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TITLE: Phase I Induction and Estrogen Metabolism in Women With and Without Breast Cancer and in Response to a Dietary Intervention

PRINCIPAL INVESTIGATOR: James R. Hebert, Sc.D.

CONTRACTING ORGANIZATION: University of South Carolina
Columbia, South Carolina 29208

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James R. Hebert, Sc.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of South Carolina
Columbia, South Carolina 29208

E-Mail: jhebert@sph.sc.edu

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13. ABSTRACT ***(Maximum 200 Words)***
This combined case-comparison study and randomized controlled trial (RCT) of 90 women is based on our prior epidemiologic work (1-6) indicating that vegetables in the Brassica genus (e.g., broccoli, cauliflower, brussel sprouts) can modify estrogen metabolism by causing 17β- Estradiol (E2) to be metabolized to 2-hydroxyestrone (2HE) rather than 16α-hydroxyestrone (16HE) thus producing a cascade of effects protective against breast cancer (2). Our plan is to enroll 45 postmenopausal women with breast cancer and 45 age-matched disease-free women and to compare them on: 1) AhR activation and its various protein products relevant to cancer including CYP1B1, PAI-2, and IL-1□; and 2) levels of relevant estrogens, E2, 2HE, and 16HE.

The RCT will examine the effect of an intensive Brassica-rich diet intervention on AhR activation, its protein products, and estrogen metabolites in these women. This study is beginning its second full year of funded activity. All protocols for the collection of data are finalized and we have begun to recruit participants. The baseline data are being collected for case-comparison study and the first of four intervention cycles for the RCT will begin early in 2002. Specific accomplishments are described in the following narrative, in parallel with the original Statement of Work.

1. Fowke JH, Longcope C, Hebert JR. Macronutrient intake and estrogen metabolism in healthy postmenopausal women. Breast Cancer Res Treat 2001; 65:1-10.
2. Fowke JH, Longcope C, Hebert JR. Brassica vegetable consumption shifts estrogen metabolism in healthy postmenopausal women. Cancer Epidemiol Biomark Prev 2000; 9:773-779.
3. Hebert JR, Hurley TG, Ma Y. The effect of dietary exposures on recurrence and mortality in early stage breast cancer. Breast Cancer Res Treat 1998; 51:17-28.
4. Hebert JR, Rosen A. Nutritional, socioeconomic, and reproductive factors in relation to female breast cancer mortality: findings from a cross-national study. Cancer Detect Prevent 1996; 20:234-44.
5. Hebert JR, Wynder EL. Dietary fat and the risk of breast cancer. N Engl J Med 1987; 317:165-166.
6. Hebert JR, Toporoff E. Dietary exposures and other factors of possible prognostic significance in relation to tumor size and nodal involvement in early-stage breast cancer. Int J Epidemiol 1989; 18:518-526.

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Phase I Induction and Estrogen Metabolism in Women With and Without Breast Cancer and in Response to a Dietary Intervention

Annual Report: Year 2

This study is beginning its second full year of funded activity. All protocols for the collection of data are finalized and we have begun to recruit participants. The first of four intervention cycles will begin early in 2002. Specific accomplishments are described in the following narrative, in parallel with the original Statement of Work.

Introduction

Work by our group and others provide the scientific basis of this study (1-11). Cross-national studies of breast cancer rates and studies of migrants indicate that environmental factors are responsible for large population-level differences in breast cancer rates and rates of change over time. In a study of 46 countries, we found that over 90% of breast cancer mortality could be accounted for mainly by dietary factors (12). On a per-calorie basis, the strongest effect in the data was the protective effect of cabbage. There is some evidence that vegetables in the *Brassica* genus, like cabbage and broccoli, modify estrogen metabolism by causing 17β- Estradiol (E2) to be metabolized to 2-hydroxyestrone (2HE) rather than 16α-hydroxyestrone (16HE). Relative to 2HE, 16HE appears more likely to cause cancer and breast cancer patients have a lower ratio of these metabolites than do disease-free controls. It has further been shown that the P450 enzyme CYP1B1 is present in tumor but not normal breast tissue. The indole glucosinolates (IGSL), which are contained in high concentrations in Brassica vegetables, induce a number of protein products that can shift E2 metabolism away from 16HE and towards 2HE. AhR activation also induces immune system factors such as interleukin-1β (IL-1β) and other proteins, such as plasminogen activator inhibitor-2 (PAI-2), a protease inhibitor that has been associated with inhibition of tumor invasiveness (metastasis).

