through reduction of circulating sex hormone levels. Breast cancer risk may be reduced in a population that consumes n-3 FA and a low fat diet at levels currently recommended by United States Department of Health and Human Services and the United States Department of Agriculture (362).



**Table 2-1: Macronutrient composition of the HF, LF, and LFn3 diets<sup>1</sup>**

	HF	LF	LFn3
		<i>g/% energy</i>	
Carbohydrate	233/45	329/65	317/62
Protein	77/15	76/15	77/15
Fat	92/40	46/20	54/23
		<i>g</i>	
Fiber	22.8	23.9	25.2
Cholesterol	0.209	0.203	0.210
SFA	28.2	13.4	14.8
MUFA	28.7	13.9	16.2
PUFA	28.0	13.3	16.1
18:3n-3	1.40	0.85	5.36
20:5n-3	0	0	0.46
22:6n-3	0.03	0.03	0.99
n-3 fatty acids	1.43	0.88	6.82

<sup>1</sup> Composition of diets per 8368 kJ (calculated).

**Table 2-2: Sample menu per 8368 kJ for the HF, LF and LFn3 interventions.**

Menu Items		HF	LF	LFn3
		<i>g</i>		
Breakfast:	Grapefruit Juice	130	200	200
	Oat cereal	40	32	35
	w/ Sugar	0	6	6
	Bran Muffin	75	60	60
	w/ Butter	10	4	4
	Skim Milk	240	250	230
Lunch:	Turkey Sandwich			
	Whole Wheat Bread	60	60	60
	Turkey Breast	70	55	45
	Butter	6	0	5
	Mayonnaise, Regular	10	6	0
	Salad dressing, Fat Free	0	10	10
	Egg yolk	2	5	4
	Flax oil	0	0	3
	Lettuce	20	20	20
	Tomato	50	50	50
	Pretzels	0	25	0
	Potato Chips	35	0	0
	Applesauce	140	180	200

Dinner:	Baked Orange Roughy	140	90	0
	Baked Salmon	0	0	75
	w/ Lemon Juice	5	5	5
	White Rice	140	110	110
	Broccoli	80	80	80
	Lettuce/Carrot Salad	50/15	50/15	50/15
	Fat Free French Dressing	15	20	15
	w/Corn Oil	7	5	0
	w/Flax Oil	0	0	5
	Whole Wheat Bread	26	40	40
	w/Butter	6	6	0
	Angel Food Cake	0	50	50
	w/Strawberries/Sugar	120/0	120/6	120/6
Snack:	Wheat crackers	10	20 (Regular)	30 (Low Fat)
	Mozzarella Cheese	0	30	40
	Cheddar Cheese	20	0	0
	Gumdrops	0	30	15
	Grape Juice	0	120	0

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**Table 2-3: Baseline characteristics of subjects<sup>1</sup>**

Age, <i>y</i>	57 ± 6
Body wt, <i>kg</i>	75 ± 11
Height, <i>m</i>	1.64 ± 0.06
BMI, <i>kg/m<sup>2</sup></i>	28 ± 4
Systolic blood pressure, <i>mmHg</i>	120 ± 16
Diastolic blood pressure, <i>mmHg</i>	73 ± 11
FSH <sup>2</sup> , <i>mIU/mL</i>	73 ± 22
Ethnicity, <i>n (%)</i>	
White	15 (88)
Hispanic	1 (6)
African-American	1 (6)

<sup>1</sup>All values are means ± SD except ethnic groups, which are n (%); n=17

<sup>2</sup>Screening FSH values for all women < 55 y; n = 11.

**Table 2-4: Daily baseline diet of study participants reported by Diet History Questionnaire<sup>1</sup>**

Total energy, <i>kJ</i>	7533 ± 2672
	<i>g (% energy)</i>
Protein	73 ± 30 (16 ± 2)
Carbohydrate	232 ± 81 (52 ± 7)
Total fat	66 ± 27 (33 ± 5)
SFA	22 ± 10 (11 ± 3)
MUFA	25 ± 10 (12 ± 2)
PUFA	14 ± 5 (7 ± 1)
18:2n-6	13 ± 4 (6.5 ± 2.0)
18:3n-3	1.2 ± 0.4 (0.6 ± 0.2)
20:4n-6	0.09 ± 0.05 (0.045 ± 0.025)
20:5n-3	0.02 ± 0.01 (0.010 ± 0.005)
22:6n-3	0.05 ± 0.04 (0.025 ± 0.020)
	<i>g</i>
Dietary fiber	19 ± 7
Cholesterol	0.177 ± 0.096

<sup>1</sup>All values are means ± SD; n =17.

**Table 2-5: Plasma phospholipid fatty acid values for the HF, LF and LFn3 diets (percent composition)<sup>1</sup>**

	HF n = 17	LF n = 16 <sup>2</sup>	LFn3 n = 17	P-value <sup>3</sup>
<b>18:2n-6</b>				
Baseline	21.08 ± 0.65	21.45 ± 0.66	22.07 ± 0.65	0.28
8 weeks	23.43 ± 0.67 <sup>a*</sup>	20.65 ± 0.68 <sup>b</sup>	18.67 ± 0.67 <sup>c*</sup>	< 0.0001
P-value <sup>4</sup>	0.0012	0.29	< 0.0001	
<b>18:3n-3</b>				
Baseline	0.30 ± 0.07	0.34 ± 0.07	0.24 ± 0.07	0.34
8 weeks	0.21 ± 0.04 <sup>a</sup>	0.24 ± 0.04 <sup>a</sup>	0.55 ± 0.04 <sup>b*</sup>	< 0.0001
P-value <sup>4</sup>	0.16	0.15	< 0.0001	
<b>20:4n-6</b>				
Baseline	9.11 ± 0.46	9.09 ± 0.47	9.54 ± 0.46	0.38
8 weeks	8.96 ± 0.44 <sup>a</sup>	10.30 ± 0.45 <sup>b*</sup>	8.06 ± 0.44 <sup>c*</sup>	< 0.0001
P-value <sup>4</sup>	0.63	< 0.001	< 0.0001	
<b>20:5n-3</b>				
Baseline	0.84 ± 0.12	0.86 ± 0.12	0.73 ± 0.12	0.55
8 weeks	0.57 ± 0.13 <sup>a*</sup>	0.73 ± 0.14 <sup>a</sup>	2.38 ± 0.13 <sup>b*</sup>	< 0.0001
P-value <sup>4</sup>	0.05	0.41	< 0.0001	
<b>22:6n-3</b>				
Baseline	2.26 ± 0.18	2.16 ± 0.18	2.23 ± 0.18	0.85

8 weeks	1.99 ± 0.25 <sup>a</sup>	2.63 ± 0.25 <sup>b*</sup>	4.39 ± 0.25 <sup>c*</sup>	< 0.0001
P-value <sup>4</sup>	0.15	0.02	< 0.0001	
Total n-3				
Baseline	4.46 ± 0.32	4.47 ± 0.32	4.26 ± 0.32	0.70
8 weeks	3.66 ± 0.41 <sup>a*</sup>	4.71 ± 0.42 <sup>b</sup>	8.86 ± 0.41 <sup>c*</sup>	< 0.0001
P-value <sup>4</sup>	0.03	0.50	< 0.0001	
Total n-6				
Baseline	34.05 ± 0.65	34.33 ± 0.67	35.25 ± 0.65	0.24
8 weeks	35.63 ± 0.63 <sup>a*</sup>	35.01 ± 0.65 <sup>a</sup>	29.61 ± 0.64 <sup>b*</sup>	< 0.0001
P-value <sup>4</sup>	0.03	0.33	< 0.0001	
n-6 : n-3 ratio				
Baseline	8.22 ± 0.55	8.27 ± 0.56	8.78 ± 0.55	0.42
8 weeks	10.43 ± 0.62 <sup>a*</sup>	8.01 ± 0.64 <sup>b</sup>	3.74 ± 0.62 <sup>c*</sup>	< 0.0001
P-value <sup>4</sup>	0.002	0.59	< 0.0001	

<sup>1</sup>All values are LS means ± standard error. Differences are within rows; differing letters denote significant differences.

<sup>2</sup>One subject was missing the LF diet, thus n = 16 for LF.

<sup>3</sup>P-value for effect of treatment across the three diets. Diets were compared using a general linear mixed model. Values with differing letters as a superscript are significantly different at P < 0.05.

<sup>4</sup>P-value for paired t-test comparing baseline and 8 week means within each diet.

\*8 week mean is significantly different from baseline mean at P < 0.05.

**Table 2-6: Baseline and 8 week concentrations of urinary eicosanoids for the HF, LF, and LFn3 diets<sup>1</sup>**

	HF	LF	LFn3	P-value <sup>4</sup>
	n = 15 <sup>2</sup>	n = 15 <sup>3</sup>	n = 16	
PGE-M	<i>ng/day</i>			
Baseline	231 (173, 307) <sup>a</sup>	306 (230, 409) <sup>b</sup>	250 (188, 332) <sup>ab</sup>	0.05
8 weeks <sup>5</sup>	356 (256, 496) <sup>a</sup>	173 (124, 241) <sup>b</sup>	246 (178, 339) <sup>ab</sup>	0.02
P-value <sup>6</sup>	0.16	0.86	0.44	
TxB-M				
Baseline	786 (573, 1079)	1076 (783, 1479)	984 (721, 1341)	0.20
8 weeks	1101 (868, 1397)*	1147 (903, 1456)	1003 (792, 1269)	0.38
P-value <sup>6</sup>	0.01	0.37	0.64	

<sup>1</sup>All values are geometric means (95% CI).

<sup>2</sup>One subject was missing urines for HF diet, thus n = 15 for HF.

<sup>3</sup>One subject was missing urines for LF diet, thus n = 15 for LF.

<sup>4</sup>P-value for effect of treatment across the three diets. Diets were compared using a general linear mixed model. Values with differing letters as a superscript are significantly different at P < 0.05.

<sup>5</sup>8 week means are adjusted for baseline PGE-M.

<sup>6</sup>P-value for paired t-test comparing baseline and 8 week means within each diet.

\*8 week mean is significantly different from baseline mean at P < 0.05.



**Table 2-7: Plasma sex hormone and SHBG profile at baseline and after 8 weeks of the HF, LF and LFn3 diets<sup>1</sup>**

	HF	LF <sup>2</sup>	LFn3	P-value <sup>3</sup>
	n = 17	n = 16	n = 17	
<hr/>				
E2	<i>pmol/L</i>			
Baseline	35.0 ± 5.1 <sup>a</sup>	51.1 ± 5.4 <sup>b</sup>	35.3 ± 5.1 <sup>a</sup>	0.02
8 weeks <sup>4</sup>	52.8 ± 6.8*	35.6 ± 7.6	39.8 ± 6.9	0.11
P-value <sup>5</sup>	0.03	0.10	0.59	
E1				
Baseline	111.3 ± 11.0	107.4 ± 11.3	103.1 ± 11.0	0.25
8 weeks	104.2 ± 8.9	105.0 ± 8.9	95.9 ± 8.8	0.14
P-value <sup>5</sup>	0.39	0.81	0.40	
FSH	<i>IU/L</i>			
Baseline	56.8 ± 6.3	58.8 ± 6.3	55.9 ± 6.3	0.43
8 weeks	54.9 ± 6.5	54.2 ± 6.5*	54.6 ± 6.4	0.95
P-value <sup>5</sup>	0.43	0.03	0.53	
E1-S	<i>nmol/L</i>			
Baseline	1.05 ± 0.10	1.04 ± 0.11	1.13 ± 0.11	0.48
8 weeks	1.10 ± 0.11	1.05 ± 0.11	1.12 ± 0.11	0.75
P-value <sup>5</sup>	0.58	0.66	0.90	
A				
Baseline	2.30 ± 0.26	2.71 ± 0.26	2.53 ± 0.26	0.10

8 weeks	2.52 ± 0.30	2.70 ± 0.30	2.64 ± 0.30	0.69
P-value <sup>5</sup>	0.30	0.98	0.63	
DHEA				
Baseline	22.67 ± 2.45	26.10 ± 2.48	24.56 ± 2.46	0.10
8 weeks	24.67 ± 2.69	26.18 ± 2.70	25.95 ± 2.65	0.80
P-value <sup>5</sup>	0.34	0.94	0.52	
SHBG				
Baseline	29.02 ± 3.10	29.64 ± 3.12	29.14 ± 3.10	0.92
8 weeks	29.81 ± 2.95	26.50 ± 2.95	27.13 ± 2.94	0.06
P-value <sup>5</sup>	0.60	0.06	0.19	
T				
Baseline	1.14 ± 0.10	1.21 ± 0.10	1.21 ± 0.10	0.59
8 weeks	1.15 ± 0.12	1.23 ± 0.12	1.21 ± 0.11	0.54
P-value <sup>5</sup>	0.84	0.75	0.99	
DHEAS <i>μmol/L</i>				
Baseline	1.55 (1.05, 2.30)	1.71 (1.15, 2.54)	1.53 (1.03, 2.26)	0.42
8 weeks	1.69 (1.16, 2.47)	1.70 (1.16, 2.48)	1.65(1.13, 2.40)	0.91
P-value <sup>5</sup>	0.28	0.70	0.47	
Free E2 Index, <i>x1000</i>				
Baseline	1.5 ± 0.4	2.2 ± 0.4	1.6 ± 0.4	0.17
8 weeks	2.0 ± 0.4	1.8 ± 0.4	1.8 ± 0.3	0.62
P-value <sup>5</sup>	0.09	0.34	0.63	

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Free T Index, <i>x100</i>				
Baseline	4.85 ± 0.77	4.83 ± 0.77	5.16 ± 0.77	0.68
8 weeks	4.75 ± 0.75	5.64 ± 0.75	5.18 ± 0.75	0.10
P-value <sup>5</sup>	0.82	0.08	0.97	

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<sup>1</sup> All values are least squares means ± SEM except for DHEAS, which is the geometric mean (95% CI).

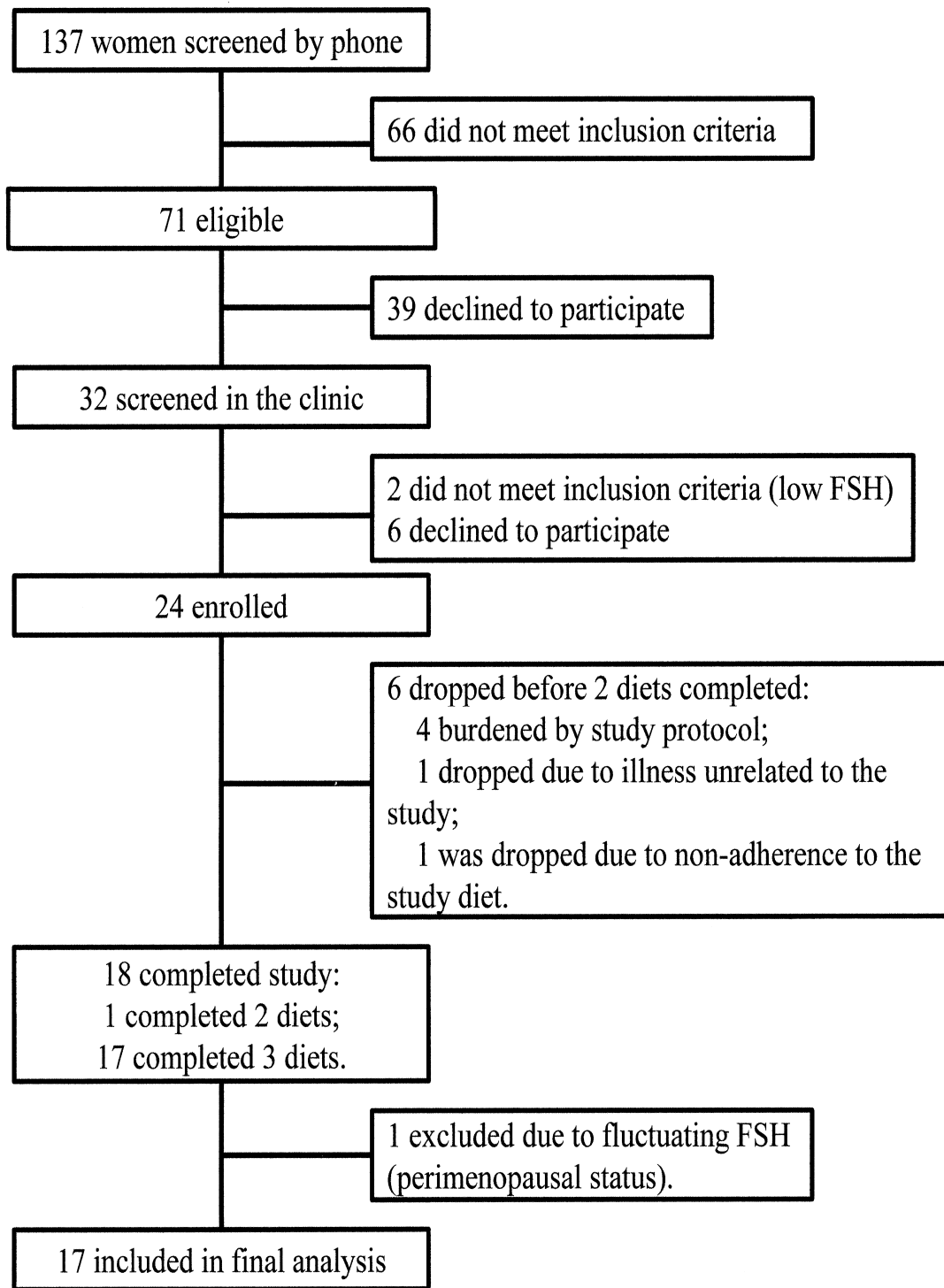
<sup>2</sup>One subject was excluded from E2, E1-S, and free E2 index in the LF, thus n=15 for these endpoints.

<sup>3</sup>P-value for effect of treatment across the three diets evaluated using a general linear mixed model. Values with differing letters as a superscript are significantly different at P < 0.05.

<sup>4</sup>8 week means were adjusted for baseline estradiol.

<sup>5</sup>P-value for paired t-test comparing baseline and 8 week means within each diet.

\*8 week mean is significantly different from baseline mean at P < 0.05.



**Figure 2-1: Flow of participants from screening to final analysis**

**Chapter 3:**  
**Total Dietary Fat and Omega-3 Fatty Acids Have Modest Effects on Urinary Sex  
Hormones in Postmenopausal Women**

Total fat and omega-3 fatty acids (n-3) in the diet may affect breast cancer risk by altering estrogen metabolism. The purpose of this study was to elucidate the effects of differing total fat and n-3 content of diets on a panel of urinary estrogens and metabolites. A controlled, cross-over feeding trial was conducted in postmenopausal women using three test diets: a high fat diet (HF; 40% energy from fat), a low fat diet (LF; 20% of energy from fat) and a low fat, high n-3 diet (LFn3; 23% energy from fat; 3% n-3 fatty acids) for 8 week periods. Urinary hormone concentrations for 16 women were compared among diets using a linear mixed model, and within diet comparisons were made using paired t-tests. Urinary excretion of estrone (E1) was greater after the LF and LFn3 compared to the HF ( $P = 0.004$ ). E1 excretion was increased from baseline within the LF only ( $P = 0.02$ ). Total E1 + Estradiol (E2) + Estriol (E3) increased from baseline with LF ( $P = 0.02$ ) and was greater than the other two diets at 8 weeks ( $P = 0.03$ ). No clinically significant alterations in sex hormone metabolism as measured by urinary estrogens and their metabolites were observed in postmenopausal women who consumed a low fat dietary intervention alone or with additional n-3 fatty acids. These data support previous observations that a low fat diet increases urinary estrogen excretion, but the effects of these minimal changes on breast cancer risk are unknown.

## **I. Introduction**

Dietary fat has been extensively, yet inconclusively, studied in relation to breast cancer risk. Prospective cohort studies have given weak confirmation of the positive associations between dietary fat and breast cancer demonstrated by international correlation studies and animal experiments (36, 347). Relatively high concentrations of estrogens in the blood and urine are associated with increased risk of breast cancer development in postmenopausal women (131), and one hypothesis is that the increased risk of breast cancer associated with high fat diet is mediated by sex hormone metabolism. Low fat dietary interventions resulted in significant reductions in circulating estrogens in some studies in postmenopausal women (37, 180, 182), however these interventions did not differentiate between the types of dietary fat consumed. Of particular interest in relation to breast cancer risk are omega-3 (n-3) fatty acids, which may suppress tumorigenesis by inhibition of the synthesis of inflammatory eicosanoids derived from arachidonic acid and subsequent suppression of aromatase and thus conversion of androgens to estrogens (56).

In addition to promoting growth via interaction with estrogen receptors, circulating estrogens are metabolized via hydroxylation reactions that result in an array of metabolites with varying biological activity relative to the parent estrogens, with some of the metabolites having genotoxic activity (114). Hydroxylation of estrogens (estradiol, E2 and estrone, E1) at the 2- position yields 2-hydroxy E2 (2OH-E2) and 2-hydroxy E1 (2OH-E1), which have reduced affinities for human estrogen receptors relative to E2 (363) and lack tumorigenic activity partially due to inactivation and clearance facilitated by O-methylation via catechol-O-methyltransferase to 2-methoxy E2 (2-ME2) and 2-methoxy-E1 (2-ME1) (364). 2-ME2 has antiproliferative, antiangiogenic, and antimetastatic properties potentially due to its disruptive effects on tubulin polymerization, and thus inhibition of mitosis, angiogenesis and cell migration (142, 143, 365). In contrast, the 4-hydroxy estrogens are considered to be carcinogenic. The small amounts of 4-hydroxy E2 (4OH-E2) and 4-hydroxy E1 (4OH-E1)

metabolites generated in oxidative metabolism can form quinone intermediates that bind to DNA to form depurinating adducts which may result in cancer initiation (116, 366).

Another product of hydroxylation of estrogens, 16 $\alpha$ -hydroxy E1 (16 $\alpha$ OH-E1), is a potent mitogen, tumor initiator, and tumor promoter *in vitro* (150). 16 $\alpha$ OH-E1 can covalently bind to the estrogen receptor  $\alpha$  and the receptor/16 $\alpha$ OH-E1 complex localizes to the nucleus, indicating that the complex affects gene transcription (151). Fishman et al demonstrated that 16 $\alpha$ -hydroxylation of estrogens was enhanced in women with breast cancer relative to healthy controls (154, 155). The ratio of 2OH-E1 (low risk) to 16 $\alpha$ OH-E1 (high risk) concentrations has been investigated as an index of breast cancer risk. A higher ratio of 2OH-E1 to 16 $\alpha$ OH-E1 (2:16 $\alpha$ OH-E1 ratio) indicated decreased breast cancer risk in some studies (156, 157), however, other investigations do not support this association (162, 165).

Metabolism of urinary estrogens and metabolites has not been extensively studied in relation to dietary fat and n-3 fatty acids interventions in postmenopausal women. A study in a small number (n = 6) of premenopausal women demonstrated decreased excretion of 16 $\alpha$ -hydroxylated estrogens in the urine following a low fat (25% energy from fat) dietary intervention (185). 16 $\alpha$ -hydroxylation was reduced by n-3 supplementation in a pilot trial (188); however, supplementation with docosahexaenoic acid (DHA)-rich algae oil had no effect on the 2:16 $\alpha$ OH-E1 ratio in postmenopausal women (189). The purpose of the present study was to determine the effects of diets with varying amounts of fat and n-3 on a panel of urinary estrogens and estrogen metabolites in healthy postmenopausal women. We hypothesized that compared to a high fat diet, diets low in fat or low in fat and high in n-3 fatty acids would decrease individual and total estrogens and decrease the carcinogenic 4-hydroxy metabolites and 16 $\alpha$ OH-E1.