Specific Aims

The two objectives of this proposal are to evaluate the products of AhR activation against the risk of breast cancer, and to investigate the ability of *Brassica* vegetables to reduce breast cancer risk. Women will be recruited from among those who have undergone a diagnostic biopsy at SCCC following a suspicious mammogram. The plan is to enroll 45 postmenopausal women who have had breast cancer and 45 age-matched women found to be disease free. The first study, conducted at the time the women enter the study, will compare the 45 breast cancer patients and the 45 high-risk healthy women on: 1) AhR activation and its various protein products relevant to cancer including CYP1B1, PAI-2, and IL-1□; and 2) levels of relevant estrogens, E2, 2HE, and 16HE. The second study will examine the effect of an intensive Brassica-rich diet intervention on AhR activation, its protein products, and estrogen metabolites in these 90 women. Measurement of all study parameters will be made at times corresponding to the baseline period and post-intervention. Blood and fasting morning urine samples will be collected for measurement of the estrogens, and levels of PAI-2 and IL-1□. Adipose tissue for

assay of CYP1B1 will be collected from routine open biopsy at the time of recruitment and from a fine needle biopsy of the contralateral breast at follow-up. Diet will be assessed by use of validated diet assessment instruments. Compliance also will be assessed by levels of isothiocyanates and dithiocarbamates in urines. Statistical analyses of the data will consist of t-tests and analysis of variance of mean levels of the parameters specified in the three groups at baseline. T-tests of change and regression analyses (e.g., repeat measures ANOVA) will focus both change and relative change in the intervention trial. Post hoc analyses will examine the effect of the indole carbinols by fitting the data as continuous, which takes into account varying levels of compliance.

Distinctive subject terms

- Brassica vegetables- vegetables belonging to the Brassica genus including cabbage, broccoli, cauliflower, spinach, collards, and Brussels sprouts
- Brassica diet- consuming an intensive Brassica-rich diet
- Indole glucosinates (IGSL)- Dietary indoles are contained in Brassica vegetables and converted in the body to aryl hydrocarbon receptor (AhR) agonists that bind to AhR and induce CYP1 enzymes.
- Aryl hydrocarbon receptor (AhR)- has a role in inducing protein products that can shift E2 metabolism away from 16HE and towards 2HE. It has a role in inducing immune system factors (e.g., interleukin-1□ and other proteins (e.g., plasminogen activator inhibitor-2)
- Hydroxyestrone- two forms of this hormone are created using the 17□-Estradiol precursor (e2) including 2-Hydroxyestrone (2HE, less toxic form) and 16α-Hydroxyestrone(16HE, more toxic form)
- Cytochrome P1B1 (CYP1B1)- a phase I enzyme present in tumor but not in breast tissue

The primary hypotheses are:

1. Examine if there are differences in AhR and its protein products, including CYP1B1, PAI-2, and IL-1□ and estrogen metabolites at baseline in two subsets of women who have undergone diagnostic open breast biopsy at SCCC;
2. If intensive Brassica vegetable intake can alter levels of these products and estrogen metabolites through intensive dietary intervention on Brassica vegetable intake; and
3. If there is a relationship between CYP1B1 and estrogen metabolites, both cross-sectionally and longitudinally.

Work Accomplished

We have modified all questionnaires being used and obtained access to the Palmetto Health Association's (PHA) tumor registry database. We are also beginning active recruitment of potential study participants. Potential women have been identified from the PHA's Tumor Registry. The study information card and letters have been mailed and women will be contacted by phone during the first week of November to schedule a clinical visit. The first of four intervention cycles will begin shortly after the first of the year, as we avoid running intensive dietary interventions during the holiday season.

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Task 1: Run-in Phase, Months 1-12:

a. Inventory and finalize all assessment instruments and data collection protocols.

Assessment instruments have been inventoried and are available for use. Final versions of all assessment instruments have been produced, as stipulated in the protocol. Copies of these instruments are included in the appendix.

Below is a list of instruments being utilized.

Baseline questionnaire Measures include: Background and Demographic Data: age; sex; marital status; education; number of children; number and dates of pregnancies; breast feeding history: (months for each child); and menopausal status (including surgical menopause). Personal Health History: present medical/psychiatric history and treatment (including history of exposure to estrogens, oral contraceptives, unusual menstrual problems). Family Health History: history of breast cancer; history of other cancers. General Self Care: sleep; exercise frequency; and smoking status.

Besides data collected on the baseline instrument we will also administer these other questionnaires:

- Marlowe-Crowe Social Desirability (MCSD) scale (Personal Reaction Inventory)
- Social Approval Scale
- Multiple 24-Hour Recall Phone Interviews [note that we have changed to this method as it appears to ease participant burden and is associated with lower overall measurement error (13).
- Vegetable and Fruit Questionnaire [the paper validating this was published recently (14)
- Monitoring questionnaire
- Intervention Course Book, which includes intervention descriptions, food preparation methods, a cook book, telephone numbers of study personnel, and a brief description of the purposes of the study

New data collection protocols have been developed to fully utilize all resources under development at USC. As part of standard recruitment procedures, we mail an introductory letter and consent form to potential participants. We follow-up this letter with a telephone calls, and answer any questions regarding the study. As part of recruitment, a meeting is scheduled at the study center located within the South Carolina Cancer Center (SCCC). The SCCC facility includes an interview room, sample processing lab, and calibrated scales and measurement instruments. At the meeting, participants have the opportunity to ask additional questions regarding the consent form. After obtaining consent, we obtain a urine sample, blood sample, buccal cells, body size measurements, and participants complete the baseline questionnaire. Follow-up measurements are collected using a similar mechanism. Additionally, near the end of the intervention a clinic appointment is scheduled for collection of breast biopsy material.

b. Review baseline questionnaires for completeness and for content validity.