## **II. Subjects and Methods**

### **A. Experimental Protocol**

Complete details of the study design and dietary treatments are described elsewhere [Orr et al, in review]. The effects of three controlled test diets [a high fat diet (HF, 45% energy as fat), a low fat (LF, 20% energy as fat) and a low fat, high n-3 diet (LFn3, 23% energy as fat)] on urinary estrogen metabolism were compared in a randomized, cross-over trial. Subjects consumed their first assigned test diet for 8 weeks followed by a wash-out period of 6-12 weeks during which time the participants consumed their normal diets. The participants consumed the next diet for 8 weeks followed by another wash-out period and then consumed the final diet treatment for 8 weeks. The test diets were prepared in the metabolic kitchen of the University of Minnesota General Clinical Research Center (GCRC). All meals (breakfast, lunch, dinner, and a snack) were provided by the study and participants were instructed to consume all of the test meals and not to consume any calorie-containing foods from outside the study for the duration of each 8 week feeding period. Subjects recorded any deviations from the test diets (parts of study meals not consumed or foods consumed from outside the study) daily on a compliance form when they picked up their meals. Daily weight was also recorded by the subjects and monitored by the study personnel.

The study was approved by the U.S. Army Medical Research and Materiel Command's Human Subjects Research Review Board and the University of Minnesota Committee for the Use of Human Subjects in Research. Written informed consent was provided by all study subjects.

### **B. Subjects**

The participants of this trial participated in a study of the effects of the test diets on plasma sex hormones and urinary eicosanoids. Subject characteristics and recruitment are discussed elsewhere [Orr et al, in review]. Participants were postmenopausal women ( $\geq 1$  year without menstruation and a screening follicle stimulating hormone concentration of  $> 23$  IU/L if less than 55 years of age); age 45 –

70 years old; had a body mass index of 19 – 32 kg/m<sup>2</sup>; had not lost or gained more than 5 pounds in the 6 months preceding the study; were willing to suspend the use of all over-the-counter and prescription non-steroidal anti-inflammatory drugs for the extent of the trial; and had not used fish oil supplements or hormone replacement therapy for 2 months prior to the trial. Subject were excluded if they had known disease process including diabetes mellitus, had a personal history of cancer, used prescription medications (excluding high blood pressure medication), or had both ovaries removed. A medical screening questionnaire was used to determine health status.

Seventeen subjects completed all three dietary treatments and one subject completed two treatments (missing LF diet treatment). One subject was excluded from the analysis because her follicle stimulating hormone concentration was intermittently in the premenopausal range following blood sample analysis in the previous study [Orr et al, in review]. Another subject was excluded due to improperly collected urine specimens. One subject was missing urine samples for the HF. In total, 15 subjects completed the HF, 15 subjects completed the LF, and 16 subjects completed the LFn3 and are included in the final analysis.

### **C. Dietary Treatments**

Details on the test diets are provided elsewhere [Orr et al, in review]. The three dietary treatments were prepared in the metabolic kitchen of the University of Minnesota GCRC using common foods. The HF (40% energy as fat, 15% energy as protein, and 45% energy as carbohydrate) and the LF (20% energy as fat, 15% energy as protein, and 65% energy as carbohydrate) each were designed to contain nominal n-3 fatty acids and a 1:1:1 ratio of saturated to monounsaturated to polyunsaturated fatty acids but different total amounts of dietary fat. The LFn3 (23% energy as fat, 15% energy as protein, and 62% energy as carbohydrate) had a fatty acid composition similar to the LF but had an additional 3% energy from n-3 fatty acids from animal and vegetable food sources such as salmon, walnuts and flax seed oil. Nutrient analysis software (Nutritionist V) was used to determine the nutritional content of the test diets.

#### **D. Urine Collection**

Subjects collected two consecutive 24-hour urine specimens, which included all voids beginning after the first morning void and commencing after the first morning void 48 hours later, at weeks 0 and 8 of each dietary treatment period. Opaque 3.5L containers including 3.5g ascorbic acid were provided for urine collection. Subjects were instructed to keep the urine cold on ice or in the refrigerator until bringing it to the GCRC for processing. Urine was pooled and sodium azide was added to 0.1%. Aliquots were stored at -20°C until analysis.

#### **E. Estrogen Metabolite Assay by LC/MS-MS**

In recent years, other research labs have developed methods to analyze a wide variety of steroid compounds at parts per billion (ng/ml) levels by LC-MS (367, 368). In our laboratory, LC-MS/MS-based methods have successfully replaced GC-MS based and immunoassays for estrogen metabolite analysis in human urine samples.

Unless otherwise noted, all standard laboratory chemicals were obtained from Fisher Scientific (Pittsburg, PA). Frozen, preserved (with sodium azide and sodium ascorbate) urine samples were thawed at room temperature, thoroughly mixed by vortex to ensure homogeneity and centrifuged at 5° C for 5 minutes. Duplicate 1.0 ml aliquots of urine were added to clean, silanized screw-top test tubes. 10 µL of the deuterated-Estrogen Metabolite (d-EM) working internal standard solution (ISS; C/D/N Isotopes, Inc., Pointe-Claire, Quebec, Canada) containing representative analytes of each class of compounds was added to the aliquots, followed by 1.0 mL of freshly prepared enzymatic hydrolysis buffer containing 2 mg of L-ascorbic acid, 5 µL of  $\alpha$ -glucuronidase/sulfatase from *Helix pomatia* (Type H-2; Sigma-Aldrich, St Louis, MO), and 1.0 mL of 0.15 M sodium acetate buffer (pH 4.1) and incubated overnight at 37 °C.

After hydrolysis, the samples were extracted 3 times with ethyl ether and the organic extracts were pooled and evaporated to dryness at 40 °C under nitrogen. 25µL of 0.1M sodium bicarbonate buffer with 0.1% ascorbic acid (pH at 9.0) and 25 µL of dansyl chloride solution (3 mg/mL in acetone; Lancaster Synthesis, Inc., Pelham, NH)

were added to each dried sample. After vortexing, each sample was heated at 65 °C for 6 minutes to form the EM and d-EM dansyl derivatives. Calibration standards (Steraloids, Inc., Newport, RI) and quality control samples were similarly hydrolyzed, extracted, and derivatized.

The chromatographic separation was performed on a 100 x 0.5mm (i.d.) Zorbax SB-C18 column 1.8µm particle size (Agilent Technologies, Santa Clara, CA). The mobile phase consisted of two eluents, solvent A (50 mL/L acetonitrile + 950 mL/L H<sub>2</sub>O containing 1 mL/L formic acid) and solvent B (475 mL/L acetonitrile + 475 mL/L methanol + 50 mL/L H<sub>2</sub>O containing 1 mL/L formic acid). LC/MS-MS analysis was performed on a Thermo Electron Quantum Discovery Max Triple Quadrupole LC-MS/MS Instrument. Quantitative analysis was performed using Thermo Electron Xcalibur proprietary software. All samples from a given subject were analyzed in the same batch. Intra-batch coefficients of variation varied from 5.1 – 12.2% among analytes. Urinary hormone data was analyzed in nmol/day and in nmol/mg creatinine and statistical analysis results did not differ, so the data is expressed as nmol/day.

#### **F. Statistical Analysis**

Data for urinary sex hormone metabolites was not normally distributed; therefore values were log-transformed and are expressed as geometric means and 95% confidence intervals. SAS Proc Mixed (SAS version 9.2, SAS Institute Inc., Cary, NC; 2002-2008) was used to fit a mixed effects linear model for each urinary hormone or metabolite with a random effect for subject to account for multiple correlated measurements within each subject. These proc mixed effect models handled isolated missing values and were used to assess period and carryover effects. The diet randomization groups were unbalanced. Least squares means generated from the mixed model were compared between treatments. Paired t-tests were applied to within diet differences. For 2OH-E1, a statistically significant difference was observed among baseline means; therefore baseline 2OH-E1 was included in the model for the 8 week analysis of 2OH-E1. A P-value of < 0.05 was considered statistically significant.

### III. Results

The study participants were slightly overweight (mean BMI 27 kg/m<sup>2</sup>, Table 3-1) middle-aged women (mean age 58 years). The participants were mostly white (88%). Baseline reported dietary intake and compliance with the test diets was reported previously (Orr et al, in review). Although mean weight did not differ among treatments at baseline or 8 weeks, there was a slight but statistically significant decrease in mean weight with all three diets (-1.1 kg with the HF, -1.3 kg with the LF, and -0.7 kg with the LFn3).

Urinary 4OH-E2 was undetectable in the urines from these postmenopausal women, so 4OH-E2 is not included in this report even though it was one of the estrogen metabolites assayed.

There were baseline differences in mean urinary 2OH-E1 ( $P = 0.02$ ) (Table 3-2); therefore the 8 week means of 2OH-E1 were adjusted for baseline 2OH-E1. There were no significant differences within or among diets for this metabolite.

Urinary excretion of E1 was significantly greater after both the LF and LFn3 compared to the HF ( $P = 0.004$ , Table 3-2), although E1 excretion was increased from baseline with the LF only ( $P = 0.02$ ). Excretion of the sum of E1, E2 and E3 ( $E1 + E2 + E3$ ) was also significantly greater with the LF than the HF at 8 weeks ( $P = 0.03$ ) and  $E1 + E2 + E3$  increased significantly from baseline to 8 weeks with the LF ( $P = 0.02$ ). There were also trends for reduced urinary E2 excretion with the HF relative to the LF and LFn3 ( $P = 0.12$ ) and for increased excretion of Estriol (E3) with the LF ( $P = 0.12$ ). Urinary excretion of 2OH-E1, 2OH-E2, 2-ME1, 2-ME2, 4-ME1, 4-ME2, 16 $\alpha$ OH-E1, 2:16 $\alpha$ OH-E1 ratio, ratio of 2OH-E1 + 2OH-E2 to 16 $\alpha$ OH-E1 (2OHE:16 $\alpha$ OH-E1 ratio), and total estrogens were not significantly different among the three test diets following 8 weeks of treatment.

#### **IV. Discussion**

Even though urinary estrone excretion was significantly greater with the LF and LFn3, there was little overall change in urinary hormone metabolism following the 8-week controlled dietary interventions involved in this study. The sum of all estrogens and metabolites assayed in this study (Total Estrogens) did not differ among diets following 8 weeks of feeding. In a previous manuscript we reported the effects of the three test diets on plasma sex hormones. In the blood, E2 concentration increased significantly with the HF ( $P = 0.03$ ) and decreased slightly, but non-significantly with the LF ( $P = 0.10$ ) [Orr et al, in review]. Because we saw small but significant changes in sex hormones in the blood, we anticipated changes in urinary sex hormone metabolism. Taken together, our study results indicate that total estrogens in the blood were reduced and urinary excretion of estrogens was increased in postmenopausal women after 8 weeks of the LF and LFn3 relative to the HF. We saw no change in urinary sex hormone metabolite excretion even though changes in the parent hormones E1 and E2 were observed in the current study. The variability of the urinary hormone analysis was high, and interpretation of the results may be hindered by our small sample size.

We anticipated that the LF and LFn3 would reduce excretion of estrogens and metabolites in our study sample in accordance with the hypothesis that a low fat diet alters the profile of sex hormones in a direction of reduced breast cancer risk. According to a quantitative review of 1 prospective cohort study and 7 case-control studies of urinary excretion of estrogens (defined as E1 + E2 + E3 or E1 + E2) in postmenopausal women by Thomas and colleagues, increased excretion of urinary estrogens was associated with increased risk of breast cancer (131). Decreased excretion of estrogens was also observed in postmenopausal women from Asian nations with relatively low breast cancer incidence and concomitantly low intake of dietary fat (167, 168, 173). However, a controlled feeding study in men by Dorgan et al reported increased excretion of urinary E1 and E2 after a low fat/high fiber diet relative to a high fat/low fiber diet in healthy men (186). While their result of increased urinary estrogen

excretion is similar to our study, the total fat intake differed (18.8% vs. 41% en fat) in the Dorgan et al study as well as the polyunsaturated to saturated fat ratio (1.3 for the low fat diet vs. 0.6 for the high fat diet). Additionally the fiber intake differed (2.0g fiber/MJ in the high fat diet vs. 4.6g fiber/MJ in the low fat diet), making it difficult to attribute the change in estrogen excretion to any one of the variables alone, in particular the dietary fat content. In the current study, the foods used to plan the diets were consistent among diets, including the amount of dietary fiber across diets, with the addition of high n-3 foods in the LFn3. The saturated to monounsaturated to polyunsaturated fat ratio was 1:1:1 for the HF and LF. The LF and LFn3 differed by additional 3% en from n-3 fatty acids (compensated by a 3% reduction in energy from carbohydrate) in the LFn3. Therefore, our results were due mainly to changes in the amount (HF vs. LF) or type (LF vs. LFn3) of dietary fat in the diets.

Reduction in total urinary estrogen excretion was observed following a 6 month Mediterranean diet intervention in postmenopausal women in Italy compared to a control group even though concentrations of individual hormones and metabolites were not different between the intervention group and controls (187). However, the Mediterranean diet intervention differed from our interventions primarily because the Mediterranean diet was not a controlled intervention and diet data were collected with 24-hour recalls and food frequency questionnaires, while our study subjects consumed premeasured, known quantities of nutrients. The Mediterranean diet intervention included a number of changes including restricted intake of animal fat and refined carbohydrate and inclusions of whole grains and fish among other traditional Mediterranean foods, again making it difficult to attribute the changes to any single dietary attribute.

In addition to the amount of fat in the diet, n-3 fatty acids may play a role in breast cancer progression. Greenland Eskimos, whose traditional diet was particularly high in n-3 fatty acids (369), have historically had a low incidence of breast cancer (370, 371). However, this risk has increased as Westernized and nontraditional foods have become more prevalent in their diets (372, 373). Few studies have investigated the

relationship of n-3 and urinary sex hormone metabolism in humans. Urinary  $16\alpha\text{OH-E1}$  was reduced in the n-3 fatty acid supplement arm in a pilot trial of high-risk women (188). In a study by Wu et al (189) postmenopausal vegetarian women consumed 2.14 g/day of DHA oil in the form of supplements derived from algae oil. There was no significant change in urinary  $2:16\alpha\text{OH-E1}$  ratio following the intervention even though EPA and DHA composition of LDL particles was significantly increased following the intervention. In the current study,  $2:16\alpha\text{OH-E1}$  ratio was also not altered following 8 weeks of each dietary intervention, although there was a trend for increased  $2\text{OH-E1}$  following 8 weeks of the LFn3 relative to the LF and HF.

## **V. Conclusion**

In conclusion, the results of this study indicate that urinary sex hormone metabolism was modestly altered in postmenopausal women by a low fat dietary intervention alone or with additional n-3 fatty acids. Our results support previous observations that a low fat diet intervention is accompanied by increased urinary estrogen excretion. However, in light of the paucity of change in the overall pattern of excretion of urinary hormones, it is unknown what effect this change would have on breast cancer risk.



**Table 3-1: Characteristics of the study subjects at screening\*.**

Age (years)	56 ± 6
Body weight (kg)	74 ± 11
Height (cm)	165 ± 5
BMI (kg/m <sup>2</sup> )	27 ± 4
Systolic blood pressure (mmHg)	118 ± 15
Diastolic blood pressure (mmHg)	71 ± 8
FSH (mIU/mL) <sup>†</sup>	73 ± 22
Ethnicity (n (%))	
White	14 (88)
Hispanic	1 (6)
African-American	1 (6)

\*All values are means ± standard deviations except ethnic groups, which are n (%); n = 16.

<sup>†</sup>Screening FSH values for all women ≤ 55 years old; n = 11

**Table 3-2: Baseline and 8 weeks concentrations of urinary estrogens and metabolites for the HF, LF and LFn3\*.**

	HF n = 15 <sup>†</sup>	LF n = 15 <sup>‡</sup>	LFn3 n = 16	p-value <sup>§</sup>
<b>Estrone (E1) (nmol/day)</b>				
Baseline	6.7 (4.8, 9.5)	7.2 (5.1, 10.1)	8.1 (5.8, 11.5)	0.36
8 weeks	6.3 (4.4, 9.1) <sup>a</sup>	9.9 (6.9, 14.2) <sup>b**</sup>	8.5 (5.9, 12.1) <sup>b</sup>	0.004
p-value <sup>¶</sup>	0.39	0.02	0.92	
<b>Estradiol (E2) (nmol/day)</b>				
Baseline	2.0 (1.0, 3.8)	2.2 (1.2, 4.3)	1.4 (0.7, 2.6)	0.36
8 weeks	1.0 (0.5, 2.1)	2.1 (1.0, 4.4)	1.7 (0.8, 3.4)	0.12
p-value <sup>¶</sup>	0.37	0.47	0.83	
<b>Estriol (E3) (nmol/day)</b>				
Baseline	7.2 (3.1, 16.8)	8.5 (3.6, 19.9)	10.8 (4.7, 25.0)	0.62
8 weeks	11.8 (6.3, 22.0)	14.3 (7.7, 26.7)	11.7 (6.3, 21.5)	0.79
p-value <sup>¶</sup>	0.84	0.12	0.64	
<b>E1 + E2 + E3 (nmol/day)</b>				
Baseline	22.9 (15.3, 34.4)	24.8 (16.6, 37.1)	23.0 (15.4, 34.2)	0.77
8 weeks	21.5 (14.3, 32.2) <sup>a</sup>	35.7 (23.9, 53.4) <sup>b**</sup>	26.8 (18.1, 39.8) <sup>ab</sup>	0.03
p-value <sup>¶</sup>	0.51	0.02	0.60	
<b>2-hydroxyestrone (2OH-E1) (nmol/day)</b>				
Baseline	22.7 (16.5, 31.2) <sup>ab</sup>	25.0 (18.2, 34.4) <sup>a</sup>	18.5 (13.5, 25.3) <sup>b</sup>	0.02
8 weeks <sup>††</sup>	17.6 (13.7, 22.7)	20.4 (15.8, 26.3)	24.1 (18.8, 30.9)	0.23
p-value <sup>¶</sup>	0.19	0.81	0.29	
<b>2-hydroxyestradiol (2OH-E2) (nmol/day)</b>				
Baseline	1.6 (0.6, 4.5)	2.2 (0.8, 5.9)	3.1 (1.2, 8.3)	0.57
8 weeks	2.4 (1.0, 5.8)	2.6, (1.1, 6.4)	2.7 (1.2, 6.5)	0.96
p-value <sup>¶</sup>	0.38	0.60	0.32	
<b>2-hydroxy estrogens (2OHE) <sup>‡‡</sup> (nmol/day)</b>				

Baseline	28.7 (20.5, 40.0)	32.7 (23.4, 45.7)	27.3 (19.6, 37.9)	0.46
8 weeks	23.9 (16.6, 34.3)	31.2 (21.7, 44.8)	28.4 (19.9, 40.6)	0.21
p-value <sup>¶</sup>	0.91	0.79	0.91	
2-methoxyestrone (2-ME1)(nmol/day)				
Baseline	1.6 (0.7, 3.8)	3.4 (1.5, 7.8)	3.1 (1.4, 7.1)	0.21
8 weeks	1.4 (0.7, 3.1)	2.1 (0.9, 4.5)	3.3 (1.5, 7.1)	0.23
p-value <sup>¶</sup>	0.98	0.48	0.91	
2-methoxyestradiol (2-ME2) (nmol/day)				
Baseline	1.3 (0.6, 3.3)	3.9 (1.6, 9.4)	1.7 (0.7, 3.9)	0.09
8 weeks	0.9 (0.4, 2.1)	1.0 (0.4, 2.3)	1.7 (0.8, 4.0)	0.39
p-value <sup>¶</sup>	0.84	0.08	0.57	
4-methoxyestrone (4-ME1) (nmol/day)				
Baseline	0.34 (0.15, 0.77)	0.74 (0.32, 1.70)	0.70 (0.31, 1.58)	0.28
8 weeks	0.38 (0.15, 0.93)	0.40 (0.16, 0.98)	0.65 (0.27, 1.56)	0.51
p-value <sup>¶</sup>	0.19	0.65	0.61	
4-methoxyestradiol (4-ME2) (nmol/day)				
Baseline	0.41 (0.17, 0.99)	0.90 (0.37, 2.21)	0.78 (0.33, 1.86)	0.30
8 weeks	0.62 (0.27, 1.42)	0.69 (0.30, 1.60)	0.94 (0.42, 2.12)	0.73
p-value <sup>¶</sup>	0.58	0.13	0.84	
4-hydroxyestrone (4OH-E1) (nmol/day)				
Baseline	0.78 (0.51, 1.21)	1.08 (0.70, 1.68)	1.17 (0.76, 1.80)	0.21
8 weeks	0.92 (0.66, 1.27)	1.05 (0.76, 1.45)	1.28 (0.93, 1.75)	0.25
p-value <sup>¶</sup>	0.64	0.93	0.50	
16 $\alpha$ -hydroxyestrone (16 $\alpha$ OH-E1) (nmol/day)				
Baseline	2.03 (1.10, 3.75)	2.39 (1.29, 4.44)	1.70 (0.93, 3.11)	0.62
8 weeks	1.50 (0.82, 2.74)	2.34 (1.27, 4.29)	2.03 (1.12, 3.65)	0.52
p-value <sup>¶</sup>	0.47	0.22	0.32	

Total Estrogens (nmol/day)

Baseline	73.7 (52.7, 100.1)	83.7 (60.9, 115.0)	72.8 (53.1, 99.6)	0.38
8 weeks	65.5 (45.8, 93.8)	86.4 (60.7, 123.0)	80.6 (57.0, 113.9)	0.27
p-value <sup>¶</sup>	0.78	0.54	0.40	

2:16 $\alpha$ OH-E1 ratio (nmol/day)

Baseline	11.3 (5.4, 23.6)	10.5 (5.0, 21.9)	10.8 (5.3, 22.4)	0.98
8 weeks	11.9 (5.7, 25.2)	10.0 (4.7, 21.3)	10.4 (5.0, 21.7)	0.90
p-value <sup>¶</sup>	0.90	0.12	0.72	

2OHE<sup>‡‡</sup>:16 $\alpha$ OH-E1 ratio (nmol/day)

Baseline	14.3 (6.7, 30.5)	13.6 (6.4, 29.1)	16.0 (7.6, 33.6)	0.93
8 weeks	15.9 (7.8, 32.5)	13.7 (6.7, 28.0)	14.0 (7.0, 28.2)	0.91
p-value <sup>¶</sup>	0.92	0.46	0.28	

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\*All values are geometric means (95% confidence interval).

<sup>†</sup>One subject was missing urines for HF diet, thus n = 15 for HF.

<sup>‡</sup>One subject was missing urines for LF diet, thus n = 15 for LF.

<sup>§</sup>p-value for effect of treatment across the three diets; values with differing letters as a superscript are significantly different.

<sup>¶</sup>p-value for paired t-test comparing baseline and 8 weeks means within each diet.

\*\*8 weeks mean is significantly different from baseline mean.

<sup>††</sup>8 weeks means were adjusted for baseline 2OH-E1.

<sup>‡‡</sup>2OHE = 2OH-E1 + 2OH-E2.