All instrument materials have been thoroughly reviewed and validated.

c. Revise baseline questionnaire to assess demographic, health history, and family health history, as necessary.

The Baseline Questionnaire has been expanded to include a more complete description of each participant's health history and demographic status. This expansion followed the move to USC, and the greater population diversity in SC as compared to Massachusetts. The questionnaire has been pilot tested, and appears to be sufficiently clear and complete.

d. Hire and train the Research Assistant.

Several personnel have been hired in order to complete this, and other, research projects. Dr. Jay Fowke has left to join Vanderbilt University faculty as a Research Assistant Professor. Dr. James Hebert will remain the Principal Investigator for the project and Dr. Jay Fowke will remain Co-Investigator Dr. Stephanie Muga will replace Dr. Mark Davis as Co-Investigator for the study. Mary Modayil has joined the USC doctoral program in the Department of Epidemiology and is acting as Project Coordinator, and will be largely responsible for the day-to-day operations of the project. Thomas Hurley functions as a full-time data manager. His primary responsibility focuses on developing the tracking databases necessary for ensuring complete recruitment and data collection. Additionally, he is responsible for questionnaire maintenance, questionnaire development, and data entry. Yasmin Khan, Krystal Hanrahan, and Tiffany Barker are Masters students in the Department of Epidemiology. Their primary responsibility will be to assist Dr. James Hebert in contacting potentially eligible participants, mailings, and data management.

e. Develop the study data management systems, using a combination of Lotus Notes, Microsoft Excel, and EpiInfo.

As mentioned in the previous report, we have developed an improved data management system using optical scanning technology and the Teleform software package. Lotus Notes was not used in this study as we have moved to more universally recognized solutions. All questionnaires are now optically scanned, thus avoiding operator error associated with keypunching data, and greatly speeding the data entry process. Optically scanned data are directly transferred to a SAS dataset for analysis, thus eliminating most of the need for EpiInfo.

f. Develop the tracking database in Microsoft Access and Microsoft Excel based on our experience with other intervention studies in the Department of Epidemiology and Biostatistics.

We are in the process of refining an extensive database system, which links directly with the clinical hospital patient bases and other ongoing cancer studies. This data management system is able to rapidly identify potentially eligible women receiving care at one of the

cancer centers. This information is converted to the study-specific tracking system, used for maintaining records of recruitment, participant status, and data collection.

g. Train staff in all data-related and clinic-based procedures.

We have trained staff to conduct all data-related procedures. Dr. Hebert, Mr. Hurley, and Ms. Modayil are responsible for the overall data management and statistical analysis. Mr. Hurley, the data manager, has received formal training in the Teleform software package and extensive experience using the SAS software package. The graduate research assistants have been trained in the application of Teleform and they are developing the skills necessary to perform many routine SAS data management operations. They also have been trained to collect body size measurements using standard and systematic protocols, as well as in urine and buccal cell collection, sample preparation, and storage protocols. The biopsy collection protocol will be conducted by one of the members of the Radiology Department with the PHA hospital network.

h. Develop and finalize all laboratory procedures to be used in the trial.

The majority of laboratory procedures will be conducted by Dr. Stephanie Muga at USC. With the exception of the CYP1B1 assay, all necessary laboratory protocols are commercially available as kits. Members of Dr. Muga's lab have extensive experience in forming radioimmunoassays and enzyme immunoassays as required through use of these kits.

i. Finalize all biological sample collection and storage procedures to be used in the study.

All biological sample collection and storage procedures for urine, blood, and buccal cells are finalized. The biopsy collection protocol has been developed in order to maximize volume of epithelial cells from breast tissue, due to new published findings suggesting better methods to detect CYP1B1 in breast tissue. The assay protocol is almost finalized with the help of Dr. Muga's lab with the goal of increasing sensitivity of the antibody to the CYP1B1.

j. Establish recruitment procedures for women entering the study, including pre-screen for certain criteria such as menopausal status.

Recruitment procedures have been established and we are beginning to recruit women. We are mailing an initial recruitment card to height awareness of the study. This is being followed by an information letter relating more study details. Both of these recruitment methods also mention the upcoming phone interview during which we are collecting pre-screening information on personal characteristics, diet, medication use, and health history. We will identify women seeking a screening mammogram at one of the clinical centers within the PHA. We have developed the data management system such that we will be able to identify women who receive a negative screening (healthy) and women who eventually are diagnosed with breast cancer.

k. Finalize the intervention protocol.

We have finalized the intervention protocol, based on our experiences with past dietary interventions. An intervention syllabus has been generated, listing specific content and topics for each class. The dietician, Leigh Hart has been hired for the first intervention cycle. Mrs. Hart will lead weekly group discussions on incorporating Brassica vegetables into a daily diet, menu planning, and preparing quick healthy meals. Intervention materials have been generated, including a course booklet, 3-day diet diaries, a brief vegetable questionnaire, a brief monitoring questionnaire designed to measure adverse reactions or changes in health-related behaviors, and a recipe book. Dietary goals have been set. Rapid conversion of self-reported compliance levels will allow participants to monitor compliance relative to peers. We have identified several dieticians in Columbia who are sufficiently skilled to lead the intervention, and we are confident in our ability to hire such an intervention leader at the appropriate time.

Task 2: Recruitment, Months 12-24:

a. Identify women who could be eligible for the study from among those visiting the Breast Clinic at Richland Memorial Hospital for the purpose of an open biopsy as a part of a diagnostic work up following a suspicious mammogram. We have also identified former breast cancer cases from the PHA Tumor Registry Database who may be eligible to take part in this study. We will primarily sample from this registry for the first cycles of the intervention. We have mailed recruitment information to these women and have begun phone interviews.

b.

We have put into place procedures for recruitment through the PHA clinical services. We will be able to identify women receiving breast biopsy procedures and who could be eligible for the study among those visiting the PHA participating hospitals. Recruitment has begun (October to November 2001).

b. Among those who say they are willing to participate, determine eligibility using the 18 criteria listed in section 4.1 of the proposal.

We have developed a simple eligibility screening form suitable for use in the large-scale screening of potential participants during a telephone interview.

c. Abstract medical records for relevant health history and pathology data. The PHA Tumor Registry contains information on pathology and the history of the first course of treatment for women with a previous diagnosis of the disease. For women currently visiting the Breast Clinic at Richland Memorial Hospital or Baptist Hospital, we are able to link their medical records with eligibility criteria in order to enroll them into this study.
d. Randomize to either intervention or control. Inform woman of this.
e. Enroll the consecutive eligible women who have histologically confirmed stage I or II cancer of the breast.
f. Enroll consecutive eligible women who are disease free and meet all eligibility requirements of the study and are matched to the cases on age (±5 years).

g. Schedule the first clinic appointment for the purposes of collecting all of the blood and urine specimens and taking the anthropometric measurements.
h. Ensure that the open biopsy material is processed and sent to Dr. Muga's laboratory.
i. Collect data on lifestyle, demographic, and health (family and personal history) plus psychosocial factors as outlined in 4.4.3.
j. If in the intervention, schedule the individual and group sessions with the dietitian.

Task 3: Intervention / Passive Follow Up in the Controls, Months 14-28 (all items subsumed here are on-going):

Ensure that the intervention is delivered according to the protocol.

a. Through collaboration with a local cardiac rehabilitation center, we have access to an appropriate conference room and adjoining teaching kitchen.

b. Encourage women randomized to the intervention to attend all of the sessions.
c. Stay in contact with the control group to assure compliance with the follow-up measures.
d. Schedule the follow-up visit at the Breast Clinic for the blood, urine, and anthropometric data collection.
e. Schedule the visit for the needle biopsy at the Breast Clinic.
f. Assure that all self-assessments are completed at follow up.

Task 4: Data Entry, Verification and Interim Analyses, Months 12-28 (all items subsumed here are on-going):

a. Assure that all data are immediately read into the tracking and analytic databases.
b. Flag all outlier and illogical responses.
c. Verify all outlier and illogical responses, re-contacting participants, if necessary.
d. Conduct simple descriptive analyses (e.g., cross-tabulations and univariate statistics).

Task 5: Final Data Analyses, months 28-36:

a. Perform all exploratory analyses to test for adherence to model assumptions.
b. Perform all necessary data manipulations (e.g., log transforming all non-normal and heteroschedastic data).
c. Test study hypotheses.
d. Conduct post-hoc analyses of study data.
e. Prepare manuscripts.
f. Archive datasets for future analyses and future patient follow-up.
g. Plan for future studies.

Key Research Accomplishments are all subsumed under the Task List, as noted above.

Reportable Outcomes, in addition to those things noted above, include one paper of relevance to this study using isothiocyanate excretion as a biological marker of *Brassica* vegetable

consumption (14). A copy of this is included in the appendix. We also have produced a large number of measurement instruments that are included in the Appendix as well.

Conclusion: After experiencing delays with study start up due to issues around Human Use, this study is now on track in terms of research deliverables.