**Chapter 4:**  
**Low Fat Diet with Omega-3 Fatty Acids Increases Plasma Insulin-like Growth  
Factor Concentration in Healthy Postmenopausal Women**

The insulin-like growth factor pathway plays a central role in the normal and abnormal growth of tissues; however, the nutritional determinants of insulin-like growth factor I (IGF-I) and its binding proteins in normal individuals are not well-defined. The purpose of this study was to determine the effects of three test diets with varying types and amounts of dietary fat on circulating IGF-I, IGF binding protein-3 (IGFBP-3), insulin and glucose concentrations. Three test diets: high fat diet (HF; 40% energy as fat), low fat diet (LF; 20% energy as fat) and low fat, high omega-3 fatty acids diet (LFn3; 23% energy as fat) were tested in a randomized cross-over designed controlled dietary feeding trial in healthy postmenopausal women. Plasma IGF-I, IGFBP-3, insulin, glucose and ratio of IGF-I:IGFBP-3 concentrations were measured in response to the diets. Insulin sensitivity was calculated using the homeostatic model assessment of insulin resistance (HOMA-IR). 8 weeks of the LFn3 diet increased circulating IGF-I ( $P < 0.001$ ) and IGFBP-3 ( $P = 0.01$ ) and the LF diet increased IGFBP-3 ( $P = 0.04$ ) resulting in trends towards an increased IGF-I:IGFBP-3 ratio with the LFn3 diet and a decreased IGF-I:IGFBP-3 ratio with the LF diet ( $P = 0.13$  for both comparisons). Insulin, glucose and HOMA-IR were not altered by the interventions. Low fat diet with a high percentage of n-3 fatty acids may increase circulating IGF-I concentrations without adversely affecting insulin sensitivity in healthy individuals.

## **I. Introduction**

Insulin-like growth factor I (IGF-I) is a peptide hormone predominantly secreted by the liver in response to pituitary-derived growth hormone (GH) (190). IGF-I is generated to a lesser degree in peripheral tissues and acts in an autocrine and paracrine fashion in these tissues (191). In the blood, approximately 90% of IGF-I is complexed with insulin-like growth factor binding protein-3 (IGFBP-3; one of six IGF binding proteins) and acid labile subunit in a 1:1:1 ratio which increases the half-life of IGF-I (192). IGF-I is involved in both the normal and neoplastic growth of tissues via mediation of cell proliferation, cell cycle progression and programmed cell death (190, 191, 199) and inhibition of the IGF signaling pathway is a target for cancer therapy (207). In contrast, aging is associated with decreased GH and IGF-I concentrations as well as decreased bone mineral density and lean body tissue and increased adiposity along with a higher risk vascular profile associated with increased cardiovascular mortality and morbidity (272).

IGF-I levels are markedly reduced with malnutrition (374, 375), protein and calorie restriction (193) and in cancer cachexia (194), however the nutritional determinants of IGF-I and its binding proteins are less well-defined in normal, adequately-fed individuals. Cross-sectional studies have shown associations between concentrations of IGF-I and IGFBP-3 and dietary fat intake as assessed by food frequency questionnaire (242, 290, 376), although all studies did not report an association (287-289). Vegans who reportedly consumed significantly more polyunsaturated fat than meat-eaters and vegetarians had reduced concentrations of IGF-I (377), while intake of n-3 fatty acids was associated with increased concentrations of IGFBP-3 (242).

Insulin modulates the bioavailability of IGF-1 by reducing levels of IGF binding proteins, modulating GH receptor density on liver cells, and stimulating hepatic IGF-1 synthesis (192, 378). High total fat intake was positively associated with fasting insulin concentrations (298, 299) and negatively associated with insulin sensitivity (300) in cross-sectional studies in non-diabetic individuals. Low fat/high carbohydrate diets improved insulin sensitivity (301-303) and fasting insulin (181, 301, 304) concentrations

in several intervention studies relative to a high fat (302, 303) or habitual (181, 301, 304) diet in healthy individuals.

We previously reported the effects of three test diets with varying amounts and types of dietary fat on circulating sex hormones (Orr et al, in review). The purpose of the current study was to determine the effects of these diets on components of the IGF pathway in healthy postmenopausal women. Circulating levels of IGF-I, IGFBP-3, insulin and glucose were measured in response to the diets. Insulin sensitivity was assessed using the homeostatic model assessment of insulin resistance (HOMA-IR) (379, 380).

## **II. Subjects and Methods**

### **A. Experimental Protocol**

This study is part of a trial that was designed to determine the effect of three test diets with varying amounts of dietary fat and n-3 fatty acids on plasma sex hormone profile and urinary eicosanoids in postmenopausal women [Orr et al, in review]. Controlled high fat (45% energy from fat; HF), low fat (20% energy from fat; LF) and low fat, high n-3 fatty acids (23% energy from fat; 3% of energy from n-3 fatty acids; LFn3) diets were provided to subjects in a randomized, cross-over fashion. Each diet was provided for 8 weeks with a washout period of 6-12 weeks between diets. During the washout periods, the subjects consumed their habitual diets. During the intervention periods, study participants picked up packaged study meals (breakfast, lunch, dinner, and a snack) that were prepared in the metabolic kitchen of the University of Minnesota General Clinical Research Center. Meals were bagged for pick-up each day on weekdays and for 3-day weekend pick-up. Subjects recorded any foods that were consumed in addition to the study meals and any foods from the prepared study meals that were not consumed on a daily compliance questionnaire that was monitored by the study staff. At meal pick-up times subjects also recorded their daily weight.

The University of Minnesota Committee for the Use of Human Subjects in Research and the U.S. Army Medical Research and Materiel Command's Human



Subjects Research Review Board approved the protocol for the study. All participants gave written informed consent prior to enrollment in the study.

## **B. Subjects**

Postmenopausal women were recruited from Minneapolis/St Paul, MN and the surrounding area. Details on study recruitment were detailed previously [Orr et al, in review]. Participants were 45-70 years old and postmenopausal ( $\geq 1$  year since last menstrual period plus follicle stimulating hormone concentration  $\geq 23$  IU/L at screening or  $\geq 55$  years old); had a body mass index of 19-32 kg/m<sup>2</sup> with no significant weight change in the 6 months prior to study participation; were willing to refrain from taking non-steroidal anti-inflammatory drugs and aspirin during the course of the study; and had not taken hormone replacement therapy or fish oil supplements for 2 months prior to study enrollment. Potential participants were excluded if they were current smokers, had hormone-related cancer in the past, used non-steroidal anti-inflammatory drugs or prescription medication (excluding high blood pressure medication), had a bilateral oophorectomy (an exclusion criteria pertaining to the larger study on circulating sex hormones in the same study subjects) or had diagnosed chronic concurrent disease (e.g. diabetes mellitus, inflammatory disease). A medical history screening questionnaire was used to exclude participants with known disease and those taking medications prohibited by the study protocol.

A total of 17 subjects completed all aspects of the study. An additional subject completed two diet treatments (missing LF diet period). Following biological sample processing, one subject was excluded from the analysis due to multiple fasting glucose measurements  $\geq 200$  mg/dL (indicating the presence of Type II diabetes). One subject was excluded from the analysis for the LFn3 diet (missing samples). One subject was excluded from the LFn3 diet analysis for insulin and HOMA-IR due to an inexplicably high insulin value ( $> 5$  standard deviations above the mean) at week 8.

### **C. Dietary Treatments**

The three test diets have been described in detail previously [Orr et al, in review]. Briefly, the three diets were isoenergetic high fat (HF; 40% energy from fat, 15% energy from protein, 45% energy from carbohydrate), low fat (LF; 20% energy from fat, 15% energy from protein, and 65% energy from carbohydrate) and low fat, high n-3 (LFn3; 23% energy from fat, 15% energy from protein, and 62% energy from carbohydrate) diets prepared from common, commercially available foods. The HF and LF diets contained minimal n-3 fatty acids and had similar proportions of saturated to monounsaturated to polyunsaturated fatty acids (1:1:1). The LFn3 diet included 3% of energy from n-3 fatty acids in foods naturally rich in these fats (salmon, flax seed oil and walnuts). Nutrients in the test diets were determined using nutrient analysis software (Nutritionist V).

The Harris-Benedict equation [ $655.1 + 9.56 \times \text{weight (kg)} + 1.85 \times \text{height (cm)} - 4.68 \times \text{age (y)}$ ] multiplied by an activity factor (1.4 – 1.7, mean 1.6) was used to predict an energy level appropriate for weight maintenance for each subject. The Registered Dietitian made a clinical assessment of activity level based on reported work and exercise habits (381). Total energy of the diets was increased or decreased by 840 kJ (200 kcal) if a subject's weight fluctuated by 1.0 kg. Deviation from the study diets was calculated from the daily compliance questionnaires and was reported previously [Orr et al, in review].

### **D. Plasma Collection and Analysis**

Blood was drawn from fasting subjects between 7am and 10am at 0 and 8 weeks of each experimental diet period. Blood samples were centrifuged and plasma was flash frozen on dry ice and stored in 1mL aliquots at -80°C until analysis. Samples were assayed for IGF-I and IGFBP-3 using enzyme-linked immunosorbent assay kits (Diagnostics Systems Laboratories, Austin, TX) and samples were assayed for insulin by enzyme-linked immunosorbent assay kits (IBL America, Minneapolis, MN). Glucose was assayed using colorimetric assay kits (Cayman Chemical Company, Ann

Arbor, MI). All samples from a given subject were assayed in the same batch. Intra-assay coefficients of variations were 5.9% for IGF-I, 3.3% for IGFBP-3, 4.3% for insulin, and 0.5% for glucose. Inter-batch variation was assessed using an internal control blood sample, and inter-batch coefficients of variation were 14.0% for IGF-I, 12.7% for IGFBP-3, 7.0% for insulin, and 7.4% for glucose. IGF-I:IGFBP-3 molar ratio was calculated as  $(0.130 \times \text{IGF-I concentration [ng/mL]}) / (0.036 \times \text{IGFBP-3 concentration [ng/mL]})$  (382). HOMA-IR, calculated as  $(\text{fasting plasma glucose [mmol/L]} \times \text{fasting plasma insulin [mU/L]}) / 22.5$ , was used as a measure of insulin resistance (379, 380).

#### **E. Statistical Analysis**

A general linear mixed model (SAS Proc Mixed, SAS® 9.2, Cary, NC: SAS Institute Inc., 2002-2008) was fit to the data for each of the primary endpoints. The correlation of multiple measurements within each subject was accounted for by use of the random effect option for subject. The resulting model allowed for missing values and for monitoring of carryover and period effects. The randomization groups were unbalanced. Least squares means generated from the mixed model were the basis of pairwise comparisons between diets. The baseline and 8 week means were compared within diet treatments using paired t-tests. A *P*-value of < 0.05 denotes statistical significance.

### **III. Results**

Baseline reported dietary intake and characteristics of the study subjects were reported previously [Orr et al, in review]. At the study baseline, the subjects were  $57 \pm 6$  years of age with a body mass index of  $28 \pm 4 \text{ kg/m}^2$ . The study diets were well-tolerated by the study subjects and reported deviation from the treatment diets was estimated from the daily compliance questionnaires to be less than 1% of total calories and less than 0.5% for n-3 intake, as reported previously. Baseline body weight did not differ among the three diets (*P* = 0.83) although mean body weight decreased

significantly from baseline within all diets (-1.1 kg with the HF,  $P < 0.001$ ; -1.3 kg with the LF,  $P < 0.0001$ ; and -0.7 kg with the LFn3,  $P = 0.02$ ) although weights did not differ among the diets at 8 weeks ( $P = 0.46$ ).

Consumption of the LFn3 resulted in increased IGF-I ( $P < 0.001$ , Table 4-1) and IGFBP-3 ( $P = 0.01$ ) while consumption of the LF increased IGFBP-3 only ( $P = 0.04$ ). Because of these changes, the ratio of IGF-I:IGFBP-3 increased with the LFn3 and decreased with the LF, although the changes in the ratio were not statistically significant ( $P = 0.13$  for both comparisons). Of note, the concentration of IGF-I at baseline with the LFn3 diet was non-significantly reduced ( $P = 0.24$ ) relative to the other two diets, therefore this may have contributed to the magnitude of the increase observed. No changes within or among the diets were observed with insulin, glucose, or HOMA-IR.

#### **IV. Discussion**

The results of this well-controlled dietary feeding study in healthy, non-diabetic postmenopausal women indicate that circulating IGF-I and IGFBP-3 concentrations were both increased by a diet low in fat containing 3% of energy from omega-3 fatty acids, resulting in a trend towards increased IGF-I:IGFBP-3 ratio. As both IGF-I and IGFBP-3 are GH-dependent, these results may indicate a greater effect of the LFn3 diet compared to the LF diet on growth hormone levels. Few data are available regarding the effects of n-3 fatty acids on circulating IGF-I concentrations, however a cross-sectional study in men and women in Singapore reported an association between increased IGFBP-3 and high intake of n-3 fatty acids (242).

The effects of a low fat, high fiber diet on serum IGF-I, IGFBP-3, insulin, and glucose were the focus of an ancillary study involving 750 subjects who participated in the Polyp Prevention Trial (PPT) (294). The investigators observed no significant differences in IGF-I, IGFBP-3, insulin or glucose concentrations at one year or four years follow-up in the intervention group compared to baseline, or the control group, although the intervention group achieved self-reported reductions in dietary fat from 35% of energy to 22.7% of energy and an increase in fiber intake from 18.9 g/day to

33.6 g/day at four years. The diets in the current study were controlled and not self-reported, therefore the results are not likely affected by reporting errors. There was also no change in IGF-I, IGFBP-1 and IGFBP-3 after a 12-month low fat/high fiber dietary intervention in premenopausal women conducted by Gann et al (295). Neither the PPT authors nor Gann et al addressed n-3 fatty acid composition of the diets. Our low fat dietary intervention was much shorter in duration than both the PPT and Gann et al trial and it is unknown whether the increase in IGFBP-3 that we observed with the LF diet or the increases in IGF-I and IGFBP-3 that we observed with the LFn3 diet are transitory changes or whether concentrations could be altered long-term by a diet low in total fat and high in n-3 fatty acids. This point deserves further investigation in a longer-term trial.

There was no indication that insulin sensitivity was altered by the test diets in this study, although the study subjects were healthy, non-diabetic individuals. However, insulin sensitivity-related endpoints did change following the multifactor intervention of the Diet and Androgens (DIANA) Randomized Trial (292). The subjects in the DIANA trial intervention group were instructed to alter a number of dietary factors simultaneously and follow a Mediterranean/macrobiotic type diet, which among other aims, intended to reduce animal fat intake and increase n-3 fatty acids intake. Following 4 months of the intervention, the subjects reported decreased intake of animal protein, total fat (reduced from 37.1 to 30.8% of energy), animal fat, saturated fat, monounsaturated fat, cholesterol, and total energy and increased intake of vegetable protein, vegetable fat, polyunsaturated fat, and carbohydrates (183). While IGF-I and IGFBP-3 concentrations were not altered in the intervention group, concentrations of IGFBP-1 and -2 were significantly increased, and C-peptide and fasting glucose decreased significantly. There was no change in fasting insulin with the intervention, although area under the curve of insulin during the oral glucose tolerance test was significantly reduced in the intervention group (292). Importantly, there was also a significant deficit in energy intake in the DIANA trial intervention group and significant changes attributed to the intervention were attenuated when adjustments for weight and

waist circumference were applied. Therefore the effects observed with the intervention may be due more to weight loss and the accompanying body composition changes than to the change in dietary composition. Our subjects lost a small but statistically significant amount of weight with each diet treatment, however the amount of weight lost and mean weights at 8 weeks of each treatment did not differ.

IGF-I concentrations decrease with protein and calorie restriction and with malnutrition (285). Increasing protein intake in adequately-fed individuals may also affect IGF-I and IGF binding protein concentrations. Addition of a soy protein supplement to a low fat/high fiber dietary intervention or usual diet reduced IGFBP-3 concentrations and increased IGF-I:IGFBP-3 ratio in premenopausal women (295). In post-menopausal women, addition of either a 40g/day milk protein or soy protein supplement to their usual diets increased circulating IGF-I concentrations, although the women concomitantly reduced their protein intake from other sources and ended the 3-month study with a similar total protein intake to baseline (383). Similarly, IGF-I concentrations were significantly increased by consumption of soy protein supplements in healthy men (384) and prostate cancer patients (385). In the present study, the diets were designed so that the percentage of energy from protein was equivalent (15% of energy) among the three test diets, therefore the significant changes that we observed in IGF-I (LFn3) and IGFBP-3 (LF and LFn3) were not due to differential protein intake.

IGF-I concentrations decrease with age, and the effects of aging (decreased bone mineral density and lean body tissue, increased fat mass, and a high-risk cardiovascular profile) mirror the symptoms of GH deficiency (272). Increasing the concentration of IGF-I with pharmacological GH replacement is accompanied by a number of symptoms related to water retention (274), and therefore the possibility of raising IGF-I concentrations with a dietary intervention is desirable. Further studies employing the addition of n-3 fatty acids to the diet and assessment of IGF-I concentrations and effects on the symptoms of aging are warranted.

On the other hand, there have been reports of associations between IGF-I and breast cancer risk. This association appears to depend on menopausal status, with most

studies showing no association between relatively increased IGF-I concentrations and breast cancer risk in postmenopausal women (203). Current data do not suggest that the increase in IGF-I with the LFn3 diet in our postmenopausal female study participants is likely to increase risk of breast cancer.

The results of this trial should be regarded with caution due to the small sample size. Even though our study was a cross-over trial, imparting stronger statistical power than if each subject had completed only one dietary treatment, the sample size is still regarded as being relatively small and differences in the endpoints measured may not have been detected because of the high variability and small sample size.

## **V. Conclusion**

The results of this controlled dietary intervention in postmenopausal women indicate that the addition of n-3 fatty acids to a low fat diet increases IGF-I and IGFBP-3 concentrations while low fat diet with no additional n-3 fatty acids increases IGFBP-3 only. The effects of increasing IGF-I concentrations on cancer risk is unknown, however, an increase in circulating IGF-I may be beneficial in preventing the reduced bone and lean mass associated with aging.

**Table 4-1: Baseline and 8 weeks values for plasma glucose, insulin, IGF-I, IGFBP-3, HOMA-IR, and IGF-I:IGFBP-3 ratio for the HF, LF, and LFn3<sup>1</sup>.**

	HF n = 17	LF n = 16	LFn3 <sup>2</sup> n = 16	P-value <sup>3</sup>
Glucose (mg/dL)				
Baseline	107.7 ± 5.4	107.3 ± 5.5	104.1 ± 5.5	0.43
8 weeks	107.2 ± 4.3	102.1 ± 4.5	109.0 ± 4.5	0.47
P-value <sup>4</sup>	0.92	0.20	0.36	
Insulin (mIU/L)				
Baseline	10.6 ± 1.0	11.0 ± 1.0	10.2 ± 1.0	0.56
8 weeks	10.4 ± 1.1	10.6 ± 1.1	11.0 ± 1.1	0.60
P-value <sup>4</sup>	0.83	0.66	0.31	
IGF-I (ng/mL)				
Baseline	151.1 ± 11.3	150.7 ± 11.5	136.9 ± 11.5	0.24
8 weeks	148.5 ± 12.2	155.9 ± 12.4	164.5 ± 12.4*	0.10
P-value <sup>4</sup>	0.74	0.61	< 0.001	
IGFBP-3 (ng/mL)				
Baseline	5084 ± 400	4641 ± 415	5097 ± 415	0.60
8 weeks	5362 ± 428	4990 ± 445*	5553 ± 444*	0.60
P-value <sup>4</sup>	0.10	0.04	0.01	
HOMA-IR				
Baseline	2.9 ± 0.4	3.1 ± 0.4	2.8 ± 0.4	0.42
8 weeks	2.8 ± 0.3	2.7 ± 0.3	3.1 ± 0.3	0.26
P-value <sup>4</sup>	0.66	0.19	0.31	
IGF-I:IGFBP-3 ratio				
Baseline	0.120 ± 0.016	0.129 ± 0.017	0.112 ± 0.017	0.42
8 weeks	0.111 ± 0.016	0.119 ± 0.016	0.123 ± 0.016	0.74
P-value <sup>4</sup>	0.20	0.13	0.13	

<sup>1</sup>All values are LS means ± standard errors.



<sup>2</sup>One subject was excluded from insulin and HOMA-IR analysis in the LFn3, thus n=15 for these endpoints.

<sup>3</sup>*P*-value for effect of treatment across the three diets. Values with differing letters as a superscript are significantly different.

<sup>4</sup>*P*-value for paired t-test comparing baseline and 8 weeks means within each diet.

\*8 weeks mean is significantly different from baseline mean.

**Chapter 5:**  
**Dissertation Summary**

## Dissertation Summary

The purpose of this dissertation research was to determine the effects of altering amount and type of dietary fat on markers of breast cancer risk in healthy postmenopausal women. The greatest effects on reduction of breast cancer risk markers were anticipated with the LFn3 diet.

High fat diet significantly increased excretion of urinary eicosanoids and increased plasma E2. Effect of the LF and LFn3 were more modest. The LFn3 diet significantly increased PLFA n-3 concentrations, although the expected concomitant magnitude of reduction in plasma estrogens was not observed. The LFn3 diet did not alter E2 in a meaningful way although there was a trend towards decreased in E1. Urinary excretion of E1 was significantly increased by both the LF diet and the LFn3 diet relative to the HF, although in the context of no significant changes in any of the other urinary endpoints or in total estrogen excretion, this change is likely not clinically significant for the reduction of breast cancer risk. Therefore, the HF diet appears to have increased breast cancer risk markers, while the results are less conclusive for the LF diet and the LFn3 diet.

The analysis of the effects of the three test diets on endpoints related to the IGF-I pathway was largely exploratory, and a surprising result was that the LFn3 diet increased plasma IGF-I and IGFBP-3 with a resulting trend for increased IGF-I:IGFBP-3 ratio. The LF diet also increased IGFBP-3 but a trend for decreased IGF-I:IGFBP-3 ratio resulted. The three test diets had little effect on insulin sensitivity endpoints in the healthy, non-diabetic postmenopausal women in the trial. The effect of increased IGF-I in our study subject is unknown in relation to breast cancer risk because there is little evidence in the epidemiological literature that increased concentrations of IGF-I are associated with breast cancer risk; however increased IGF-I may be useful in reversing or preventing the effects of aging in postmenopausal women, including loss of bone mineral density and lean muscle mass.

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**Appendix 1:**  
**Menus**

**Menu 2000 Kcal - Day 1**

<i>Menu</i>	<i>HF</i>	<i>LF</i>	<i>LFn3</i>
<b>Breakfast</b>			
Grapefruit Juice	130g	200 g	200 g
Cheerios	40g	32g	35g
w/ Sugar	0g	6g	6g
Bran Muffin	75g (HF)	60g (LF)	60g (LF)
w/ Butter	10g	4g	4g
Skim Milk	240g	250g	230g
<b>Lunch:</b>			
Turkey Sandwich			
Whole Wheat Bread	60g	60g	60g
Turkey Breast	70g	55g	45g
Butter	6g	0	5g
Mayonnaise, Regular	10g	6g	0g
Miracle Whip	0	10g (FF)	10g (FF)
Egg yolk	2g	5g	4 g
Flax oil	0g	0	3g
Lettuce	20 g	20 g	20 g
Tomato	50 g	50 g	50 g
Pretzels	0	25g	0
Potato Chips	35g	0	0
Applesauce	140g	180g	200g
<b>Dinner:</b>			
Orange Roughy	140g	90g	0
Baked Salmon	0	0	75g
w/Cajun Spice/Lemon Juice	Dash/5g	Dash/5g	Dash/5g
White Rice	140g	110g	110g
Broccoli	80g	80g	80g
Lettuce/Carrot Salad	50g/15g	50g/15g	50g/15g
Fat Free French Dressing	15g	20g	15g
w/Corn Oil	7g	5g	0
w/Flax Oil	0	0	5g
Whole Wheat Bread	26g	40g	40g
w/Butter	6g	6g	0
Angel Food Cake	0	50g	50g
w/Strawberries/Sugar	120g/0	120g/6g	120g/6g
			0
<b>Snack</b>			
Wheat Thins	10g	20g (Regular)	30g (Reduced Fat)

Mozzarella Cheese	0	30g	40g
Cheddar Cheese	20g	0	0
Gumdrops	0	30g	15g
Grape Juice	0	120g	0

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***Nutrient Composition (Calculated)***

<b><i>Kcal</i></b>	2020	2011	2015
<b><i>CHO (g/%)</i></b>	233g/45%	329g/65%	317g/62%
<b><i>Pro (g/%)</i></b>	77g/15%	76g/15%	77g/15%
<b><i>Fat (g/%)</i></b>	92g/40%	46g/20%	54g/23%
<b><i>Fiber (g)</i></b>	22.8g	23.9g	25.2g
<b><i>Chol (mg)</i></b>	209mg	203mg	210mg
<b><i>SFA (g)</i></b>	28.2g	13.4g	14.8g
<b><i>MUFA (g)</i></b>	28.7g	13.9g	16.2g
<b><i>PUFA (g)</i></b>	28.0g	13.3g	16.1g
<b><i>18:3 (g)</i></b>	1.40g	0.85g	5.36g
<b><i>20:5 (g)</i></b>	0g	0g	0.46g
<b><i>22:6 (g)</i></b>	0.03g	.03g	0.99g
<b><i>Omega 3 FA (g)</i></b>	1.43g	0.88g	6.82g

**Menu 2000 Kcal - Day 2**

<i>Menu</i>	<i>HF</i>	<i>LF</i>	<i>LFn3</i>
<b>Breakfast</b>			
Wheaties	25g	35g	45g
w/ Sugar	4g	6g	6g
Orange	100g	150g	150g
w/ Sugar	0	0	5g
Orange Muffin	55g (HF)	50g (LF)	50g (FLn3)
w/ Butter/Margarine	4g (M)	5g (B)	3g (B)
w/ Preserves	0	15g	0
Milk-Skim/2%	200g (2%)	200g (S)	240g (S)
<b>Lunch:</b>			
Chicken/Tuna Sandwich			
Whole Wheat Bread	50g	65g	65g
Lettuce/Tomato	20g/0	20g/50g	20g/50g
Cheddar Cheese`	15g	0	20g
Miracle Whip-Reg/FF	10g (Reg)	15g (FF)	15g (FF)
w/ Egg Yolk	0	4g	4g
w/ Flax Oil	0	0	4g
Chicken Breast/Tuna	60g (C)	70g (C)	70g (T)
Margarine	4g	0	0
Carrots	60g	60g	60g
Pineapple	100g	100g	100g
w/ Sugar	0	5g	5g
<b>Dinner:</b>			
Spaghetti with Meat Sauce			
Ragu Lite	110g	150g	150g
w/ Corn Oil	8g	0	0
Spaghetti	80g	130g	150g
Mushrooms	50g	50g	50g
Lean Ground Beef	65g	60g	40g
French Bread/White Bread	25g (W)	40g (F)	40g (F)
w/ Butter	7g	5g	0
Green Beans	100g	100g	100g
Lettuce Salad	70g	75g	75g
w/ Italian Dressing-Reg/FF	20g (Reg)	15g (FF)	15g (FF)
w/ Fat Free Italian Dressing	0	15g	15g
w/ Safflower Oil/Flax Oil	0	2g (S)	3g (F)
w/ Egg Yolk/Egg White	0	0	4.5g/15g

	Peaches	90g	100g	100g
	w/ Sugar	0	5g	5g
<b>Snack</b>	Blondies	50g (HF)	50g (LF)	50g (LFn3)
	Milk Chocolate Candy	15g	0	10g
	2% Milk	180g	0	0
	Gumdrops	0	35g	20g

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**Nutrient Composition (Calculated)**

<b>Kcal</b>	2026	2006	2036
<b>CHO</b>			
<b>(g/%)</b>	230g/45%	327g/65%	323g/62%
<b>Pro (g/%)</b>	77g/15%	77g/15%	76g/15%
<b>Fat (g/%)</b>	92g/40%	46g/20%	52g/23%
<b>Fiber (g)</b>	21.6g	27.2g	30.5g
<b>Chol (mg)</b>	204.1mg	201.3mg	213mg
<b>SFA (g)</b>	26.9g	12.4g	14.2g
<b>MUFA (g)</b>	30.4g	14.7g	14.4g
<b>PUFA (g)</b>	27.3g	12.99	14.2g
<b>18:3 (g)</b>	1.21g	.577g	6.786g
<b>20:5 (g)</b>	.006g	.008g	0.176g
<b>22:6 (g)</b>	.013g	.020g	.451g
<b>Omega 3 FA (g)</b>	1.23g	.605g	7.413g

**Menu 2000 Kcal - Day 3**

<i>Menu</i>	<i>HF</i>	<i>LF</i>	<i>LFn3</i>
<b>Breakfast</b>			
Pineapple Juice	120g	150g	140g
Wheat Chex	30g	30g	30g
w/ Sugar	0	4g	4g
WW Bread/WW Bagel	50g (Bread)	60g (Bagel)	60g (Bagel)
w/ Cream Cheese-Reg/Lite	30g (Reg)	20g (Lite)	30g (Lite)
Diet Jelly/Preserves	10g (J)	20g (P)	10g (P)
Milk-Skim/2%	120g (2%)	200g (S)	200g (S)
<b>Lunch:</b>			
Chicken/Salmon Pasta Salad			
Macaroni	80g	80g	80g
Chicken Breast/Salmon	80g (C)	55g (C)	65g (S)
Green Onion	10g	10g	10g
Diced Celery	10g	10g	10g
Peas	15g	15g	15g
Garlic Powder/Salt/Pepper	Dash	Dash	Dash
Lemon Juice	2g	2g	2g
Mayonnaise	25g	9g	0
Fat Free Miracle Whip	0	20g	30g
w/ Egg Yolk	0	7g	7g
Whole Wheat Bread	25g	60g	30g
w/ Butter/Flax Butter	10g (B)	4g (B)	5g (F)
Blueberries	100g	100g	100g
w/ Sugar	0	4g	4g
Oatmeal Walnut Cookie	30g	30g	30g
<b>Dinner:</b>			
Beef Tenderloin	65g	60g	75g
Mashed Potatoes	100g	140g	140g
w/ Butter/Olive Oil	5g/4g	0	0
Corn	80g	120g	120g
Whole Wheat Bread	25g	30g	30g
w/ Butter/Margarine	9g (B)	4g (M)	0
Lettuce Salad	60g	60g	60g
w/ Ranch Dressing-Reg/FF	15g (Reg)	15g (FF)	15g (FF)
w/ Corn Oil/Flaxseed Oil	0	3g (C)	3g (F)
Fruit Cocktail	90g	100g	100g
w/ Sugar	0	4g	0

Snack	Brownie	60g (HF)	60g (LF)	50g (LFn3)
	Raisins	0	25g	30g
<b><i>Nutrient Composition (Calculated)</i></b>				
<b><i>Kcal</i></b>		2010	2011	2009
<b><i>CHO (g/%)</i></b>		232g	334g/65%	313g/61%
<b><i>Pro (g/%)</i></b>		75g	77g/15%	79g/15%
<b><i>Fat (g/%)</i></b>		92g	47g/20%	54g/24%
<b><i>Fiber (g)</i></b>		21.2g	27.6g	27.4g
<b><i>Chol (mg)</i></b>		241.3mg	218.7mg	231.8mg
<b><i>SFA (g)</i></b>		28.5g	13.4g	15.2g
<b><i>MUFA (g)</i></b>		28.6g	14.98g	16.1g
<b><i>PUFA (g)</i></b>		27.8g	12.8g	17.5g
<b><i>18:3 (g)</i></b>		1.886g	1.172g	5.073g
<b><i>20:5 (g)</i></b>		.008g	.006g	.449g
<b><i>22:6 (g)</i></b>		.016g	.019g	.955g
<b><i>Omega 3 FA (g)</i></b>		3.311g	1.197g	6.472g



**Menu 2000 Kcal - Day 4**

<i>Menu</i>	<i>HF</i>	<i>LF</i>	<i>LFn3</i>
<b>Breakfast</b>			
Grape Juice	80g	100g	80g
Rice Krispies	25g	30g	30g
Whole Wheat English Muffin	40g	60g	60g
w/ Butter/Margarine	6g (B)	3g (B)	3g (B)/3g (M)
w/ Preserves	0	15g	15g
Skim Milk	240g	200g	200g
<b>Lunch:</b>			
Roast Beef Sandwich			
White Bread	50g	50g	50g
Roast Beef, Deli	40g	65g	65g
Mayonnaise, Reg	22g	6g	0
Fat Free Miracle Whip	0	10g	10g
Flax Butter	0	0	4g
American Cheese	30g	0	0
Lettuce	20g	20g	20g
Tomato	50g	50g	50g
Fritos/Pretzels	30g (F)	25g (P)	25g (P)
Peaches	90g	100g	100g
Blondies	0	55g (LF)	55g (LF)
<b>Dinner:</b>			
Fish/Salmon Cakes	180g (F)	200g (F)	200g (S)
w/ Sour Cream Sauce-Reg/Lite	25g (Reg)	20g (Lite)	20g (Lite)
w/ Dill/Salt/Pepper/Lemon	Dash	Dash	Dash
Brown Rice	110g	120g	100g
w/ Butter	3g	0	0
Carrots	100g	100g	100g
Lettuce Salad	70g	70g	70g
w/ Egg White	25g	0	0
w/ French Dressing-Low Cal/FF	15g (Low Cal)	15g (FF)	15g (FF)
w/ Corn Oil/ Flaxseed Oil	8g (C)	3g (C)	6g (F)
Whole Wheat Bread	25g	60g	60g
w/ Butter/Flax Butter	5g (B)	5g (B)	3g (F)
Pears	80g	120g	120g
<b>Snack</b>			
Skim Milk	240g	0	0
Yoplait Lite	0	190g	150g

Oatmeal Walnut Cookie	30g	0	0
Dates	0	40g	40g
<b><i>Nutrient Composition (Calculated)</i></b>			
<b><i>Kcal</i></b>	2003	2013	2041
<b><i>CHO (g%)</i></b>	229g/45%	330g/65%	319g/62%
<b><i>Pro (g%)</i></b>	73g/15%	73g/15%	73g/14%
<b><i>Fat (g%)</i></b>	91g/40%	46g/20%	54g/24%
<b><i>Fiber (g)</i></b>	17.7g	23.6g	23.2g
<b><i>Chol (mg)</i></b>	240.96mg	196.6mg	209.6mg
<b><i>SFA (g)</i></b>	28.5g	13.4g	14.3g
<b><i>MUFA (g)</i></b>	27.97g	14.9g	17.5g
<b><i>PUFA (g)</i></b>	28.7g	13.97g	18.4g
<b><i>18:3 (g)</i></b>	2.429g	.864g	5.749g
<b><i>20:5 (g)</i></b>	.002g	.002g	.338g
<b><i>22:6 (g)</i></b>	.008g	.008g	0.72
<b><i>Omega 3 FA (g)</i></b>	2.439g	.874g	6.807g

**Menu 2000 Kcal - Day 5**

<i>Menu</i>	<i>HF</i>	<i>LF</i>	<i>LFn3</i>
<b>Breakfast</b>			
Orange Juice	120g	220g	220g
Shredded Wheat	25g	35g	35g
w/ Sugar	0	6g	6g
Whole Wheat Bagel	60g	60g	60g
w/ Cream Cheese-Reg/FF	30g (Reg)	30g (FF)	30g (FF)
w/ Preserves	0	20g	20g
w/ Margarine	5g	0	0
Skim Milk	120g	200g	140g
<b>Lunch:</b>			
Chicken/Salmon Salad Sandwich			
Cracked Wheat Bread	50g	60g	60g
Chicken Breast/Salmon	60g (C)	45g (C)	60g (S)
Miracle Whip-Reg/FF	30g (Reg)	7g (Reg)/10g (FF)	10g (FF)
Egg Yolk/Flax Oil	0	7g (Yolk)	7g (Yolk)/4g (Oil)
Lettuce Leaf	20g	20g	20g
Carrot/Cucumber	60g/40g	60g/40g	60g/40g
Raspberries	80g	110g	110g
w/ Sugar	0	6g	6g
Brownie	50g (HF)	40g (LF)	40g (LF)
<b>Dinner:</b>			
Meatloaf	100g	80g	80g
w/ Fat Free Gravy	40g	40g	40g
w/ Safflower Oil/Flax Oil	7g (S)	3g (S)	3g (F)
Mashed Potato	100g	140g	140g
Green Beans	100g	100g	100g
Spinach Salad	70g	70g	70g
w/ Fat free Ranch	15g	15g	15g
w/ Safflower Oil/Flax Oil	7g (S)	4g (S)	3g (F)
Whole Wheat Roll	25g	45g	30g
w/ Butter	8g	0	0
Pineapple	80g	120g	120g
<b>Snack</b>			
Grape Juice	0	120g	120g
Low Fat Triscuits	25g	40g	40g
Mozzarella Cheese	40g	25g	30g

**Nutrient Composition (Calculated)**

<b>Kcal</b>	1979	2009	2000
<b>CHO</b>			
<b>(g/%)</b>	232g/45.5%	332g/64%	320g/62%
<b>Pro (g/%)</b>	76g/15%	80g/15%	77g/15%
<b>Fat (g/%)</b>	89g/39.5%	48g/21%	54g/23%
<b>Fiber (g)</b>	32.7g	40.6g	39.5g
<b>Chol (mg)</b>	198.7mg	198.4mg	198.1mg
<b>SFA (g)</b>	27.3g	13.5g	14.8g
<b>MUFA (g)</b>	27.4g	15.7g	18.0g
<b>PUFA (g)</b>	27.7g	14.3g	15.8g
<b>18:3 (g)</b>	1.327g	.778g	5.824g
<b>20:5 (g)</b>	.006g	.005g	.415g
<b>22:6 (g)</b>	.013g	.018g	.883g
<b>Omega 3 FA (g)</b>	1.346g	.963g	7.122g

**Appendix 2:**  
**Study Participant Recruitment, Screening and Education Materials**



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## Dietary Fat and Breast Cancer Risk Study

- Postmenopausal women, 45-70 years, are needed for a 1-year study of the effects of dietary fat on factors related to breast cancer risk.
- The Principal Investigator is Susan Raatz, PhD, RD, Assistant Professor of Medicine, University of Minnesota, 424 Harvard St SE, M251, Minneapolis, MN 55455

- 
- Healthy postmenopausal women, ages 45-70 years
  - Subjects should have had at least 1 year since their last menstrual period and not be using hormone replacement therapy
  - Non smokers
  - No prescription drug use
  - Not currently taking fish oil or flax oil supplements
  - Study will provide all meals for three 8-week periods
  - Test diets will vary in fat and omega-3 fatty acid content
  - You will be asked to provide blood and urine samples for analysis

University of Minnesota  
General Clinical Research Center  
Call 612-626-5159 for additional information

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## II. P914 Eligibility Screening Questionnaire

Name: \_\_\_\_\_

### **P914: Dietary Fat, Eicosanoids, and Breast Cancer Risk**

#### **Recruitment Screening**

We are evaluating the effect of dietary fat intake on breast cancer risk by evaluating its effect on sex hormone metabolism. The study is being conducted by Susan Raatz, PhD, RD at the University of Minnesota. All study visits will occur at the General Clinical Research Center.

At baseline, the beginning of the study, a screening visit will be required at the General Clinical Research Center. At this visit we will determine whether or not you are eligible for participation in the study. We will provide some questionnaires and review them with you after you fill them out. The questionnaires will provide information about your general health, average level of physical activity, typical dietary habits and the nutrients you consume. We do not require volunteers to meet any set standards, other than age, gender and postmenopausal status. However, there are factors that might disqualify your participation in the study. For example, certain medications might influence the nutrient metabolism we are attempting to study. We will also attempt to identify life-factors that could make compliance with the requirements of the study difficult for you. We appreciate your help reaching our research goals, and want you to enjoy the experience.

Once your eligibility in the study has been assured, you will be randomly chosen to receive one of three diets. The three diets will be similar in most regards, but there will be differences in the levels of dietary fat, and particularly the omega-3 fatty acid content. It is very important that you consume only the foods that we provide for you, with no other foods from outside of the study. You will be required to eat the foods we provide to you for eight consecutive weeks. During these 8 weeks you will need to come to the GCRC daily to pick up your food. After the 8-week period you can unwind from eating foods that have been chosen for you and go back to enjoying your usual diet for 8 weeks. You will repeat this cycle two (2) more times for a total of

3 times, with eight weeks of study diet alternating with eight weeks of your normal eating. At baseline we will ask you to come to the center two days in a row to provide blood and urine samples. We will require that you return to the General Research Center to repeat these measurements twice more, after 4 and 8 weeks time. We will use these samples to determine how certain chemical compounds are being created in your body as a result of the test diets.

We need to ask you some questions over the telephone to help determine your eligibility for participation.



914 Eligibility Screen

ELIGIBLE

NOT ELIGIBLE

Name: \_\_\_\_\_ Date: \_\_\_\_\_

**Inclusion Criteria**

If the answers to all of the following are Yes, the subject qualifies for participation.

YES      NO

\_\_\_\_      \_\_\_\_ 1. Age 45-70 years at the time of enrollment.

Date of Birth: \_\_\_\_\_

\_\_\_\_      \_\_\_\_ 2. Postmenopausal women who have had at least one year since their last menstrual cycle and not using hormone replacement therapy.

Date of Last Menstrual Cycle: \_\_\_\_\_

Menstrual Status:    Regular    Irregular    None

Menopausal

Menses Stopped:

Natural Menopause    Hysterectomy    Oophrectomy

Estrogen Replacement within past 3 months?    Yes    No

\_\_\_\_      \_\_\_\_ 3. To meet one of the following criteria

- BMI of 19-32 (ideal or overweight to obese)

Height \_\_\_\_\_ in/cm

Weight \_\_\_\_\_ lb/kg

BMI \_\_\_\_\_ (kg/m<sup>2</sup>)

\_\_\_\_      \_\_\_\_ 4. Stable Body Weight for Past 6 Months.

- \_\_\_\_\_ 5. Good Health
- \_\_\_\_\_ 6. Nonsmoker
- \_\_\_\_\_ 7. Willing to discontinue use of over-the-counter medications with anti-prostaglandin activity such as aspirin and ibuprofen or non-steroidal anti-inflammatory medications.
- \_\_\_\_\_ 8. Consumption of a "Typical" American diet with no unusual dietary practices such as compliance with a strict vegetarian diet.
- How often eat:
- Meat, Poultry, Fish or Eggs \_\_\_\_\_
- Milk and/or Dairy \_\_\_\_\_
- Fruit and Fruit Juice \_\_\_\_\_
- Vegetables \_\_\_\_\_
- Breads, Cereals, Pasta, Rice \_\_\_\_\_
- \_\_\_\_\_ 9. Willing to comply with study protocol.
- \_\_\_\_\_ 10. Planning to remain in the Minneapolis Area for the next year.

**EXCLUSION CRITERIA**

If the answers to any of the following are Yes, the subject does not qualify for participation.

YES            NO

- \_\_\_\_\_ 1. Active Medical Problem
- Medical Problems:

Medication Use:

Steroid Medications            Yes    No

Aspirin                                      Yes    No  
Ibuprofen/NSAID                        Yes    No  
Other Medication                        Yes    No

\*Thyroid replacement and high blood pressure meds ok\*

**ADDITIONAL INFORMATION (IF ELIGIBLE)**

Address:

Phone:(Hm)\_\_\_\_\_ (Ofc)\_\_\_\_\_ (Cell)\_\_\_\_\_

Fairview Med Rec # \_\_\_\_\_ Emergency Contact \_\_\_\_\_

SS# \_\_\_\_\_ Phone# \_\_\_\_\_

Marital Status: \_\_\_\_\_ Ethnicity: \_\_\_\_\_

**Screening Visit Date:** \_\_\_\_\_

**Baseline Visit 1:** \_\_\_\_\_ **2:** \_\_\_\_\_

**Diet 1: Week 4 Visit 1:** \_\_\_\_\_ **2:** \_\_\_\_\_

**Week 8 Visit 1:** \_\_\_\_\_ **2:** \_\_\_\_\_

**Diet 2: Week 4 Visit 1:** \_\_\_\_\_ **2:** \_\_\_\_\_

**Week 8 Visit 1:** \_\_\_\_\_ **2:** \_\_\_\_\_

**Diet 3: Week 4 Visit 1:** \_\_\_\_\_ **2:** \_\_\_\_\_

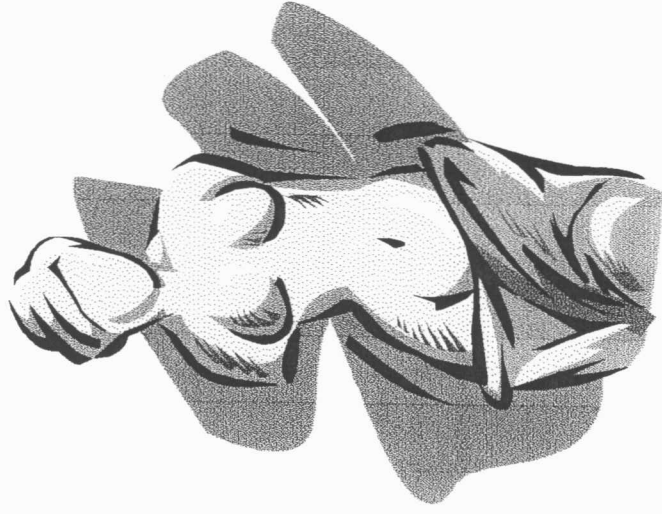
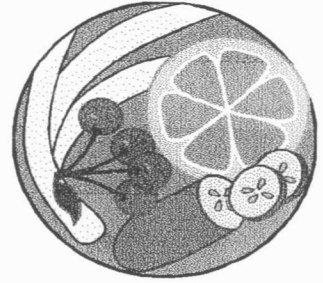
**Week 8 Visit 1:** \_\_\_\_\_ **2:** \_\_\_\_\_

## Meal Guidelines

- Plan to pick up your meals at the Clinical Research Center after 4:30 PM. Your dinner, evening snack, and the next day's breakfast and lunch will be bagged for you.
  - You must eat all of the food you get within the day it is planned for you.
  - Refrain from eating any foods other than those we provide for you. If you eat or drink anything extra, please be sure to tell us about it.
- You may have calorie-free beverages with or between meals: coffee, tea, mineral water, or diet soda. The milk planned with your meals may be used in coffee or tea - just don't add anything other than what we give you.
- We will adjust your calorie level if you are feeling hungry or are losing weight.
  - You will be weighed every day to evaluate if you are getting enough calories.
  - Do not add any condiments to your food items. You may add a small amount of salt and pepper or spices if you want to.
  - Be sure to eat all of the food you get - use your bread products to wipe your plate clean!