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1. Hebert JR, Toporoff E. Dietary exposures and other factors of possible prognostic significance in relation to tumor size and nodal involvement in early-stage breast cancer. Int J Epidemiol 1989; 18:518-526.
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Appendices

Appendix 1: Curriculum Vitae and Journal Article	Stephanie Muga - Biosketch Journal Article by Jay Fowke
Appendix 2: Assessment Instruments	Baseline Questionnaire Vegetable and Fruit Questionnaire Side-Effects and Reactions Form
Appendix 3: Recruitment and Consent	Recruitment Card Letter of Introduction Consent Form Phone Script
Appendix 4: Collection & Processing	Urine Blood* Buccal Cells Body Size Measurements Measurements Form
Appendix 5: Intervention Materials	Draft Syllabus* Food Lists and Dietary Goals* 24-HR Recall Script

*Indicates these are unchanged from those filed in the previous report.

Appendix 1
Curriculum Vitae and Manuscript

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Stephanie J. Muga, Ph.D.	Research Assistant Professor

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.*)

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Univ. of North Carolina-CH, Chapel Hill, NC	B.S.	1986	Biology
Univ. of Texas-Austin, Austin, TX	Ph.D.	1995	Biochemistry
Univ. of Texas-Austin, Austin, TX	Post-Doc.	1995-1996	Nutrition
UT MD Anderson Cancer Center, Smithville, TX	Post-Doc.	1996-1999	Carcinogenesis
UT MD Anderson Cancer Center, Smithville, TX	Res. Assoc	1999-2000	Carcinogenesis

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

Professional Experience:

7/95-6/96 Post-Doctoral Fellow, Department of Human Ecology, Division of Nutritional Sciences, University of Texas-Austin, Austin, TX.

7/96-8/99 Post-Doctoral Fellow, Department of Carcinogenesis, Science Park-Research Division, UT MD Anderson Cancer Center, Smithville, TX.

9/99-7/00 Research Associate, Department of Carcinogenesis, Science Park-Research Division, UT MD Anderson Cancer Center, Smithville, TX

7/00-present Research Assistant Professor, Dept. of Developmental Biology & Anatomy and SC Cancer Center, USC School of Medicine, Columbia, SC

Honors:

1996 Postdoctoral Fellowship: NIH/NCI Training Grant in Carcinogenesis and Mutagenesis, Univ. of Texas, MD Anderson Cancer Center, Science Park-Research Division, Smithville, TX

1997AACR Molecular Biology and Pathology of Neoplasia Workshop Awardee, Keystone Resort, Keystone, CO

1998-2000 Most Distinguished Postdoctoral Fellow for Excellence in Teaching. University of Texas MD Anderson Cancer Center, Science Park- Research Division. Smithville, TX

Professional Organizations:

American Chemical Society: Member 1997-present
American Association for Cancer Research (AACR): Associate Member 1998-present
Women in Cancer Research (WICR-AACR): Member 1998-present
Molecular Epidemiology Working Group (AACR): Member 2001-present

Extramural Support:

2000-01 Cancer Research Foundation of America: *PPAR activators as chemopreventive agents of UV-induced skin carcinogenesis.* Principal Investigator.

2001 South Carolina Research Initiative Grants. *Prevention of Breast and Colon Cancer in South Carolina.* Co-Principal Investigator.

Selected Publications

Thuillier, P., Anchiraico, G. J., Nickel, K. P., Maldve, R. E., Giminez-Conti, I., **Muga, S. J.**, Liu, Kai-Li, Fischer, S. M., and Belury, M. A. (2000) Peroxisome proliferator activated receptor (PPAR) alpha activators inhibit mouse skin tumor promotion. *Molecular Carcinogenesis,* Nov.; 29(3):134-42.

Kleymenova, E., **Muga, S. J.**, Fischer, S. M., and Walker, C. L. (2000) Application of HPLC-based DNA Fragment Analysis (HADF) to Molecular Carcinogenesis. *Molecular Carcinogenesis*, Oct.; 29(2), 51-58.

Muga, S. J., Thuillier, P., Pavone, A., Rundhaug, J. E., Jisaka, M., Boeglin, W., Brash, A. R., and Fischer, S. M. (2000) 8*S*-lipoxygenase products activate PPARα and induce differentiation in murine keratinocytes. *Cell Growth and Differentiation*, 11: 447-454.

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Research Projects:

Active:

PPAR activators as chemopreventive agents of UV- induced skin carcinogenesis.

Principal Investigator: Stephanie J. Muga, Ph.D. Agency: Cancer Research Foundation of America
Type: Research Grant Annual Direct Costs: $35,000
Dates: 1/15/00-1/15/01
Percent Effort: 25%

To test the effectiveness of several agents that may be useful in preventing or controlling cell proliferation in the skin thus preventing the formation of skin tumors after ultra-violet light exposure.

Cancer Prevention Drug Discovery for Breast and Colon Cancer.