## University of Minnesota Clinical Research Center Feeding Study Handbook

- There is a small galley kitchen on the nursing station in which your bagged meals will be available for pick-up.
- In case of an emergency situation, we will make arrangements that will allow you to continue with study participation.
- On days before you come in for testing [blood drawing or other tests], please eat all of your food by 8 PM and then fast until you come into the Clinical Research Center in the morning.
- If you are ill for a day or two and cannot eat all of your food, please notify the research staff immediately.



## Dietary Fat and Breast Cancer Risk Study

## Volunteers Needed

Women between the ages of 45 and 70 who are not taking any medications or hormone replacement therapy are needed for our study. Participants must be willing to consume a variety of commonly consumed foods including fish, meats, and dairy. Please call the study coordinator at 612-626-5159 to discuss participation in this study.

## 212 Research Participation

First and foremost, we would like to thank you for your participation in our research project. The advancement of nutritional science is dependent on the participation of individuals such as you. Your participation in this feeding study will provide us with information that is useful in answering our scientific questions.

The results of this study may prove to be useful

## Contact People: Important Phone Numbers

### Study Coordinator:

Lindsay Orr, PhD Student 612-626-5159

### Investigators:

Susan Raatz, PhD, RD 612-624-6642

J Bruce Redmon, MD 612-626-1960

Mindy Kurzer, PhD 612-625-8412

General Clinical Research Center

Dietitian's Office 612-624-2922

Research Kitchen 612-624-9936

Nursing Station 612-624-0104

in the development of treatment strategies for disease states or for public health recommendations that will help to reduce the incidence of disease in the general population.

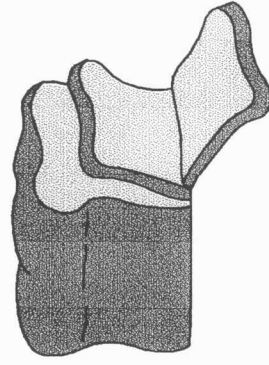
The decision to participate in a feeding study is not an easy one. You are giving up the freedom to choose the foods you want and are committing yourself to a demanding schedule for study compliance. For these things, we thank you very much. We hope this research study is a pleasant experience for you.

-The Research Team

## How do we pick the right



By evaluating your age, gender, and activity level, we pick a calorie level that meets your energy needs. The distribution of your calories may be different from what you are used to and it may take a few days to



#### IV. Health History Questionnaire

NAME: \_\_\_\_\_ DATE: \_\_\_\_\_

Age: \_\_\_\_\_ Gender: Male Female

Height: \_\_\_\_\_ cm Weight: \_\_\_\_\_ kg BMI \_\_\_\_\_

Person to contact in case of Emergency:

Name: \_\_\_\_\_ Phone: \_\_\_\_\_

Are you taking any medications or drugs?

If YES, please list medication, dose and reason:

Medication: \_\_\_\_\_ Dose: \_\_\_\_\_ Reason: \_\_\_\_\_

Medication: \_\_\_\_\_ Dose: \_\_\_\_\_ Reason: \_\_\_\_\_

Medication: \_\_\_\_\_ Dose: \_\_\_\_\_ Reason: \_\_\_\_\_

Medication: \_\_\_\_\_ Dose: \_\_\_\_\_ Reason: \_\_\_\_\_

General Health Questions:	Yes	No
History of heart problems, chest pain or stroke	<input type="checkbox"/>	<input type="checkbox"/>
Increased blood pressure	<input type="checkbox"/>	<input type="checkbox"/>
History of breathing or lung problems	<input type="checkbox"/>	<input type="checkbox"/>
Diabetes or thyroid disease	<input type="checkbox"/>	<input type="checkbox"/>
Seizures, tremors or severe headaches	<input type="checkbox"/>	<input type="checkbox"/>
Arthritis or other bone problems	<input type="checkbox"/>	<input type="checkbox"/>
Depression or other mental problems	<input type="checkbox"/>	<input type="checkbox"/>
Increased blood cholesterol	<input type="checkbox"/>	<input type="checkbox"/>
Cigarette smoking habit	<input type="checkbox"/>	<input type="checkbox"/>
Obesity (more than 20 percent over ideal body weight)	<input type="checkbox"/>	<input type="checkbox"/>
Recent surgery (last 12 months)	<input type="checkbox"/>	<input type="checkbox"/>
Last menstrual period (Date: _____)	<input type="checkbox"/>	<input type="checkbox"/>
Any other chronic illness or condition Please List:	<input type="checkbox"/>	<input type="checkbox"/>

Comments:

Reviewed by \_\_\_\_\_ Date: \_\_\_\_\_

## V. Physical Activity Assessment Form

Name:

Date:

Please indicate all the exercise you have performed during the past week. Also note for each activity the minutes spent each day performing that activity. Assess your activity level carefully as this information will be used to determine your energy needs.

TIME SPENT IN EACH ACTIVITY								
ACTIVITY	MON	TUE	WED	THU	FRI	SAT	SUN	TOTAL
Aerobic: high impact								
Aerobic: low impact								
Aerobic: step, 10" bench								
Calisthenics: light, moderate								
Calisthenics: stretching/yoga								
Cycling < 10 mph, leisure								
Cycling 10-12 mph, light								
Cycling 14-16 mph, vigorous								
Dancing: fast								
Dancing: slow								
Golf								
Rollerblading: casual								
Run: 6 min/mile								
Run: 10 min/mile								
Run: jog								
Skiing: X county								
Skiing: downhill								
Stairclimbing: machine								
Swimming: freestyle, laps								
Swimming: freestyle, slow								
Tennis								
Toning Exercise								
Treadmill: 3.0 mph, level								
Walking: slow (2 mph)								
Walking: brisk (3.5 mph)								
Weightlifting: free weights								
Weightlifting: machine								
Other								

## VI. Collection of a 24-Hour Urine

- Your Urine will be collected for a continuous 24-hour period of time. Choose the 24-hour period of time carefully so that the collection ends as close to the time you leave for the hospital or clinic as possible.
  
- When starting your 24-hour urine collection, follow these instructions:
  1. Urinate into the toilet. Do not save this sample, but record the time. This is the time your 24-hour collection starts. Your nurse will want to know this starting time.

Start time: \_\_\_\_\_

2. Save all urine after this time for exactly 24 hours.
- Exactly 24 hours after the collection began, urinate and place the urine in the container. This is the last urine to go into the container. Your collection is now complete. Bring the urine with you when you come to the hospital or clinic and give it to the nurse or secretary.
    - Women can urinate into a “hat” and place the urine into the container provided. Men can urinate directly into the container.



**VII. Daily Food Consumption Questionnaire**

P914:Effects of dietary fat on breast cancer risk

Participant: \_\_\_\_\_

Date: \_\_\_\_\_

Was there any food provided with your diet over the past 24 hours that you did not eat?

\_\_\_\_\_ YES      \_\_\_\_\_ NO

If yes, please list the foods or beverages you did not consume and the proportion of the item that you did not eat:

Food/Beverage	Amount	Time
---------------	--------	------

Did you eat or drink any foods in addition to the diet you were provided?

\_\_\_\_\_ YES      \_\_\_\_\_ NO

If yes, please list the foods or beverages you consumed, the amount of the item, and the time that you had it.

Food/Beverage	Amount	Time
---------------	--------	------

This is a sample form. Do not use for scanning.

NATIONAL INSTITUTES OF HEALTH

## Diet History Questionnaire



### GENERAL INSTRUCTIONS

- Answer each question as best you can. Estimate if you are not sure. A guess is better than leaving a blank.
- Use only a black ball-point pen. Do not use a pencil or felt-tip pen. Do not fold, staple, or tear the pages.
- Put an X in the box next to your answer.
- If you make any changes, cross out the incorrect answer and put an X in the box next to the correct answer. Also draw a circle around the correct answer.
- If you mark NEVER, NO, or DON'T KNOW for a question, please follow any arrows or instructions that direct you to the next question.

BEFORE TURNING THE PAGE, PLEASE COMPLETE THE FOLLOWING QUESTIONS.

Today's date:

MONTH	DAY	YEAR
<input type="checkbox"/> Jan		<input type="checkbox"/> 2007
<input type="checkbox"/> Feb		<input type="checkbox"/> 2008
<input type="checkbox"/> Mar	<input type="checkbox"/> 0 <input type="checkbox"/> 0	<input type="checkbox"/> 2009
<input type="checkbox"/> Apr	<input type="checkbox"/> 1 <input type="checkbox"/> 1	<input type="checkbox"/> 2010
<input type="checkbox"/> May	<input type="checkbox"/> 2 <input type="checkbox"/> 2	<input type="checkbox"/> 2011
<input type="checkbox"/> Jun	<input type="checkbox"/> 3 <input type="checkbox"/> 3	
<input type="checkbox"/> Jul	<input type="checkbox"/> 4 <input type="checkbox"/> 4	
<input type="checkbox"/> Aug	<input type="checkbox"/> 5 <input type="checkbox"/> 5	
<input type="checkbox"/> Sep	<input type="checkbox"/> 6 <input type="checkbox"/> 6	
<input type="checkbox"/> Oct	<input type="checkbox"/> 7 <input type="checkbox"/> 7	
<input type="checkbox"/> Nov	<input type="checkbox"/> 8 <input type="checkbox"/> 8	
<input type="checkbox"/> Dec	<input type="checkbox"/> 9 <input type="checkbox"/> 9	

In what month were you born?

<input type="checkbox"/> Jan
<input type="checkbox"/> Feb
<input type="checkbox"/> Mar
<input type="checkbox"/> Apr
<input type="checkbox"/> May
<input type="checkbox"/> Jun
<input type="checkbox"/> Jul
<input type="checkbox"/> Aug
<input type="checkbox"/> Sep
<input type="checkbox"/> Oct
<input type="checkbox"/> Nov
<input type="checkbox"/> Dec

In what year were you born?

19	<input type="checkbox"/> 0	<input type="checkbox"/> 0
	<input type="checkbox"/> 1	<input type="checkbox"/> 1
	<input type="checkbox"/> 2	<input type="checkbox"/> 2
	<input type="checkbox"/> 3	<input type="checkbox"/> 3
	<input type="checkbox"/> 4	<input type="checkbox"/> 4
	<input type="checkbox"/> 5	<input type="checkbox"/> 5
	<input type="checkbox"/> 6	<input type="checkbox"/> 6
	<input type="checkbox"/> 7	<input type="checkbox"/> 7
	<input type="checkbox"/> 8	<input type="checkbox"/> 8
	<input type="checkbox"/> 9	<input type="checkbox"/> 9

Are you male or female?

Male  
 Female

BAR CODE LABEL OR SUBJECT ID  
HERE

--	--	--	--	--	--	--	--	--	--	--

**This is a sample form. Do not use for scanning.**

1. Over the past 12 months, how often did you drink **tomato juice or vegetable juice**?

- NEVER (GO TO QUESTION 2)
- |   |  |
|---|--|
| <input type="checkbox"/> 1 time per month or less | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 2-3 times per month      | <input type="checkbox"/> 2-3 times per day       |
| <input type="checkbox"/> 1-2 times per week       | <input type="checkbox"/> 4-5 times per day       |
| <input type="checkbox"/> 3-4 times per week       | <input type="checkbox"/> 6 or more times per day |
| <input type="checkbox"/> 5-6 times per week       |  |

1a. Each time you drank **tomato juice or vegetable juice**, how much did you usually drink?

- Less than ¼ cup (6 ounces)  
 ¼ to 1¼ cups (6 to 10 ounces)  
 More than 1¼ cups (10 ounces)

2. Over the past 12 months, how often did you drink **orange juice or grapefruit juice**?

- NEVER (GO TO QUESTION 3)
- |   |  |
|---|--|
| <input type="checkbox"/> 1 time per month or less | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 2-3 times per month      | <input type="checkbox"/> 2-3 times per day       |
| <input type="checkbox"/> 1-2 times per week       | <input type="checkbox"/> 4-5 times per day       |
| <input type="checkbox"/> 3-4 times per week       | <input type="checkbox"/> 6 or more times per day |
| <input type="checkbox"/> 5-6 times per week       |  |

2a. Each time you drank **orange juice or grapefruit juice**, how much did you usually drink?

- Less than ¼ cup (6 ounces)  
 ¼ to 1¼ cups (6 to 10 ounces)  
 More than 1¼ cups (10 ounces)

3. Over the past 12 months, how often did you drink **other 100% fruit juice or 100% fruit juice mixtures** (such as apple, grape, pineapple, or others)?

- NEVER (GO TO QUESTION 4)
- |   |  |
|---|--|
| <input type="checkbox"/> 1 time per month or less | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 2-3 times per month      | <input type="checkbox"/> 2-3 times per day       |
| <input type="checkbox"/> 1-2 times per week       | <input type="checkbox"/> 4-5 times per day       |
| <input type="checkbox"/> 3-4 times per week       | <input type="checkbox"/> 6 or more times per day |
| <input type="checkbox"/> 5-6 times per week       |  |

3a. Each time you drank **other fruit juice or fruit juice mixtures**, how much did you usually drink?

- Less than ¼ cup (6 ounces)  
 ¼ to 1½ cups (6 to 12 ounces)  
 More than 1½ cups (12 ounces)

Question 4 appears in the next column

Over the past 12 months...

4. How often did you drink other **fruit drinks** (such as cranberry cocktail, Hi-C, lemonade, or Kool-Aid, diet or regular)?

- NEVER (GO TO QUESTION 5)
- |   |  |
|---|--|
| <input type="checkbox"/> 1 time per month or less | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 2-3 times per month      | <input type="checkbox"/> 2-3 times per day       |
| <input type="checkbox"/> 1-2 times per week       | <input type="checkbox"/> 4-5 times per day       |
| <input type="checkbox"/> 3-4 times per week       | <input type="checkbox"/> 6 or more times per day |
| <input type="checkbox"/> 5-6 times per week       |  |

4a. Each time you drank **fruit drinks**, how much did you usually drink?

- Less than 1 cup (8 ounces)  
 1 to 2 cups (8 to 16 ounces)  
 More than 2 cups (16 ounces)

4b. How often were your fruit drinks **diet or sugar-free drinks**?

- Almost never or never  
 About ¼ of the time  
 About ½ of the time  
 About ¾ of the time  
 Almost always or always

5. How often did you drink **milk as a beverage** (NOT in coffee, NOT in cereal)? (Please include chocolate milk and hot chocolate.)

- NEVER (GO TO QUESTION 6)
- |   |  |
|---|--|
| <input type="checkbox"/> 1 time per month or less | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 2-3 times per month      | <input type="checkbox"/> 2-3 times per day       |
| <input type="checkbox"/> 1-2 times per week       | <input type="checkbox"/> 4-5 times per day       |
| <input type="checkbox"/> 3-4 times per week       | <input type="checkbox"/> 6 or more times per day |
| <input type="checkbox"/> 5-6 times per week       |  |

5a. Each time you drank **milk as a beverage**, how much did you usually drink?

- Less than 1 cup (8 ounces)  
 1 to 1½ cups (8 to 12 ounces)  
 More than 1½ cups (12 ounces)

5b. What kind of **milk** did you usually drink?

- Whole milk  
 2% fat milk  
 1% fat milk  
 Skim, nonfat, or ½% fat milk  
 Soy milk  
 Rice milk  
 Other

Question 6 appears on the next page

This is a sample form. Do not use for scanning.

Over the **past 12 months**...

6. How often did you drink **meal replacement, energy, or high-protein beverages** such as Instant Breakfast, Ensure, Slimfast, Sustacal or others?
- NEVER (GO TO QUESTION 7)
- |   |  |
|---|--|
| <input type="checkbox"/> 1 time per month or less | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 2-3 times per month      | <input type="checkbox"/> 2-3 times per day       |
| <input type="checkbox"/> 1-2 times per week       | <input type="checkbox"/> 4-5 times per day       |
| <input type="checkbox"/> 3-4 times per week       | <input type="checkbox"/> 6 or more times per day |
| <input type="checkbox"/> 5-6 times per week       |  |

- 6a. Each time you drank **meal replacement beverages**, how much did you usually drink?
- Less than 1 cup (8 ounces)  
 1 to 1½ cups (8 to 12 ounces)  
 More than 1½ cups (12 ounces)

7. Over the past 12 months, did you drink **soft drinks, soda, or pop**?

- NO (GO TO QUESTION 8)  
 YES

- 7a. How often did you drink **soft drinks, soda, or pop IN THE SUMMER**?
- NEVER
- |   |  |
|---|--|
| <input type="checkbox"/> 1 time per month or less | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 2-3 times per month      | <input type="checkbox"/> 2-3 times per day       |
| <input type="checkbox"/> 1-2 times per week       | <input type="checkbox"/> 4-5 times per day       |
| <input type="checkbox"/> 3-4 times per week       | <input type="checkbox"/> 6 or more times per day |
| <input type="checkbox"/> 5-6 times per week       |  |

- 7b. How often did you drink **soft drinks, soda, or pop DURING THE REST OF THE YEAR**?
- NEVER
- |   |  |
|---|--|
| <input type="checkbox"/> 1 time per month or less | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 2-3 times per month      | <input type="checkbox"/> 2-3 times per day       |
| <input type="checkbox"/> 1-2 times per week       | <input type="checkbox"/> 4-5 times per day       |
| <input type="checkbox"/> 3-4 times per week       | <input type="checkbox"/> 6 or more times per day |
| <input type="checkbox"/> 5-6 times per week       |  |

- 7c. Each time you drank **soft drinks, soda, or pop**, how much did you usually drink?
- Less than 12 ounces or less than 1 can or bottle  
 12 to 16 ounces or 1 can or bottle  
 More than 16 ounces or more than 1 can or bottle

Question 8 appears in the next column

- 7d. How often were these soft drinks, soda, or pop **diet or sugar-free**?

- Almost never or never  
 About ¼ of the time  
 About ½ of the time  
 About ¾ of the time  
 Almost always or always

- 7e. How often were these soft drinks, soda, or pop **caffeine-free**?

- Almost never or never  
 About ¼ of the time  
 About ½ of the time  
 About ¾ of the time  
 Almost always or always

8. Over the **past 12 months**, did you drink **beer**?

- NO (GO TO QUESTION 9)  
 YES

- 8a. How often did you drink **beer IN THE SUMMER**?

- NEVER
- |   |  |
|---|--|
| <input type="checkbox"/> 1 time per month or less | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 2-3 times per month      | <input type="checkbox"/> 2-3 times per day       |
| <input type="checkbox"/> 1-2 times per week       | <input type="checkbox"/> 4-5 times per day       |
| <input type="checkbox"/> 3-4 times per week       | <input type="checkbox"/> 6 or more times per day |
| <input type="checkbox"/> 5-6 times per week       |  |

- 8b. How often did you drink **beer DURING THE REST OF THE YEAR**?

- NEVER
- |   |  |
|---|--|
| <input type="checkbox"/> 1 time per month or less | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 2-3 times per month      | <input type="checkbox"/> 2-3 times per day       |
| <input type="checkbox"/> 1-2 times per week       | <input type="checkbox"/> 4-5 times per day       |
| <input type="checkbox"/> 3-4 times per week       | <input type="checkbox"/> 6 or more times per day |
| <input type="checkbox"/> 5-6 times per week       |  |

- 8c. Each time you drank **beer**, how much did you usually drink?

- Less than a 12-ounce can or bottle  
 1 to 3 12-ounce cans or bottles  
 More than 3 12-ounce cans or bottles



Question 9 appears on the next page

This is a sample form. Do not use for scanning.

Over the past 12 months...

9. How often did you drink **wine** or **wine coolers**?

- NEVER (GO TO QUESTION 10)
- |   |  |
|---|--|
| <input type="checkbox"/> 1 time per month or less | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 2-3 times per month      | <input type="checkbox"/> 2-3 times per day       |
| <input type="checkbox"/> 1-2 times per week       | <input type="checkbox"/> 4-5 times per day       |
| <input type="checkbox"/> 3-4 times per week       | <input type="checkbox"/> 6 or more times per day |
| <input type="checkbox"/> 5-6 times per week       |  |

9a. Each time you drank **wine** or **wine coolers**, how much did you usually drink?

- Less than 5 ounces or less than 1 glass  
 5 to 12 ounces or 1 to 2 glasses  
 More than 12 ounces or more than 2 glasses

10. How often did you drink **liquor** or **mixed drinks**?

- NEVER (GO TO QUESTION 11)
- |   |  |
|---|--|
| <input type="checkbox"/> 1 time per month or less | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 2-3 times per month      | <input type="checkbox"/> 2-3 times per day       |
| <input type="checkbox"/> 1-2 times per week       | <input type="checkbox"/> 4-5 times per day       |
| <input type="checkbox"/> 3-4 times per week       | <input type="checkbox"/> 6 or more times per day |
| <input type="checkbox"/> 5-6 times per week       |  |

10a. Each time you drank **liquor** or **mixed drinks**, how much did you usually drink?

- Less than 1 shot of liquor  
 1 to 3 shots of liquor  
 More than 3 shots of liquor

11. Over the past 12 months, did you eat **oatmeal**, **grits**, or **other cooked cereal**?

- NO (GO TO QUESTION 12)  
 YES

11a. How often did you eat **oatmeal**, **grits**, or **other cooked cereal** IN THE WINTER?

- NEVER
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per winter  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per winter | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month      | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month   | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week       | <input type="checkbox"/> 2 or more times per day |

Question 12 appears in the next column

11b. How often did you eat **oatmeal**, **grits**, or **other cooked cereal** DURING THE REST OF THE YEAR?

- NEVER
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

11c. Each time you ate **oatmeal**, **grits**, or **other cooked cereal**, how much did you usually eat?

- Less than ¼ cup  
 ¼ to 1¼ cups  
 More than 1¼ cups

12. How often did you eat **cold cereal**?

- NEVER (GO TO QUESTION 13)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

12a. Each time you ate **cold cereal**, how much did you usually eat?

- Less than 1 cup  
 1 to 2½ cups  
 More than 2½ cups

12b. How often was the cold cereal you ate **Total**, **Product 19**, or **Right Start**?

- Almost never or never  
 About ¼ of the time  
 About ½ of the time  
 About ¾ of the time  
 Almost always or always

12c. How often was the cold cereal you ate **All Bran**, **Fiber One**, **100% Bran**, or **Bran Buds**?

- Almost never or never  
 About ¼ of the time  
 About ½ of the time  
 About ¾ of the time  
 Almost always or always

Question 13 appears on the next page

This is a sample form. Do not use for scanning.

Over the past 12 months...

12d. How often was the cold cereal you ate **some other bran or fiber cereal** (such as Cheerios, Shredded Wheat, Raisin Bran, Bran Flakes, Grape-Nuts, Granola, Wheaties, or Healthy Choice)?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

12e. How often was the cold cereal you ate any **other type of cold cereal** (such as Corn Flakes, Rice Krispies, Frosted Flakes, Special K, Froot Loops, Cap'n Crunch, or others)?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

12f. Was **milk** added to your cold cereal?

- NO (GO TO QUESTION 13)
- YES

12g. What kind of **milk** was usually added?

- Whole milk
- 2% fat milk
- 1% fat milk
- Skim, nonfat, or ½% fat milk
- Soy milk
- Rice milk
- Other

12h. Each time **milk** was added to your cold cereal, how much was usually added?

- Less than ½ cup
- ½ to 1 cup
- More than 1 cup

13. How often did you eat **applesauce**?

- NEVER (GO TO QUESTION 14)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

Question 14 appears in the next column

13a. Each time you ate **applesauce**, how much did you usually eat?

- Less than ½ cup
- ½ to 1 cup
- More than 1 cup

14. How often did you eat **apples**?

- NEVER (GO TO QUESTION 15)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

14a. Each time you ate **apples**, how many did you usually eat?

- Less than 1 apple
- 1 apple
- More than 1 apple

15. How often did you eat **pears** (fresh, canned, or frozen)?

- NEVER (GO TO QUESTION 16)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

15a. Each time you ate **pears**, how many did you usually eat?

- Less than 1 pear
- 1 pear
- More than 1 pear

16. How often did you eat **bananas**?

- NEVER (GO TO QUESTION 17)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day



5

Question 17 appears on the next page

This is a sample form. Do not use for scanning.

Over the past 12 months...

16a. Each time you ate **bananas**, how many did you usually eat?

- Less than 1 banana
- 1 banana
- More than 1 banana

17. How often did you eat **dried fruit**, such as prunes or raisins (not including dried apricots)?

- NEVER (GO TO QUESTION 18)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

17a. Each time you ate **dried fruit**, how much did you usually eat (not including dried apricots)?

- Less than 2 tablespoons
- 2 to 5 tablespoons
- More than 5 tablespoons

18. Over the past 12 months, did you eat **peaches, nectarines, or plums**?

- NO (GO TO QUESTION 19)
- YES

18a. How often did you eat **fresh peaches, nectarines, or plums WHEN IN SEASON**?

- NEVER
- 1-6 times per season
- 7-11 times per season
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

18b. How often did you eat **peaches, nectarines, or plums** (fresh, canned, or frozen) **DURING THE REST OF THE YEAR**?

- NEVER
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

Question 19 appears in the next column

18c. Each time you ate **peaches, nectarines, or plums**, how much did you usually eat?

- Less than 1 fruit or less than 1/2 cup
- 1 to 2 fruits or 1/2 to 3/4 cup
- More than 2 fruits or more than 3/4 cup

19. How often did you eat **grapes**?

- NEVER (GO TO QUESTION 20)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

19a. Each time you ate **grapes**, how much did you usually eat?

- Less than 1/2 cup or less than 10 grapes
- 1/2 to 1 cup or 10 to 30 grapes
- More than 1 cup or more than 30 grapes

20. Over the past 12 months, did you eat **cantaloupe**?

- NO (GO TO QUESTION 21)
- YES

20a. How often did you eat **fresh cantaloupe WHEN IN SEASON**?

- NEVER
- 1-6 times per season
- 7-11 times per season
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

20b. How often did you eat **fresh or frozen cantaloupe DURING THE REST OF THE YEAR**?

- NEVER
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

Question 21 appears on the next page

This is a sample form. Do not use for scanning.

Over the past 12 months...

20c. Each time you ate **cantaloupe**, how much did you usually eat?

- Less than ¼ melon or less than ½ cup
- ¼ melon or ½ to 1 cup
- More than ¼ melon or more than 1 cup

21. Over the past 12 months, did you eat **melon, other than cantaloupe** (such as watermelon or honeydew)?

NO (GO TO QUESTION 22)

YES

21a. How often did you eat **fresh melon, other than cantaloupe** (such as watermelon or honeydew) **WHEN IN SEASON?**

- NEVER
- 1-6 times per season
- 7-11 times per season
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

21b. How often did you eat **fresh or frozen melon, other than cantaloupe** (such as watermelon or honeydew) **DURING THE REST OF THE YEAR?**

- NEVER
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

21c. Each time you ate **melon other than cantaloupe**, how much did you usually eat?

- Less than ½ cup or 1 small wedge
- ½ to 2 cups or 1 medium wedge
- More than 2 cups or 1 large wedge



Question 22 appears in the next column

22. Over the past 12 months, did you eat **strawberries?**

NO (GO TO QUESTION 23)

YES

22a. How often did you eat **fresh strawberries WHEN IN SEASON?**

- NEVER
- 1-6 times per season
- 7-11 times per season
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

22b. How often did you eat **fresh or frozen strawberries DURING THE REST OF THE YEAR?**

- NEVER
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

22c. Each time you ate **strawberries**, how much did you usually eat?

- Less than ¼ cup or less than 3 berries
- ¼ to ¾ cup or 3 to 8 berries
- More than ¾ cup or more than 8 berries

23. Over the past 12 months, did you eat **oranges, tangerines, or tangelos?**

NO (GO TO QUESTION 24)

YES

23a. How often did you eat **fresh oranges, tangerines, or tangelos WHEN IN SEASON?**

- NEVER
- 1-6 times per season
- 7-11 times per season
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

Question 24 appears on the next page



This is a sample form. Do not use for scanning.

Over the past 12 months...

23b. How often did you eat **oranges, tangerines, or tangelos** (fresh or canned) **DURING THE REST OF THE YEAR?**

- NEVER
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

23c. Each time you ate **oranges, tangerines, or tangelos**, how many did you usually eat?

- Less than 1 fruit  
 1 fruit  
 More than 1 fruit

24. Over the past 12 months, did you eat **grapefruit**?

NO (GO TO QUESTION 25)

YES

24a. How often did you eat **fresh grapefruit** **WHEN IN SEASON?**

- NEVER
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per season  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per season | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month      | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month   | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week       | <input type="checkbox"/> 2 or more times per day |

24b. How often did you eat **grapefruit** (fresh or canned) **DURING THE REST OF THE YEAR?**

- NEVER
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

24c. Each time you ate **grapefruit**, how much did you usually eat?

- Less than 1/2 grapefruit  
 1/2 grapefruit  
 More than 1/2 grapefruit

Question 25 appears in the next column

25. How often did you eat **other kinds of fruit**?

- NEVER (GO TO QUESTION 26)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

25a. Each time you ate **other kinds of fruit**, how much did you usually eat?

- Less than 1/4 cup  
 1/4 to 1/2 cup  
 More than 1/4 cup

26. How often did you eat **COOKED greens** (such as spinach, turnip, collard, mustard, chard, or kale)?

NEVER (GO TO QUESTION 27)

- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

26a. Each time you ate **COOKED greens**, how much did you usually eat?

- Less than 1/2 cup  
 1/2 to 1 cup  
 More than 1 cup

27. How often did you eat **RAW greens** (such as spinach, turnip, collard, mustard, chard, or kale)?  
*(We will ask about lettuce later.)*

NEVER (GO TO QUESTION 28)

- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

27a. Each time you ate **RAW greens**, how much did you usually eat?

- Less than 1/2 cup  
 1/2 to 1 cup  
 More than 1 cup

Question 28 appears on the next page

This is a sample form. Do not use for scanning.

Over the past 12 months...

28. How often did you eat **coleslaw**?

- NEVER (GO TO QUESTION 29)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

28a. Each time you ate **coleslaw**, how much did you usually eat?

- Less than ¼ cup  
 ¼ to ½ cup  
 More than ½ cup

29. How often did you eat **sauerkraut** or **cabbage** (other than coleslaw)?

- NEVER (GO TO QUESTION 30)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

29a. Each time you ate **sauerkraut** or **cabbage**, how much did you usually eat?

- Less than ¼ cup  
 ¼ to 1 cup  
 More than 1 cup

30. How often did you eat **carrots** (fresh, canned, or frozen)?

- NEVER (GO TO QUESTION 31)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

30a. Each time you ate **carrots**, how much did you usually eat?

- Less than ¼ cup or less than 2 baby carrots  
 ¼ to ½ cup or 2 to 5 baby carrots  
 More than ½ cup or more than 5 baby carrots

Question 31 appears in the next column

31. How often did you eat **string beans** or **green beans** (fresh, canned, or frozen)?

- NEVER (GO TO QUESTION 32)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

31a. Each time you ate **string beans** or **green beans**, how much did you usually eat?

- Less than ¼ cup  
 ¼ to 1 cup  
 More than 1 cup

32. How often did you eat **peas** (fresh, canned, or frozen)?

- NEVER (GO TO QUESTION 33)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

32a. Each time you ate **peas**, how much did you usually eat?

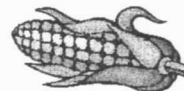
- Less than ¼ cup  
 ¼ to ½ cup  
 More than ½ cup

33. Over the past 12 months, did you eat **corn**?

- NO (GO TO QUESTION 34)  
 YES

33a. How often did you eat **fresh corn** **WHEN IN SEASON**?

- NEVER
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per season  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per season | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month      | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month   | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week       | <input type="checkbox"/> 2 or more times per day |



Question 34 appears on the next page

This is a sample form. Do not use for scanning.

Over the **past 12 months**...

33b. How often did you eat **corn** (fresh, canned, or frozen) **DURING THE REST OF THE YEAR?**

- NEVER
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

33c. Each time you ate **corn**, how much did you usually eat?

- Less than 1 ear or less than 1/2 cup  
 1 ear or 1/2 to 1 cup  
 More than 1 ear or more than 1 cup

34. Over the **past 12 months**, how often did you eat **broccoli** (fresh or frozen)?

- NEVER (GO TO QUESTION 35)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

34a. Each time you ate **broccoli**, how much did you usually eat?

- Less than 1/4 cup  
 1/4 to 1 cup  
 More than 1 cup

35. How often did you eat **cauliflower** or **Brussels sprouts** (fresh or frozen)?

- NEVER (GO TO QUESTION 36)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

35a. Each time you ate **cauliflower** or **Brussels sprouts**, how much did you usually eat?

- Less than 1/4 cup  
 1/4 to 1/2 cup  
 More than 1/2 cup

Question 36 appears in the next column

36. How often did you eat mixed **vegetables**?

- NEVER (GO TO QUESTION 37)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

36a. Each time you ate mixed **vegetables**, how much did you usually eat?

- Less than 1/2 cup  
 1/2 to 1 cup  
 More than 1 cup

37. How often did you eat **onions**?

- NEVER (GO TO QUESTION 38)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

37a. Each time you ate **onions**, how much did you usually eat?

- Less than 1 slice or less than 1 tablespoon  
 1 slice or 1 to 4 tablespoons  
 More than 1 slice or more than 4 tablespoons

38. Now think about all the **cooked vegetables** you ate in the **past 12 months** and how they were prepared. How often were your vegetables **COOKED WITH** some sort of fat, including oil spray? (*Please do not include potatoes.*)

- NEVER (GO TO QUESTION 39)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |



10 Question 39 appears on the next page

This is a sample form. Do not use for scanning.

Over the past 12 months...

38a. Which fats were usually added to your vegetables **DURING COOKING**? (Please do not include potatoes. **Mark all that apply.**)

- |  |   |
|--|---|
| <input type="checkbox"/> Margarine (including low-fat) | <input type="checkbox"/> Corn oil                         |
| <input type="checkbox"/> Butter (including low-fat)    | <input type="checkbox"/> Canola or rapeseed oil           |
| <input type="checkbox"/> Lard, fatback, or bacon fat   | <input type="checkbox"/> Oil spray, such as Pam or others |
| <input type="checkbox"/> Olive oil                     | <input type="checkbox"/> Other kinds of oils              |
|  | <input type="checkbox"/> None of the above                |

39. Now, thinking again about all the **cooked vegetables** you ate in the past 12 months, how often was some sort of fat, sauce, or dressing added **AFTER COOKING OR AT THE TABLE**? (Please do not include potatoes.)

- NEVER (GO TO QUESTION 40)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 2 times per day         |
| <input type="checkbox"/> 1-2 times per week  | <input type="checkbox"/> 3 or more times per day |

39a. Which fats, sauces, or dressings were usually added **AFTER COOKING OR AT THE TABLE**? (Please do not include potatoes. **Mark all that apply.**)

- |  |   |
|--|---|
| <input type="checkbox"/> Margarine (including low-fat) | <input type="checkbox"/> Salad dressing |
| <input type="checkbox"/> Butter (including low-fat)    | <input type="checkbox"/> Cheese sauce   |
| <input type="checkbox"/> Lard, fatback, or bacon fat   | <input type="checkbox"/> White sauce    |
|  | <input type="checkbox"/> Other          |

39b. If margarine, butter, lard, fatback, or bacon fat was added to your cooked vegetables **AFTER COOKING OR AT THE TABLE**, how much did you usually add?

- Did not usually add these
- Less than 1 teaspoon
- 1 to 3 teaspoons
- More than 3 teaspoons

39c. If salad dressing, cheese sauce, or white sauce was added to your cooked vegetables **AFTER COOKING OR AT THE TABLE**, how much did you usually add?

- Did not usually add these
- Less than 1 tablespoon
- 1 to 3 tablespoons
- More than 3 tablespoons

Question 40 appears in the next column

40. Over the past 12 months, how often did you eat **sweet peppers** (green, red, or yellow)?

- NEVER (GO TO QUESTION 41)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

40a. Each time you ate **sweet peppers**, how much did you usually eat?

- Less than 1/4 pepper
- 1/4 to 1/2 pepper
- More than 1/2 pepper

41. Over the past 12 months, did you eat **fresh tomatoes** (including those in salads)?

NO (GO TO QUESTION 42)

YES

41a. How often did you eat **fresh tomatoes** (including those in salads) **WHEN IN SEASON**?

- NEVER
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per season  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per season | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month      | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month   | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week       | <input type="checkbox"/> 2 or more times per day |

41b. How often did you eat **fresh tomatoes** (including those in salads) **DURING THE REST OF THE YEAR**?

- NEVER
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

41c. Each time you ate **fresh tomatoes**, how much did you usually eat?

- Less than 1/4 tomato
- 1/4 to 1/2 tomato
- More than 1/2 tomato

Question 42 appears on the next page

This is a sample form. Do not use for scanning.

Over the **past 12 months**...

42. How often did you eat **lettuce salads** (with or without other vegetables)?

- NEVER (GO TO QUESTION 43)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

42a. Each time you ate **lettuce salads**, how much did you usually eat?

- Less than ¼ cup  
 ¼ to 1¼ cups  
 More than 1¼ cups

43. How often did you eat **salad dressing** (including low-fat) on salads?

- NEVER (GO TO QUESTION 44)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

43a. Each time you ate **salad dressing** on salads, how much did you usually eat?

- Less than 2 tablespoons  
 2 to 4 tablespoons  
 More than 4 tablespoons

44. How often did you eat **sweet potatoes** or **yams**?

- NEVER (GO TO QUESTION 45)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

44a. Each time you ate **sweet potatoes** or **yams**, how much did you usually eat?

- 1 small potato or less than ¼ cup  
 1 medium potato or ¼ to ¾ cup  
 1 large potato or more than ¾ cup

Question 45 appears in the next column

45. How often did you eat **French fries, home fries, hash browned potatoes, or tater tots**?

- NEVER (GO TO QUESTION 46)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

45a. Each time you ate **French fries, home fries, hash browned potatoes, or tater tots** how much did you usually eat?

- Less than 10 fries or less than ½ cup  
 10 to 25 fries or ½ to 1 cup  
 More than 25 fries or more than 1 cup

46. How often did you eat **potato salad**?

- NEVER (GO TO QUESTION 47)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

46a. Each time you ate **potato salad**, how much did you usually eat?

- Less than ½ cup  
 ½ to 1 cup  
 More than 1 cup

47. How often did you eat **baked, boiled, or mashed potatoes**?

- NEVER (GO TO QUESTION 48)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

47a. Each time you ate **baked, boiled, or mashed potatoes**, how much did you usually eat?

- 1 small potato or less than ½ cup  
 1 medium potato or ½ to 1 cup  
 1 large potato or more than 1 cup

12 Question 48 appears on the next page

This is a sample form. Do not use for scanning.

Over the past 12 months...

47b. How often was **sour cream** (including low-fat) added to your potatoes, **EITHER IN COOKING OR AT THE TABLE?**

- Almost never or never (GO TO QUESTION 47d)
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

47c. Each time **sour cream** was added to your potatoes, how much was usually added?

- Less than 1 tablespoon
- 1 to 3 tablespoons
- More than 3 tablespoons

47d. How often was **margarine** (including low-fat) added to your potatoes, **EITHER IN COOKING OR AT THE TABLE?**

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

47e. How often was **butter** (including low-fat) added to your potatoes, **EITHER IN COOKING OR AT THE TABLE?**

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

47f. Each time **margarine** or **butter** was added to your potatoes, how much was usually added?

- Never added
- Less than 1 teaspoon
- 1 to 3 teaspoons
- More than 3 teaspoons

47g. How often was **cheese** or **cheese sauce** added to your potatoes, **EITHER IN COOKING OR AT THE TABLE?**

- Almost never or never (GO TO QUESTION 48)
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

Question 48 appears in the next column

47h. Each time **cheese** or **cheese sauce** was added to your potatoes, how much was usually added?

- Less than 1 tablespoon
- 1 to 3 tablespoons
- More than 3 tablespoons

48. How often did you eat **salsa**?

- NEVER (GO TO QUESTION 49)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

48a. Each time you ate **salsa**, how much did you usually eat?

- Less than 1 tablespoon
- 1 to 5 tablespoons
- More than 5 tablespoons

49. How often did you eat **catsup**?

- NEVER (GO TO QUESTION 50)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

49a. Each time you ate **catsup**, how much did you usually eat?

- Less than 1 teaspoon
- 1 to 6 teaspoons
- More than 6 teaspoons

50. How often did you eat **stuffing, dressing, or dumplings**?

- NEVER (GO TO QUESTION 51)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

50a. Each time you ate **stuffing, dressing, or dumplings**, how much did you usually eat?

- Less than ½ cup
- ½ to 1 cup
- More than 1 cup

Question 51 appears on the next page

This is a sample form. Do not use for scanning.

Over the past 12 months...

51. How often did you eat **chili**?

- NEVER (GO TO QUESTION 52)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

51a. Each time you ate **chili**, how much did you usually eat?

- Less than 1/2 cup  
 1/2 to 1 1/4 cups  
 More than 1 1/4 cups

52. How often did you eat **Mexican foods** (such as tacos, tostados, burritos, tamales, fajitas, enchiladas, quesadillas, and chimichangas)?

- NEVER (GO TO QUESTION 53)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

52a. Each time you ate **Mexican foods**, how much did you usually eat?

- Less than 1 taco, burrito, etc.  
 1 to 2 tacos, burritos, etc.  
 More than 2 tacos, burritos, etc.

53. How often did you eat **cooked dried beans** (such as baked beans, pintos, kidney, blackeyed peas, lima, lentils, soybeans, or refried beans)? (Please don't include bean soups or chili.)

- NEVER (GO TO QUESTION 54)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

53a. Each time you ate **beans**, how much did you usually eat?

- Less than 1/2 cup  
 1/2 to 1 cup  
 More than 1 cup

Question 54 appears in the next column

53b. How often were the beans you ate **refried beans, beans prepared with any type of fat, or with meat added**?

- Almost never or never  
 About 1/4 of the time  
 About 1/2 of the time  
 About 3/4 of the time  
 Almost always or always

54. How often did you eat **other kinds of vegetables**?

NEVER (GO TO QUESTION 55)

- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

54a. Each time you ate **other kinds of vegetables**, how much did you usually eat?

- Less than 1/4 cup  
 1/4 to 1/2 cup  
 More than 1/2 cup

55. How often did you eat **rice or other cooked grains** (such as bulgur, cracked wheat, or millet)?

NEVER (GO TO QUESTION 56)

- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

55a. Each time you ate **rice or other cooked grains**, how much did you usually eat?

- Less than 1/2 cup  
 1/2 to 1 1/2 cups  
 More than 1 1/2 cups

55b. How often was **butter, margarine, or oil** added to your rice **IN COOKING OR AT THE TABLE**?

- Almost never or never  
 About 1/4 of the time  
 About 1/2 of the time  
 About 3/4 of the time  
 Almost always or always

This is a sample form. Do not use for scanning.

Over the **past 12 months...**

56. How often did you eat **pancakes, waffles, or French toast**?

- NEVER (GO TO QUESTION 57)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

56a. Each time you ate **pancakes, waffles, or French toast**, how much did you usually eat?

- Less than 1 medium piece  
 1 to 3 medium pieces  
 More than 3 medium pieces

56b. How often was **margarine** (including low-fat) added to your **pancakes, waffles, or French toast AFTER COOKING OR AT THE TABLE**?

- Almost never or never  
 About 1/4 of the time  
 About 1/2 of the time  
 About 3/4 of the time  
 Almost always or always

56c. How often was **butter** (including low-fat) added to your **pancakes, waffles, or French toast AFTER COOKING OR AT THE TABLE**?

- Almost never or never  
 About 1/4 of the time  
 About 1/2 of the time  
 About 3/4 of the time  
 Almost always or always

56d. Each time **margarine** or **butter** was added to your **pancakes, waffles, or French toast**, how much was usually added?

- Never added  
 Less than 1 teaspoon  
 1 to 3 teaspoons  
 More than 3 teaspoons

56e. How often was **syrup** added to your **pancakes, waffles, or French toast**?

- Almost never or never (GO TO QUESTION 57)  
 About 1/4 of the time  
 About 1/2 of the time  
 About 3/4 of the time  
 Almost always or always

Question 57 appears in the next column

56f. Each time **syrup** was added to your **pancakes, waffles, or French toast**, how much was usually added?

- Less than 1 tablespoon  
 1 to 4 tablespoons  
 More than 4 tablespoons

57. How often did you eat **lasagna, stuffed shells, stuffed manicotti, ravioli, or tortellini**? (Please do not include spaghetti or other pasta.)

- NEVER (GO TO QUESTION 58)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

57a. Each time you ate **lasagna, stuffed shells, stuffed manicotti, ravioli, or tortellini**, how much did you usually eat?

- Less than 1 cup  
 1 to 2 cups  
 More than 2 cups

58. How often did you eat **macaroni and cheese**?

- NEVER (GO TO QUESTION 59)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

58a. Each time you ate **macaroni and cheese**, how much did you usually eat?

- Less than 1 cup  
 1 to 1 1/2 cups  
 More than 1 1/2 cups

59. How often did you eat **pasta salad** or **macaroni salad**?

- NEVER (GO TO QUESTION 60)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

15 Question 60 appears on the next page



This is a sample form. Do not use for scanning.

Over the past 12 months...

59a. Each time you ate **pasta salad** or **macaroni salad**, how much did you usually eat?

- Less than 1/2 cup
- 1/2 to 1 cup
- More than 1 cup

60. Other than the pastas listed in Questions 57, 58, and 59, how often did you eat **pasta, spaghetti, or other noodles**?

- NEVER (GO TO QUESTION 61)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

60a. Each time you ate **pasta, spaghetti, or other noodles**, how much did you usually eat?

- Less than 1 cup
- 1 to 3 cups
- More than 3 cups

60b. How often did you eat your pasta, spaghetti, or other noodles with **tomato sauce** or **spaghetti sauce made WITH meat**?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

60c. How often did you eat your pasta, spaghetti, or other noodles with **tomato sauce** or **spaghetti sauce made WITHOUT meat**?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

60d. How often did you eat your pasta, spaghetti, or other noodles with **margarine, butter, oil, or cream sauce**?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

Question 61 appears in the next column

61. How often did you eat **bagels** or **English muffins**?

- NEVER (GO TO INTRODUCTION TO QUESTION 62)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

61a. Each time you ate **bagels** or **English muffins**, how many did you usually eat?