Principal Investigator: Michael J. Wargovich, Ph.D. Agency: SC Research Initiative Grants
Co-Principal Investigator: Joan E. Cunningham, Ph.D. Annual Direct Costs: $115,000
Co-Principal Investigator: Stephanie J. Muga, Ph.D.
Type: Research Grant
Dates: 1/01/01-12/31/01
Percent Effort: 5%

To develop new cancer prevention strategies by evaluating novel drugs for cancer prevention and use this research to benefit South Carolinians at high risk for cancer.

"Do the Effects of Exercise on Breast Cancer Prevention Vary with Environment?"
Principal Investigator: Jane Teas, Ph.D. Agency: Department of Defense, US Army
Collaborator: Stephanie J. Muga, Ph.D. Annual Direct Costs: $50,000
Type: Research Grant
Dates: 1/01/01-12/31/02
Percent Effort: 5%
To determine those factors which regulate incidence or recurrence of breast cancer. Does exercise have a significant impact on regulating VEGF and HIF-A1 (hypoxia inducible factor 1 alpha) levels and does this contribute to the preventive effects of vitamin D metabolism on breast tumor development?

"Can Hyperbaric Oxygen Therapy Reduce Breast Cancer treatment Related Lymphedema?"
Principal Investigator: Dick Clark, CHT Agency: Palmetto Health Alliance Foundation
Consultant/Collaborator: Stephanie J. Muga, Ph.D. Annual Direct Costs: $10,600
Type: Research Grant
Dates: 9/1/2000-8/31/2001
Percent Effort: 0%
Pilot Study to investigate therapeutic potential, and the associated angiogenic and lymphagiogenic responses, of hyperbaric oxygen therapy in lymphedema.

Pending:
Dept. Of Defense: Army
Prevention of Breast Cancer By NSAIDs and Thiazolidinediones
Principal Investigator: Michael J. Wargovich, Ph.D. Agency: DOD
Co-Principal Investigator: James Hebert, Ph.D. Type: IDEA Research Grant
Co-Principal Investigator: Joan Cunningham, Ph.D.
Co-Principal Investigator: Stephanie J. Muga, Ph.D. Dates: 1/1/02-1/1/06 **PENDING**
Percent Effort (Muga): 10% Total Costs: $625,000

A joint basic science and epidemiologic study of non-steroidal anti-inflammatory drugs and anti-diabetic thiazolidinediones in the prevention of breast cancer. To determine the chemopreventive efficacy of commonly used NSAIDS (aspirin, ibuprofen, and other commonly used NSAIDS) in two genetically altered mouse models for mammary cancer. An epidemiological study will be conducted to test in South Carolina women the association between a recent history of use of NSAIDs and anti-diabetic drugs, and risk of breast cancer.

American Institute for Cancer Research
Herbal Supplements and Prevention of Colon Cancer
Principal Investigator: Michael J. Wargovich, Ph.D. Agency: AICR
Co-Investigator: Stephanie J. Muga, Ph.D. Type: Research Grant
Percent Effort (Muga): 10% Dates: 1/31/02-1/30/04 **PENDING**
Total Costs: $164,947

To determine if herbals and botanicals have the ability to modulate cyclooxygenase activity in an animal model of colorectal cancer. We postulate that certain herbals and botanicals may work in a manner similar to the non-steroidal anti-inflammatory drugs and decrease the risk for developing colon cancer.

Public Health Nutrition: 4(3), 837–846

DOI: 10.1079/PHN2000113

Using isothiocyanate excretion as a biological marker of *Brassica* vegetable consumption in epidemiological studies: evaluating the sources of variability

Jay H Fowke[1,*], Jed W Fahey[2], Katherine K Stephenson[2] and James R Hebert[1]

[1]Department of Epidemiology and Biostatistics and the Nutrition Center, School of Public Health, University of South Carolina and the South Carolina Cancer Center, Columbia, SC, USA: [2]Department of Pharmacology and Molecular Sciences, *Brassica* Chemoprotection Laboratory, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Abstract

Objective: *Brassica* vegetable consumption (e.g. broccoli) leads to excretion of isothiocyanates (ITC) in urine. We evaluated the consistency of ITC as a biomarker for dietary *Brassica* vegetable consumption across the types of vegetables and methods of preparation used in Western societies, and across consumption levels.

Design: A single-armed behavioural intervention with duplicate baseline assessment and post-intervention assessment. Urinary ITC excretion and estrogen metabolites were measured from 24-hour urine samples. Dietary intake was measured by a 24-hour recall.

Setting: The behavioural intervention facilitated daily *Brassica* intake among participants by providing peer support, food preparation instruction, guided practice in a teaching kitchen, and other information.

Subjects: Thirty-four healthy free-living postmenopausal women who recently had a negative screening mammogram at the University of Massachusetts Medical Center.