- Less than 1 bagel or English muffin
- 1 bagel or English muffin
- More than 1 bagel or English muffin

61b. How often was **margarine** (including low-fat) added to your bagels or English muffins?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

61c. How often was **butter** (including low-fat) added to your bagels or English muffins?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

61d. Each time **margarine** or **butter** was added to your bagels or English muffins, how much was usually added?

- Never added
- Less than 1 teaspoon
- 1 to 2 teaspoons
- More than 2 teaspoons

61e. How often was **cream cheese** (including low-fat) spread on your bagels or English muffins?

- Almost never or never (GO TO INTRODUCTION TO QUESTION 62)
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

16 Introduction to Question 62 appears on the next page

This is a sample form. Do not use for scanning.

Over the past 12 months...

61f. Each time **cream cheese** was added to your bagels or English muffins, how much was usually added?

- Less than 1 tablespoon
- 1 to 2 tablespoons
- More than 2 tablespoons

The next questions ask about your intake of breads other than bagels or English muffins. First, we will ask about bread you ate as part of sandwiches only. Then we will ask about all other bread you ate.

62. How often did you eat **bread**s or **rolls** AS PART OF SANDWICHES (including burger and hot dog rolls)?

- NEVER (GO TO QUESTION 63)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

62a. Each time you ate **bread**s or **rolls** AS PART OF SANDWICHES, how many did you usually eat?

- 1 slice or 1/2 roll
- 2 slices or 1 roll
- More than 2 slices or more than 1 roll

62b. How often were the breads or rolls that you used for your sandwiches **white bread** (including burger and hot dog rolls)?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

62c. How often was **mayonnaise** or **mayonnaise-type dressing** (including low-fat) added to your sandwich bread or rolls?

- Almost never or never (GO TO QUESTION 62e)
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

Question 62e appears in the next column

Question 63 appears in the next column

62d. Each time **mayonnaise** or **mayonnaise-type dressing** was added to your sandwich breads or rolls, how much was usually added?

- Less than 1 teaspoon
- 1 to 3 teaspoons
- More than 3 teaspoons

62e. How often was **margarine** (including low-fat) added to your sandwich bread or rolls?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

62f. How often was **butter** (including low-fat) added to your sandwich bread or rolls?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

62g. Each time **margarine** or **butter** was added to your sandwich breads or rolls, how much was usually added?

- Never added
- Less than 1 teaspoon
- 1 to 2 teaspoons
- More than 2 teaspoons

63. How often did you eat **bread**s or **dinner rolls**, NOT AS PART OF SANDWICHES?

- NEVER (GO TO QUESTION 64)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

63a. Each time you ate **bread**s or **dinner rolls**, NOT AS PART OF SANDWICHES, how much did you usually eat?

- 1 slice or 1 dinner roll
- 2 slices or 2 dinner rolls
- More than 2 slices or 2 dinner rolls



Question 64 appears on the next page

This is a sample form. Do not use for scanning.

Over the **past 12 months**...

63b. How often were the breads or rolls you ate **white bread**?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

63c. How often was **margarine** (including low-fat) added to your breads or rolls?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

63d. How often was **butter** (including low-fat) added to your breads or rolls?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

63e. Each time **margarine** or **butter** was added to your breads or rolls, how much was usually added?

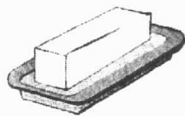
- Never added
- Less than 1 teaspoon
- 1 to 2 teaspoons
- More than 2 teaspoons

63f. How often was **cream cheese** (including low-fat) added to your breads or rolls?

- Almost never or never (GO TO QUESTION 64)
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

63g. Each time **cream cheese** was added to your breads or rolls, how much was usually added?

- Less than 1 tablespoon
- 1 to 2 tablespoons
- More than 2 tablespoons



Question 64 appears in the next column

64. How often did you eat **jam, jelly, or honey** on bagels, muffins, bread, rolls, or crackers?

- NEVER (GO TO QUESTION 65)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

64a. Each time you ate **jam, jelly, or honey**, how much did you usually eat?

- Less than 1 teaspoon
- 1 to 3 teaspoons
- More than 3 teaspoons

65. How often did you eat **peanut butter or other nut butter**?

- NEVER (GO TO QUESTION 66)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

65a. Each time you ate **peanut butter or other nut butter**, how much did you usually eat?

- Less than 1 tablespoon
- 1 to 2 tablespoons
- More than 2 tablespoons

66. How often did you eat **roast beef or steak IN SANDWICHES**?

- NEVER (GO TO QUESTION 67)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

66a. Each time you ate **roast beef or steak IN SANDWICHES**, how much did you usually eat?

- Less than 1 slice or less than 2 ounces
- 1 to 2 slices or 2 to 4 ounces
- More than 2 slices or more than 4 ounces

Question 67 appears on the next page

**This is a sample form. Do not use for scanning.**

Over the past 12 months...

67. How often did you eat **turkey** or **chicken COLD CUTS** (such as loaf, luncheon meat, turkey ham, turkey salami, or turkey pastrami)? (We will ask about other turkey or chicken later.)

- NEVER (GO TO QUESTION 68)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

67a. Each time you ate **turkey** or **chicken COLD CUTS**, how much did you usually eat?

- Less than 1 slice  
 1 to 3 slices  
 More than 3 slices

68. How often did you eat **luncheon** or **deli-style ham**? (We will ask about other ham later.)

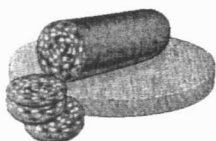
- NEVER (GO TO QUESTION 69)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

68a. Each time you ate **luncheon** or **deli-style ham**, how much did you usually eat?

- Less than 1 slice  
 1 to 3 slices  
 More than 3 slices

68b. How often was the luncheon or deli-style ham you ate **light, low-fat, or fat-free**?

- Almost never or never  
 About ¼ of the time  
 About ½ of the time  
 About ¾ of the time  
 Almost always or always



Question 69 appears in the next column

69. How often did you eat **other cold cuts** or **luncheon meats** (such as bologna, salami, corned beef, pastrami, or others, including low-fat)? (Please do not include ham, turkey, or chicken cold cuts.)

- NEVER (GO TO QUESTION 70)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

69a. Each time you ate **other cold cuts** or **luncheon meats**, how much did you usually eat?

- Less than 1 slice  
 1 to 3 slices  
 More than 3 slices

69b. How often were the other cold cuts or luncheon meats you ate **light, low-fat, or fat-free cold cuts or luncheon meats**? (Please do not include ham, turkey, or chicken cold cuts.)

- Almost never or never  
 About ¼ of the time  
 About ½ of the time  
 About ¾ of the time  
 Almost always or always

70. How often did you eat **canned tuna** (including in salads, sandwiches, or casseroles)?

- NEVER (GO TO QUESTION 71)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

70a. Each time you ate **canned tuna**, how much did you usually eat?

- Less than ¼ cup or less than 2 ounces  
 ¼ to ½ cup or 2 to 3 ounces  
 More than ½ cup or more than 3 ounces

70b. How often was the canned tuna you ate **water-packed tuna**?

- Almost never or never  
 About ¼ of the time  
 About ½ of the time  
 About ¾ of the time  
 Almost always or always

Question 71 appears on the next page

This is a sample form. Do not use for scanning.

Over the past 12 months...

70c. How often was the canned tuna you ate prepared with mayonnaise or other dressing (including low-fat)?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

71. How often did you eat **GROUND** chicken or turkey? (We will ask about other chicken and turkey later.)

- NEVER (GO TO QUESTION 72)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

71a. Each time you ate **GROUND** chicken or turkey, how much did you usually eat?

- Less than 2 ounces or less than 1/2 cup
- 2 to 4 ounces or 1/2 to 1 cup
- More than 4 ounces or more than 1 cup

72. How often did you eat **beef hamburgers** or **cheeseburgers**?

- NEVER (GO TO QUESTION 73)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

72a. Each time you ate **beef hamburgers** or **cheeseburgers**, how much did you usually eat?

- Less than 1 patty or less than 2 ounces
- 1 patty or 2 to 4 ounces
- More than 1 patty or more than 4 ounces

72b. How often were the beef hamburgers or cheeseburgers you ate made with **lean ground beef**?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

Question 73 appears in the next column

73. How often did you eat **ground beef in mixtures** (such as meatballs, casseroles, chili, or meatloaf)?

- NEVER (GO TO QUESTION 74)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

73a. Each time you ate **ground beef in mixtures**, how much did you usually eat?

- Less than 3 ounces or less than 1/2 cup
- 3 to 8 ounces or 1/2 to 1 cup
- More than 8 ounces or more than 1 cup

74. How often did you eat **hot dogs** or **frankfurters**? (Please do not include sausages or vegetarian hot dogs.)

- NEVER (GO TO QUESTION 75)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

74a. Each time you ate **hot dogs** or **frankfurters**, how many did you usually eat?

- Less than 1 hot dog
- 1 to 2 hot dogs
- More than 2 hot dogs

74b. How often were the hot dogs or frankfurters you ate **light** or **low-fat** hot dogs?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always



Question 75 appears on the next page

This is a sample form. Do not use for scanning.

Over the past 12 months...

75. How often did you eat beef mixtures such as **beef stew, beef pot pie, beef and noodles, or beef and vegetables**?

- NEVER (GO TO QUESTION 76)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

75a. Each time you ate **beef stew, beef pot pie, beef and noodles, or beef and vegetables**, how much did you usually eat?

- Less than 1 cup  
 1 to 2 cups  
 More than 2 cups

76. How often did you eat **roast beef or pot roast**?  
*(Please do not include roast beef or pot roast in sandwiches.)*

- NEVER (GO TO QUESTION 77)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

76a. Each time you ate **roast beef or pot roast** (including in mixtures), how much did you usually eat?

- Less than 2 ounces  
 2 to 5 ounces  
 More than 5 ounces

77. How often did you eat **steak** (beef)? *(Do not include steak in sandwiches)*

- NEVER (GO TO QUESTION 78)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

77a. Each time you ate **steak** (beef), how much did you usually eat?

- Less than 3 ounces  
 3 to 7 ounces  
 More than 7 ounces

Question 78 appears in the next column

77b. How often was the steak you ate **lean steak**?

- Almost never or never  
 About 1/4 of the time  
 About 1/2 of the time  
 About 3/4 of the time  
 Almost always or always

78. How often did you eat **pork or beef spareribs**?

- NEVER (GO TO QUESTION 79)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

78a. Each time you ate **pork or beef spareribs**, how much did you usually eat?

- Less than 4 ribs  
 4 to 12 ribs  
 More than 12 ribs

79. How often did you eat **roast turkey, turkey cutlets, or turkey nuggets** (including in sandwiches)?

- NEVER (GO TO QUESTION 80)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

79a. Each time you ate **roast turkey, turkey cutlets, or turkey nuggets**, how much did you usually eat? *(Please note: 4 to 8 turkey nuggets = 3 ounces.)*

- Less than 2 ounces  
 2 to 4 ounces  
 More than 4 ounces

80. How often did you eat **chicken** as part of **salads, sandwiches, casseroles, stews, or other mixtures**?

- NEVER (GO TO QUESTION 81)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

Question 81 appears on the next page

This is a sample form. Do not use for scanning.

Over the past 12 months...

80a. Each time you ate **chicken** as part of **salads, sandwiches, casseroles, stews, or other mixtures**, how much did you usually eat?

- Less than 1/2 cup
- 1/2 to 1 1/2 cups
- More than 1 1/2 cups

81. How often did you eat **baked, broiled, roasted, stewed, or fried chicken** (including nuggets)?  
(Please do not include chicken in mixtures.)

- NEVER (GO TO QUESTION 82)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

81a. Each time you ate **baked, broiled, roasted, stewed, or fried chicken** (including nuggets), how much did you usually eat?

- Less than 2 drumsticks or wings, less than 1 breast or thigh, or less than 4 nuggets
- 2 drumsticks or wings, 1 breast or thigh, or 4 to 8 nuggets
- More than 2 drumsticks or wings, more than 1 breast or thigh, or more than 8 nuggets

81b. How often was the chicken you ate **fried chicken** (including deep fried) or **chicken nuggets**?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

81c. How often was the chicken you ate **WHITE meat**?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

81d. How often did you eat chicken **WITH skin**?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

Question 82 appears in the next column

82. How often did you eat **baked ham or ham steak**?

- NEVER (GO TO QUESTION 83)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

82a. Each time you ate **baked ham or ham steak**, how much did you usually eat?

- Less than 1 ounce
- 1 to 3 ounces
- More than 3 ounces

83. How often did you eat **pork** (including chops, roasts, and in mixed dishes)? (Please do not include ham, ham steak, or sausage.)

- NEVER (GO TO QUESTION 84)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

83a. Each time you ate **pork**, how much did you usually eat?

- Less than 2 ounces or less than 1 chop
- 2 to 5 ounces or 1 chop
- More than 5 ounces or more than 1 chop

84. How often did you eat **gravy** on meat, chicken, potatoes, rice, etc.?

- NEVER (GO TO QUESTION 85)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

84a. Each time you ate **gravy** on meat, chicken, potatoes, rice, etc., how much did you usually eat?

- Less than 1/4 cup
- 1/4 to 1/2 cup
- More than 1/2 cup

22

Question 85 appears on the next page

**This is a sample form. Do not use for scanning.**

Over the past 12 months...

85. How often did you eat **liver** (all kinds) or **liverwurst**?

- NEVER (GO TO QUESTION 86)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

85a. Each time you ate **liver** or **liverwurst**, how much did you usually eat?

- Less than 1 ounce  
 1 to 4 ounces  
 More than 4 ounces

86. How often did you eat **bacon** (including low-fat)?

- NEVER (GO TO QUESTION 87)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

86a. Each time you ate **bacon**, how much did you usually eat?

- Fewer than 2 slices  
 2 to 3 slices  
 More than 3 slices

86b. How often was the bacon you ate **light, low-fat, or lean bacon**?

- Almost never or never  
 About ¼ of the time  
 About ½ of the time  
 About ¾ of the time  
 Almost always or always

87. How often did you eat **sausage** (including low-fat)?

- NEVER (GO TO QUESTION 88)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

Question 88 appears in the next column

87a. Each time you ate **sausage**, how much did you usually eat?

- Less than 1 patty or 2 links  
 1 to 3 patties or 2 to 5 links  
 More than 3 patties or 5 links

87b. How often was the sausage you ate **light, low-fat, or lean sausage**?

- Almost never or never  
 About ¼ of the time  
 About ½ of the time  
 About ¾ of the time  
 Almost always or always

88. How often did you eat **fish sticks** or **fried fish** (including fried seafood or shellfish)?

- NEVER (GO TO QUESTION 89)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

88a. Each time you ate **fish sticks** or **fried fish**, how much did you usually eat?

- Less than 2 ounces or less than 1 fillet  
 2 to 7 ounces or 1 fillet  
 More than 7 ounces or more than 1 fillet

89. How often did you eat **fish** or **seafood that was NOT FRIED** (including shellfish)?

- NEVER (GO TO INTRODUCTION TO QUESTION 90)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

89a. Each time you ate **fish** or **seafood that was NOT FRIED**, how much did you usually eat?

- Less than 2 ounces or less than 1 fillet  
 2 to 5 ounces or 1 fillet  
 More than 5 ounces or more than 1 fillet





This is a sample form. Do not use for scanning.

Over the past 12 months...

Now think about all the meat, poultry, and fish you ate in the past 12 months and how they were prepared.

90. How often was oil, butter, margarine, or other fat used to FRY, SAUTE, BASTE, OR MARINATE any meat, poultry, or fish you ate? (Please do not include deep frying.)

- NEVER (GO TO QUESTION 91)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

90a. Which of the following fats were regularly used to prepare your meat, poultry, or fish? (Mark all that apply.)

- |  |   |
|--|---|
| <input type="checkbox"/> Margarine (including low-fat) | <input type="checkbox"/> Corn oil                         |
| <input type="checkbox"/> Butter (including low-fat)    | <input type="checkbox"/> Canola or rapeseed oil           |
| <input type="checkbox"/> Lard, fatback, or bacon fat   | <input type="checkbox"/> Oil spray, such as Pam or others |
| <input type="checkbox"/> Olive oil                     | <input type="checkbox"/> Other kinds of oils              |
|  | <input type="checkbox"/> None of the above                |

91. How often did you eat tofu, soy burgers, or soy meat-substitutes?

- NEVER (GO TO QUESTION 92)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

91a. Each time you ate tofu, soy burgers, or soy meat-substitutes, how much did you usually eat?

- Less than 1/4 cup or less than 2 ounces  
 1/4 to 1/2 cup or 2 to 4 ounces  
 More than 1/2 cup or more than 4 ounces

Question 92 appears in the next column

92. Over the past 12 months, did you eat soups?

- NO (GO TO QUESTION 93)  
 YES

92a. How often did you eat soup DURING THE WINTER?

- NEVER
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per winter  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per winter | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month      | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month   | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week       | <input type="checkbox"/> 2 or more times per day |

92b. How often did you eat soup DURING THE REST OF THE YEAR?

- NEVER
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

92c. Each time you ate soup, how much did you usually eat?

- Less than 1 cup  
 1 to 2 cups  
 More than 2 cups

92d. How often were the soups you ate bean soups?

- Almost never or never  
 About 1/4 of the time  
 About 1/2 of the time  
 About 3/4 of the time  
 Almost always or always

92e. How often were the soups you ate cream soups (including chowders)?

- Almost never or never  
 About 1/4 of the time  
 About 1/2 of the time  
 About 3/4 of the time  
 Almost always or always

Question 93 appears on the next page

This is a sample form. Do not use for scanning.

Over the past 12 months...

92f. How often were the soups you ate **tomato** or **vegetable soups**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

92g. How often were the soups you ate **broth soups** (including chicken) **with** or **without noodles** or **rice**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

93. How often did you eat **pizza**?

- NEVER (GO TO QUESTION 94)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

93a. Each time you ate **pizza**, how much did you usually eat?

- Less than 1 slice or less than 1 mini pizza
- 1 to 3 slices or 1 mini pizza
- More than 3 slices or more than 1 mini pizza

93b. How often did you eat pizza with **pepperoni**, **sausage**, or **other meat**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

94. How often did you eat **crackers**?

- NEVER (GO TO QUESTION 95)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

Question 95 appears in the next column

94a. Each time you ate **crackers**, how many did you usually eat?

- Fewer than 4 crackers
- 4 to 10 crackers
- More than 10 crackers

95. How often did you eat **corn bread** or **corn muffins**?

- NEVER (GO TO QUESTION 96)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

95a. Each time you ate **corn bread** or **corn muffins**, how much did you usually eat?

- Less than 1 piece or muffin
- 1 to 2 pieces or muffins
- More than 2 pieces or muffins

96. How often did you eat **biscuits**?

- NEVER (GO TO QUESTION 97)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

96a. Each time you ate **biscuits**, how many did you usually eat?

- Fewer than 1 biscuit
- 1 to 2 biscuits
- More than 2 biscuits

97. How often did you eat **potato chips**, **tortilla chips**, or **corn chips** (including low-fat, fat-free, or low-salt)?

- NEVER (GO TO QUESTION 98)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

Question 98 appears on the next page

This is a sample form. Do not use for scanning.

Over the past 12 months...

97a. Each time you ate **potato chips, tortilla chips, or corn chips**, how much did you usually eat?

- Fewer than 10 chips or less than 1 cup
- 10 to 25 chips or 1 to 2 cups
- More than 25 chips or more than 2 cups

97b. How often were the chips you ate **Wow chips** or other **chips made with fat substitute** (Olean or Olestra)?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

97c. How often were the chips you ate other **low-fat** or **fat-free chips**?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

98. How often did you eat **popcorn** (including low-fat)?

- NEVER (GO TO QUESTION 99)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

98a. Each time you ate **popcorn**, how much did you usually eat?

- Less than 2 cups, popped
- 2 to 5 cups, popped
- More than 5 cups, popped

99. How often did you eat **pretzels**?

- NEVER (GO TO QUESTION 100)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

Question 100 appears in the next column

99a. Each time you ate **pretzels**, how many did you usually eat?

- Fewer than 5 average twists
- 5 to 20 average twists
- More than 20 average twists

100. How often did you eat **peanuts, walnuts, seeds, or other nuts**?

- NEVER (GO TO QUESTION 101)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

100a. Each time you ate **peanuts, walnuts, seeds, or other nuts**, how much did you usually eat?

- Less than 1/4 cup
- 1/4 to 1/2 cup
- More than 1/2 cup

101. How often did you eat **energy, high-protein, or breakfast bars** such as **Power Bars, Balance, Clif, or others**?

- NEVER (GO TO QUESTION 102)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

101a. Each time you ate **energy, high-protein, or breakfast bars**, how much did you usually eat?

- Less than 1 bar
- 1 bar
- More than 1 bar

102. How often did you eat **yogurt** (NOT including frozen yogurt)?

- NEVER (GO TO QUESTION 103)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

Question 103 appears on the next page

This is a sample form. Do not use for scanning.

Over the past 12 months...

102a. Each time you ate **yogurt**, how much did you usually eat?

- Less than 1/2 cup or less than 1 container
- 1/2 to 1 cup or 1 container
- More than 1 cup or more than 1 container

103. How often did you eat **cottage cheese** (including low-fat)?

- NEVER (GO TO QUESTION 104)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

103a. Each time you ate **cottage cheese**, how much did you usually eat?

- Less than 1/4 cup
- 1/4 to 1 cup
- More than 1 cup

104. How often did you eat **cheese** (including low-fat; including on cheeseburgers or in sandwiches or subs)?

- NEVER (GO TO QUESTION 105)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

104a. Each time you ate **cheese**, how much did you usually eat?

- Less than 1/2 ounce or less than 1 slice
- 1/2 to 1 1/2 ounces or 1 slice
- More than 1 1/2 ounces or more than 1 slice

104b. How often was the cheese you ate **light or low-fat cheese**?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

Question 105 appears in the next column

104c. How often was the cheese you ate **fat-free cheese**?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

105. How often did you eat **frozen yogurt, sorbet, or ices** (including low-fat or fat-free)?

- NEVER (GO TO QUESTION 106)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

105a. Each time you ate **frozen yogurt, sorbet, or ices**, how much did you usually eat?

- Less than 1/2 cup or less than 1 scoop
- 1/2 to 1 cup or 1 to 2 scoops
- More than 1 cup or more than 2 scoops

106. How often did you eat **ice cream, ice cream bars, or sherbet** (including low-fat or fat-free)?

- NEVER (GO TO QUESTION 107)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

106a. Each time you ate **ice cream, ice cream bars, or sherbet**, how much did you usually eat?

- Less than 1/2 cup or less than 1 scoop
- 1/2 to 1 1/2 cups or 1 to 2 scoops
- More than 1 1/2 cups or more than 2 scoops

106b. How often was the ice cream you ate **light, low-fat, or fat-free ice cream or sherbet**?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

Question 107 appears on the next page

This is a sample form. Do not use for scanning.

Over the **past 12 months**...

107. How often did you eat **cake** (including low-fat or fat-free)?

- NEVER (GO TO QUESTION 108)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

107a. Each time you ate **cake**, how much did you usually eat?

- Less than 1 medium piece  
 1 medium piece  
 More than 1 medium piece

107b. How often was the cake you ate **light, low-fat, or fat-free cake**?

- Almost never or never  
 About 1/4 of the time  
 About 1/2 of the time  
 About 3/4 of the time  
 Almost always or always

108. How often did you eat **cookies or brownies** (including low-fat or fat-free)?

- NEVER (GO TO QUESTION 109)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

108a. Each time you ate **cookies or brownies**, how much did you usually eat?

- Less than 2 cookies or 1 small brownie  
 2 to 4 cookies or 1 medium brownie  
 More than 4 cookies or 1 large brownie

108b. How often were the cookies or brownies you ate **light, low-fat, or fat-free cookies or brownies**?

- Almost never or never  
 About 1/4 of the time  
 About 1/2 of the time  
 About 3/4 of the time  
 Almost always or always

Question 109 appears in the next column

109. How often did you eat **doughnuts, sweet rolls, Danish, or pop-tarts**?

- NEVER (GO TO QUESTION 110)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

109a. Each time you ate **doughnuts, sweet rolls, Danish, or pop-tarts**, how much did you usually eat?

- Less than 1 piece  
 1 to 2 pieces  
 More than 2 pieces

110. How often did you eat **sweet muffins or dessert breads** (including low-fat or fat-free)?

- NEVER (GO TO QUESTION 111)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

110a. Each time you ate **sweet muffins or dessert breads**, how much did you usually eat?

- Less than 1 medium piece  
 1 medium piece  
 More than 1 medium piece

110b. How often were the sweet muffins or dessert breads you ate **light, low-fat, or fat-free sweet muffins or dessert breads**?

- Almost never or never  
 About 1/4 of the time  
 About 1/2 of the time  
 About 3/4 of the time  
 Almost always or always

111. How often did you eat **fruit crisp, cobbler, or strudel**?

- NEVER (GO TO QUESTION 112)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

Question 112 appears on the next page

This is a sample form. Do not use for scanning.

Over the past 12 months...

111a. Each time you ate **fruit crisp, cobbler, or strudel**, how much did you usually eat?

- Less than 1/2 cup
- 1/2 to 1 cup
- More than 1 cup

112. How often did you eat **pie**?

- NEVER (GO TO QUESTION 113)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

112a. Each time you ate **pie**, how much did you usually eat?

- Less than 1/4 of a pie
- About 1/4 of a pie
- More than 1/4 of a pie

The next four questions ask about the kinds of pie you ate. Please read all four questions before answering.

112b. How often were the pies you ate **fruit pie** (such as apple, blueberry, others)?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

112c. How often were the pies you ate **cream, pudding, custard, or meringue pie**?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

112d. How often were the pies you ate **pumpkin or sweet potato pie**?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

Question 113 appears in the next column

112e. How often were the pies you ate **pecan pie**?

- Almost never or never
- About 1/4 of the time
- About 1/2 of the time
- About 3/4 of the time
- Almost always or always

113. How often did you eat **chocolate candy**?

- NEVER (GO TO QUESTION 114)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

113a. Each time you ate **chocolate candy**, how much did you usually eat?

- Less than 1 average bar or less than 1 ounce
- 1 average bar or 1 to 2 ounces
- More than 1 average bar or more than 2 ounces

114. How often did you eat **other candy**?

- NEVER (GO TO QUESTION 115)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

114a. Each time you ate **other candy**, how much did you usually eat?

- Fewer than 2 pieces
- 2 to 9 pieces
- More than 9 pieces

115. How often did you eat **eggs, egg whites, or egg substitutes** (NOT counting eggs in baked goods and desserts)? (Please include eggs in salads, quiche, and soufflés.)

- NEVER (GO TO QUESTION 116)
- 1-6 times per year
- 7-11 times per year
- 1 time per month
- 2-3 times per month
- 1 time per week
- 2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2 or more times per day

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Question 116 appears on the next page

This is a sample form. Do not use for scanning.

Over the **past 12 months...**

115a. Each time you ate **eggs**, how many did you usually eat?

- 1 egg
- 2 eggs
- 3 or more eggs

115b. How often were the eggs you ate **egg substitutes**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

115c. How often were the eggs you ate **egg whites only**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

115d. How often were the eggs you ate **regular whole eggs**?

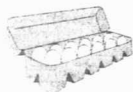
- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

115e. How often were the eggs you ate cooked in **oil, butter, or margarine**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

115f. How often were the eggs you ate part of **egg salad**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always



Question 116 appears in the next column

116. How many cups of **coffee**, caffeinated or decaffeinated, did you drink?

- NEVER (GO TO QUESTION 117)
- Less than 1 cup per month
- 1-3 cups per month
- 1 cup per week
- 2-4 cups per week
- 5-6 cups per week
- 1 cup per day
- 2-3 cups per day
- 4-5 cups per day
- 6 or more cups per day

116a. How often was the coffee you drank **decaffeinated**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

117. How many glasses of **ICED tea**, caffeinated or decaffeinated, did you drink?

- NEVER (GO TO QUESTION 118)
- Less than 1 cup per month
- 1-3 cups per month
- 1 cup per week
- 2-4 cups per week
- 5-6 cups per week
- 1 cup per day
- 2-3 cups per day
- 4-5 cups per day
- 6 or more cups per day

117a. How often was the iced tea you drank **decaffeinated or herbal tea**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

118. How many cups of **HOT tea**, caffeinated or decaffeinated, did you drink?

- NEVER (GO TO QUESTION 119)
- Less than 1 cup per month
- 1-3 cups per month
- 1 cup per week
- 2-4 cups per week
- 5-6 cups per week
- 1 cup per day
- 2-3 cups per day
- 4-5 cups per day
- 6 or more cups per day

118a. How often was the hot tea you drank **decaffeinated or herbal tea**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

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Question 119 appears on the next page

This is a sample form. Do not use for scanning.

Over the past 12 months...

119. How often did you add **sugar** or **honey** to your coffee or tea?

- NEVER (GO TO QUESTION 120)
- |   |  |
|---|--|
| <input type="checkbox"/> Less than 1 time per month | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 1-3 times per month        | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week            | <input type="checkbox"/> 2-3 times per day       |
| <input type="checkbox"/> 2-4 times per week         | <input type="checkbox"/> 4-5 times per day       |
|   | <input type="checkbox"/> 6 or more times per day |

119a. Each time **sugar** or **honey** was added to your coffee or tea, how much was usually added?

- Less than 1 teaspoon  
 1 to 3 teaspoons  
 More than 3 teaspoons

120. How often did you add **artificial sweetener** to your coffee or tea?

- NEVER (GO TO QUESTION 121)
- |   |  |
|---|--|
| <input type="checkbox"/> Less than 1 time per month | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 1-3 times per month        | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week            | <input type="checkbox"/> 2-3 times per day       |
| <input type="checkbox"/> 2-4 times per week         | <input type="checkbox"/> 4-5 times per day       |
|   | <input type="checkbox"/> 6 or more times per day |

120a. What kind of **artificial sweetener** did you usually use?

- Equal or aspartame  
 Sweet N Low or saccharin

121. How often was **non-dairy creamer** added to your coffee or tea?

- NEVER (GO TO QUESTION 122)
- |   |  |
|---|--|
| <input type="checkbox"/> Less than 1 time per month | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 1-3 times per month        | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week            | <input type="checkbox"/> 2-3 times per day       |
| <input type="checkbox"/> 2-4 times per week         | <input type="checkbox"/> 4-5 times per day       |
|   | <input type="checkbox"/> 6 or more times per day |

121a. Each time **non-dairy creamer** was added to your coffee or tea, how much was usually used?

- Less than 1 teaspoon  
 1 to 3 teaspoons  
 More than 3 teaspoons

Question 122 appears in the next column

121b. What kind of **non-dairy creamer** did you usually use?

- Regular powdered  
 Low-fat or fat-free powdered  
 Regular liquid  
 Low-fat or fat-free liquid

122. How often was **cream** or **half and half** added to your coffee or tea?

- NEVER (GO TO QUESTION 123)
- |   |  |
|---|--|
| <input type="checkbox"/> Less than 1 time per month | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 1-3 times per month        | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week            | <input type="checkbox"/> 2-3 times per day       |
| <input type="checkbox"/> 2-4 times per week         | <input type="checkbox"/> 4-5 times per day       |
|   | <input type="checkbox"/> 6 or more times per day |

122a. Each time **cream** or **half and half** was added to your coffee or tea, how much was usually added?

- Less than 1 tablespoon  
 1 to 2 tablespoons  
 More than 2 tablespoons

123. How often was **milk** added to your coffee or tea?

- NEVER (GO TO QUESTION 124)
- |   |  |
|---|--|
| <input type="checkbox"/> Less than 1 time per month | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 1-3 times per month        | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week            | <input type="checkbox"/> 2-3 times per day       |
| <input type="checkbox"/> 2-4 times per week         | <input type="checkbox"/> 4-5 times per day       |
|   | <input type="checkbox"/> 6 or more times per day |

123a. Each time **milk** was added to your coffee or tea, how much was usually added?

- Less than 1 tablespoon  
 1 to 3 tablespoons  
 More than 3 tablespoons

123b. What kind of **milk** was usually added to your coffee or tea?

- Whole milk  
 2% milk  
 1% milk  
 Skim, nonfat, or ½% milk  
 Evaporated or condensed (canned) milk  
 Soy milk  
 Rice milk  
 Other

Question 124 appears on the next page



This is a sample form. Do not use for scanning.

Over the past 12 months...

124. How often was **sugar** or **honey** added to foods you ate? (Please do not include sugar in coffee, tea, other beverages, or baked goods.)

- NEVER (GO TO INTRODUCTION TO QUESTION 125)
- |  |  |
|--|--|
| <input type="checkbox"/> 1-6 times per year  | <input type="checkbox"/> 2 times per week        |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 3-4 times per week      |
| <input type="checkbox"/> 1 time per month    | <input type="checkbox"/> 5-6 times per week      |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 1 time per day          |
| <input type="checkbox"/> 1 time per week     | <input type="checkbox"/> 2 or more times per day |

124a. Each time **sugar** or **honey** was added to foods you ate, how much was usually added?

- Less than 1 teaspoon  
 1 to 3 teaspoons  
 More than 3 teaspoons

The following questions are about the kinds of margarine, mayonnaise, sour cream, cream cheese, and salad dressing that you eat. If possible, please check the labels of these foods to help you answer.

125. Over the past 12 months, did you eat margarine?

- NO (GO TO QUESTION 126)  
 YES

125a. How often was the margarine you ate **regular-fat margarine** (stick or tub)?

- Almost never or never  
 About 1/4 of the time  
 About 1/2 of the time  
 About 3/4 of the time  
 Almost always or always

125b. How often was the margarine you ate **light** or **low-fat margarine** (stick or tub)?

- Almost never or never  
 About 1/4 of the time  
 About 1/2 of the time  
 About 3/4 of the time  
 Almost always or always

Question 126 appears in the next column

125c. How often was the margarine you ate **fat-free margarine**?

- Almost never or never  
 About 1/4 of the time  
 About 1/2 of the time  
 About 3/4 of the time  
 Almost always or always

126. Over the past 12 months, did you eat **butter**?

- NO (GO TO QUESTION 127)  
 YES

126a. How often was the butter you ate **light** or **low-fat butter**?

- Almost never or never  
 About 1/4 of the time  
 About 1/2 of the time  
 About 3/4 of the time  
 Almost always or always

127. Over the past 12 months, did you eat mayonnaise or mayonnaise-type dressing?

- NO (GO TO QUESTION 128)  
 YES

127a. How often was the mayonnaise you ate **regular-fat mayonnaise**?

- Almost never or never  
 About 1/4 of the time  
 About 1/2 of the time  
 About 3/4 of the time  
 Almost always or always

127b. How often was the mayonnaise you ate **light** or **low-fat mayonnaise**?

- Almost never or never  
 About 1/4 of the time  
 About 1/2 of the time  
 About 3/4 of the time  
 Almost always or always

32 Question 128 appears on the next page

This is a sample form. Do not use for scanning.

Over the past 12 months...

127c. How often was the mayonnaise you ate **fat-free mayonnaise**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

128. Over the past 12 months, did you eat **sour cream**?

NO (GO TO QUESTION 129)

YES

128a. How often was the sour cream you ate **regular-fat sour cream**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

128b. How often was the sour cream you ate **light, low-fat, or fat-free sour cream**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

129. Over the past 12 months, did you eat **cream cheese**?

NO (GO TO QUESTION 130)

YES

129a. How often was the cream cheese you ate **regular-fat cream cheese**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

Question 130 appears in the next column

129b. How often was the cream cheese you ate **light, low-fat, or fat-free cream cheese**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

130. Over the past 12 months, did you eat **salad dressing**?

NO (GO TO INTRODUCTION TO QUESTION 131)

YES

130a. How often was the salad dressing you ate **regular-fat salad dressing** (including oil and vinegar dressing)?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

130b. How often was the salad dressing you ate **light or low-fat salad dressing**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

130c. How often was the salad dressing you ate **fat-free salad dressing**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

The following two questions ask you to summarize your usual intake of vegetables and fruits. Please do not include salads, potatoes, or juices.

131. Over the past 12 months, how many servings of **vegetables** (not including salad or potatoes) did you eat per week or per day?

- |   |  |
|---|--|
| <input type="checkbox"/> Less than 1 per week | <input type="checkbox"/> 2 per day         |
| <input type="checkbox"/> 1-2 per week         | <input type="checkbox"/> 3 per day         |
| <input type="checkbox"/> 3-4 per week         | <input type="checkbox"/> 4 per day         |
| <input type="checkbox"/> 5-6 per week         | <input type="checkbox"/> 5 or more per day |
| <input type="checkbox"/> 1 per day            |  |

This is a sample form. Do not use for scanning.

Over the past 12 months...

132. Over the past 12 months, how many servings of **fruit** (not including juices) did you eat per week or per day?

- |   |  |
|---|--|
| <input type="checkbox"/> Less than 1 per week | <input type="checkbox"/> 2 per day         |
| <input type="checkbox"/> 1-2 per week         | <input type="checkbox"/> 3 per day         |
| <input type="checkbox"/> 3-4 per week         | <input type="checkbox"/> 4 per day         |
| <input type="checkbox"/> 5-6 per week         | <input type="checkbox"/> 5 or more per day |
| <input type="checkbox"/> 1 per day            |  |

133. Over the past month, which of the following foods did you eat **AT LEAST THREE TIMES**?  
(Mark all that apply.)

- |   |   |
|---|---|
| <input type="checkbox"/> Avocado, guacamole                                   | <input type="checkbox"/> Olives                                 |
| <input type="checkbox"/> Cheesecake   | <input type="checkbox"/> Oysters                                |
| <input type="checkbox"/> Chocolate, fudge, or butterscotch toppings or syrups | <input type="checkbox"/> Pickles or pickled vegetables or fruit |
| <input type="checkbox"/> Chow mein noodles                                    | <input type="checkbox"/> Plantains                              |
| <input type="checkbox"/> Croissants   | <input type="checkbox"/> Pork neckbones, hock, head, feet       |
| <input type="checkbox"/> Dried apricots                                       | <input type="checkbox"/> Pudding or custard                     |
| <input type="checkbox"/> Egg rolls  | <input type="checkbox"/> Veal, venison, lamb                    |
| <input type="checkbox"/> Granola bars   | <input type="checkbox"/> Whipped cream, regular                 |
| <input type="checkbox"/> Hot peppers  | <input type="checkbox"/> Whipped cream, substitute              |
| <input type="checkbox"/> Jello, gelatin                                       |   |
| <input type="checkbox"/> Milkshakes or ice-cream sodas                        | <input type="checkbox"/> NONE                                   |

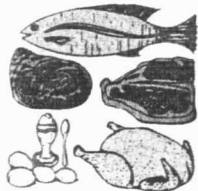
134. For **ALL** of the past 12 months, have you followed any type of **vegetarian diet**?

NO (GO TO INTRODUCTION TO QUESTION 135)

YES

134a. Which of the following foods did you **TOTALLY EXCLUDE** from your diet?  
(Mark all that apply.)

- Meat (beef, pork, lamb, etc.)
- Poultry (chicken, turkey, duck)
- Fish and seafood
- Eggs
- Dairy products (milk, cheese, etc.)



Introduction to Question 135 appears in the next column

The next questions are about your use of fiber supplements or vitamin pills.

135. Over the past 12 months, did you take any of the following types of **fiber or fiber supplements** on a regular basis (more than once per week for at least 6 of the last 12 months)?  
(Mark all that apply.)

- NO, didn't take any fiber supplements on a regular basis (GO TO QUESTION 136)
- YES, psyllium products (such as Metamucil, Fiberall, Serutan, Perdiem, Correctol)
- YES, methylcellulose/cellulose products (such as Citrucel, Unifiber)
- YES, Fibercon
- YES, Bran (such as wheat bran, oat bran, or bran wafers)

136. Over the past 12 months, did you take any **multivitamins**, such as One-a-Day-, Theragran-, or Centrum-type multivitamins (as pills, liquids, or packets)?

NO (GO TO INTRODUCTION TO QUESTION 138)

YES

137. How often did you take **One-a-day-, Theragran-, or Centrum-type** multivitamins?

- Less than 1 day per month
- 1-3 days per month
- 1-3 days per week
- 4-6 days per week
- Every day

137a. Does your **multivitamin** usually contain **minerals** (such as iron, zinc, etc.)?

- NO
- YES
- Don't know

137b. For how many years have you taken **multivitamins**?

- Less than 1 year
- 1-4 years
- 5-9 years
- 10 or more years



Introduction to Question 138 appears on the next page

This is a sample form. Do not use for scanning.

Over the past 12 months...

137c. Over the past 12 months, did you take any vitamins, minerals, or other herbal supplements other than your multivitamin?

NO

Thank you *very much* for completing this questionnaire! Because we want to be able to use all the information you have provided, we would greatly appreciate it if you would please take a moment to review each page making sure that you:

- Did not skip any pages and
- Crossed out the incorrect answer and circled the correct answer if you made any changes.

YES (GO TO INTRODUCTION TO QUESTION 138)

These last questions are about the vitamins, minerals, or herbal supplements you took that are **NOT** part of a One-a-day-, Theragran-, or Centrum-type of multivitamin.

Please include vitamins taken as part of an antioxidant supplement.

138. How often did you take **Beta-carotene** (NOT as part of a multivitamin in Question 137)?

NEVER (GO TO QUESTION 139)

- Less than 1 day per month
- 1-3 days per month
- 1-3 days per week
- 4-6 days per week
- Every day

138a. When you took **Beta-carotene**, about how much did you take in one day?

- Less than 10,000 IU
- 10,000-14,999 IU
- 15,000-19,999 IU
- 20,000-24,999 IU
- 25,000 IU or more
- Don't know

138b. For how many years have you taken **Beta-carotene**?

- Less than 1 year
- 1-4 years
- 5-9 years
- 10 or more years

Question 139 appears in the next column

139. How often did you take **Vitamin A** (NOT as part of a multivitamin in Question 137)?

NEVER (GO TO QUESTION 140)

- Less than 1 day per month
- 1-3 days per month
- 1-3 days per week
- 4-6 days per week
- Every day

139a. When you took **Vitamin A**, about how much did you take in one day?

- Less than 8,000 IU
- 8,000-9,999 IU
- 10,000-14,999 IU
- 15,000-24,999 IU
- 25,000 IU or more
- Don't know

139b. For how many years have you taken **Vitamin A**?

- Less than 1 year
- 1-4 years
- 5-9 years
- 10 or more years

140. How often did you take **Vitamin C** (NOT as part of a multivitamin in Question 137)?

NEVER (GO TO QUESTION 141)

- Less than 1 day per month
- 1-3 days per month
- 1-3 days per week
- 4-6 days per week
- Every day

140a. When you took **Vitamin C**, about how much did you take in one day?

- Less than 500 mg
- 500-999 mg
- 1,000-1,499 mg
- 1,500-1,999 mg
- 2,000 mg or more
- Don't know

140b. For how many years have you taken **Vitamin C**?

- Less than 1 year
- 1-4 years
- 5-9 years
- 10 or more years

Question 141 appears on the next page

**This is a sample form. Do not use for scanning.**

Over the past 12 months...

141. How often did you take **Vitamin E** (NOT as part of a multivitamin in Question 137)?

- NEVER (GO TO QUESTION 142)
- Less than 1 day per month  
 1-3 days per month  
 1-3 days per week  
 4-6 days per week  
 Every day

141a. When you took **Vitamin E**, about how much did you take in one day?

- Less than 400 IU  
 400-799 IU  
 800-999 IU  
 1,000 IU or more  
 Don't know

141b. For how many years have you taken **Vitamin E**?

- Less than 1 year  
 1-4 years  
 5-9 years  
 10 or more years

142. How often did you take **Calcium** or **Calcium-containing antacids** (NOT as part of a multivitamin in Question 137)?

- NEVER (GO TO QUESTION 143)
- Less than 1 day per month  
 1-3 days per month  
 1-3 days per week  
 4-6 days per week  
 Every day

142a. When you took **Calcium** or **Calcium-containing antacids**, about how much elemental calcium did you take in one day? (If possible, please check the label for elemental calcium.)

- Less than 500 mg  
 500-599 mg  
 600-999 mg  
 1,000 mg or more  
 Don't know

Question 143 appears in the next column

142b. For how many years have you taken **Calcium** or **Calcium-containing antacids**?

- Less than 1 year  
 1-4 years  
 5-9 years  
 10 or more years

The last two questions ask you about other supplements you took more than once per week.

143. Please mark any of the following **single supplements** you took more than once per week (NOT as part of a multivitamin in Question 137):

- |   |  |
|---|--|
| <input type="checkbox"/> B-6            | <input type="checkbox"/> Folic acid/folate       |
| <input type="checkbox"/> B-complex      | <input type="checkbox"/> Glucosamine             |
| <input type="checkbox"/> Brewer's yeast | <input type="checkbox"/> Hydroxytryptophan (HTP) |
| <input type="checkbox"/> Cod liver oil  | <input type="checkbox"/> Iron                    |
| <input type="checkbox"/> Coenzyme Q     | <input type="checkbox"/> Niacin                  |
| <input type="checkbox"/> Fish oil       | <input type="checkbox"/> Selenium                |
| (Omega-3 fatty acids)                   | <input type="checkbox"/> Zinc                    |

144. Please mark any of the following **herbal** or **botanical supplements** you took more than once per week.

- |   |  |
|---|--|
| <input type="checkbox"/> Aloe Vera            | <input type="checkbox"/> Ginger                      |
| <input type="checkbox"/> Astragalus           | <input type="checkbox"/> Ginkgo biloba               |
| <input type="checkbox"/> Bilberry             | <input type="checkbox"/> Ginseng (American or Asian) |
| <input type="checkbox"/> Cascara sagrada      | <input type="checkbox"/> Goldenseal                  |
| <input type="checkbox"/> Cat's claw           | <input type="checkbox"/> Grapeseed extract           |
| <input type="checkbox"/> Cayenne              | <input type="checkbox"/> Kava, kava                  |
| <input type="checkbox"/> Cranberry            | <input type="checkbox"/> Milk thistle                |
| <input type="checkbox"/> Dong Kuai (Tangkwei) | <input type="checkbox"/> Saw palmetto                |
| <input type="checkbox"/> Echinacea            | <input type="checkbox"/> Siberian ginseng            |
| <input type="checkbox"/> Evening primrose oil | <input type="checkbox"/> St. John's wort             |
| <input type="checkbox"/> Feverfew             | <input type="checkbox"/> Valerian                    |
| <input type="checkbox"/> Garlic               | <input type="checkbox"/> Other                       |

Thank you *very much* for completing this questionnaire! Because we want to be able to use all the information you have provided, we would greatly appreciate it if you would please take a moment to review each page making sure that you:

- Did not skip any pages and
- Crossed out the incorrect answer and circled the correct answer if you made any changes.