Results: Urinary ITC excretion and total *Brassica* intake followed the same pattern over the intervention. The ITC biomarker significantly predicted *Brassica* intake when *Brassica* consumption averaged about 100 g day^{-1}, but not when *Brassica* consumption averaged about 200 g day^{-1}. Urinary ITC levels were somewhat higher when more raw vegetables were consumed as compared to lightly cooked vegetables, while the types of *Brassica* consumed appeared to have only a small, non-significant effect on urinary ITC levels.

Conclusion: Urinary ITC excretion would be a good exposure biomarker among populations regularly consuming a vegetable serving/day, but may be less accurate among populations with greater intake levels or a wide range of cooking practices.

Keywords
Isothiocyanate
Biomarker
Diet
Estrogen metabolism
Dietary assessment
Brassica

A diet rich in vegetables of the family *Cruciferae*, which in the USA consists primarily of *Brassica* vegetables (e.g. broccoli, green cabbage, Brussels sprouts), may reduce the risk of many common cancers[1–8]. *Brassica* vegetables are a well-known source of glucosinolates, *N*-hydroxysulfates with a variable aglycone group containing either an alkyl, alkenyl, thioalkyl, thioalkenyl, aryl, arylalkyl or indolyl moiety[9,10]. Glucosinolates are hydrolysed to their isothiocyanate congeners, or to nitriles, thiocyanates or other compounds by myrosinase, an enzyme in plant cells and in the human gut microflora[11]. These reaction products interact with various mammalian cellular and metabolic systems that are associated with cancer risk[12–14], including Phase 2 detoxification enzymes (e.g. glutathione-*S*-transferase (GST)) that protect animals and their cells against oxidative stress, carcinogenesis and mutagenesis[15–19].

While there is an abundance of evidence illustrating a biological response to *Brassica* phytochemicals that is consistent with reduced cancer risk, there is only sparse and inconsistent prospective cohort or case–control epidemiologic evidence that *Brassica* consumption reduces cancer incidence or mortality. One explanation for this lack of epidemiological evidence might be that *Brassica* consumption is not adequately measured. Large case–control or cohort studies usually measure dietary

*Corresponding author: Email jay.fowke@palmettohealth.org

intake with a food-frequency questionnaire (FFQ). These dietary instruments query only a limited number of foods, request average portion sizes, and rely on long-term memory to recall past dietary practices[20]. Additionally, there is a growing body of evidence to suggest that data from such self-reported dietary assessment techniques are influenced by participants' characteristics, including their psychological profiles[21]. The large potential public health benefit of even a small percentage reduction in cancer incidence suggests the need for a better method to estimate glucosinolate intake in free-living study populations.

Recently, an assay was developed to measure isothiocyanates and their metabolites in human urine[11,22,23]. Biological markers for dietary intake are not susceptible to reporting errors that limit self-report, especially FFQ data[24], and therefore urinary ITC excretion might provide a less biased way of assessing *Brassica* intake in large-scale studies. In several highly controlled metabolic studies, urinary isothiocyanate excretion levels (ITC) were consistent with the amount of *Brassica* administered to the study participant[11,25–27]. Seow and colleagues found that categories of total *Brassica* intake as measured by FFQ significantly followed the trend across categories of urinary ITC levels[23]. This Asian study population consumed moderate daily amounts of *Brassica* (40 g day^{-1}), primarily as Chinese cabbage, and cooking practices were not evaluated.

There are several sources of variability that could lead to substantial error in estimating *Brassica* intake by means of urinary ITC analysis. A further evaluation of these errors could benefit research studies intending to use ITC as a biomarker for dietary intake. The glucosinolate content of *Brassica* vegetables varies across species and cultivars, and depends on numerous environmental variables such as soil conditions under which the vegetables are grown[9,15]. Not all glucosinolates are equally likely to be converted to detectable isothiocyanates[10,11]. Therefore the quality of ITC as a biomarker for *Brassica* intake may depend upon the types of vegetables consumed. In addition, post-harvest handling, plant age, vegetable preparation and individual metabolic activity further affect glucosinolate concentration, effective dose and biological activity[9,15,23,28–31].

An intensive 4-week dietary intervention was designed and implemented in order to evaluate the physiological response to increased *Brassica* vegetable consumption and to develop new functional-food assessment approaches in healthy free-living people. Since the intervention was of high intensity and relatively short duration, it provided an ideal opportunity to compare estimated *Brassica* intake from two independent sources: a well-regarded dietary assessment standard and the ITC biomarker of *Brassica* exposure. Over the three phases of the intervention, the same group of 34 participants consumed low, moderate and high amounts of *Brassica*, enabling us to evaluate the consistency between ITC excretion and self-reported *Brassica* consumption across different levels of consumption, and to evaluate the ability of ITC to track changes in dietary intake within individuals. Variability in ITC excretion with vegetable type and vegetable preparation are considered. We have previously reported that greater *Brassica* intake leads to a shift in the estrogen composition in these study participants, such that the amount of 2-hydroxyestrone increased relative to the amount of 16α-hydroxyestrone[32]. We compared urinary ITC levels, as a marker for dietary *Brassica* intake, to urinary estrogen metabolite levels known to be affected by dietary *Brassica* intake.

Materials and methods

Study participants

Several aspects of this study have been described previously[32]. Study participants were healthy, free-living, postmenopausal women who had received screening mammographic services within the past year. Participants were over 45 years of age and without a menstrual cycle during the previous 12 months. Tobacco-users or women who regularly consumed more than two alcoholic drinks per day were excluded. Additionally, subjects using any prescription or non-prescription hormones, diabetes medication, antibiotics, herbal remedies, or who were under a physician-recommended diet, were excluded. The average age of the 34 women participating in the study was 61.8 years (SD=8.1, range: 49–77). Twenty-five subjects were married and 17 had achieved at least a college degree. Fourteen women were employed, primarily in service-oriented positions such as nursing or in managerial/office jobs.

Study participants attended a series of four classes designed to facilitate the addition of *Brassica* vegetables to the daily diet. Class discussions were led by a registered dietitian, but relied on peer support and peer modelling to motivate adherence. Content focused on problem-solving skills and overcoming barriers associated with the dietary change. A strong emphasis was placed on vegetable preparation, and participants were encouraged to eat either raw or lightly steamed vegetables. Participants practised vegetable preparation skills through guided meal preparation in a teaching kitchen.

Study design and data collection

The study design and sample collection schedule are illustrated in Fig. 1. Study participants provided two 24-hour urine samples prior to the intervention period, which are referred to as 'Baseline-1' and 'Baseline-2'. Additional 24-hour urine samples were collected during the last week of the intervention phase (referred to as 'Intervention') and two weeks after the conclusion of the intervention ('Postintervention'). Both written and oral

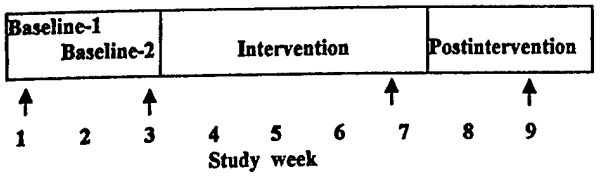

Fig. 1 Study design and data collection schedule: each arrow indicates a week during the study phase in which three 24-hour recalls were administered and a 24-hour urine sample was collected

instructions concerning 24-hour urine collection were administered to all participants. Subjects also were advised to avoid prepared mustard and horseradish during the two days prior to the urine collection, as these condiments are made from Cruciferous vegetables and have significant quantities of allyl isothiocyanate. Each opaque urine collection bottle contained 2.0 g ascorbic acid as a preservative. Urine samples were delivered to project staff within 1 day of collection, and most were delivered on the same day of collection. Upon arrival, total volumes of urine were recorded, and aliquots were stored at −80°C. Urine aliquots were shipped on dry ice to Baltimore for ITC analysis.

During each week in which urine was collected, three 24-hour diet recalls (24HR) were administered to each participant. Participants were telephoned on three randomly assigned days (two weekdays and one weekend day) and asked to describe their diet on the preceding day. A structured interview protocol was strictly followed. Highly trained registered dietitians conducted all interviews, and participants were provided a two-dimensional chart of typical foods to assist with portion size estimation. Nutrient calculations were performed using the Nutrition Data System software, developed by the Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN (Food Database: 13A; Nutrient Database: 28)[33]. There were no missing nutrient values in this analysis. Data from the 24HR within each week were averaged for each participant. Of the 408 calls assigned to the 34 participants, 401 calls were completed (98.3%). From each 24HR log, the amount (grams) of *Brassica* vegetable reported were combined within each day by vegetable type and by cooked or raw status, and then averaged across all 24HR during the week. *Brassica* vegetables reported as cooked were adjusted to reflect raw grams consumed.

Questionnaires were administered throughout the study, first to collect demographic and breast cancer-related data, then to monitor for changes in medication use, physical activity, occupational status, or alcohol use. The psychological constructs 'Social Approval' and 'Social Desirability', along with demographic and other data, were measured by questionnaire during the baseline study phase[34,35].

Isothiocyanate laboratory analysis

Samples (1.5 ml) from each urine collection were thawed and centrifuged (200*g* for 5 min at 4°C) to remove particulate matter. The cyclocondensation reaction[22] with urine was carried out in 4 ml, screw-topped glass vials in a final volume of 2.0 ml that contained 200 or 500 µl of urine and enough water to total 500 µl, 0.5 ml of 500 mM sodium borate buffer (pH 9.25), and 1.0 ml of 40 mM 1,2-benzenedithiol in methanol. The vials were flushed with N_2 gas, sealed with Teflon-lined septa, and the contents were mixed with a Vortex mixer and incubated for 2 h at 65°C. Samples were then cooled to room temperature, briefly centrifuged (350*g* for 5 min), and loaded into a Waters WISP Autosampler®. Aliquots (200 µl) of each reaction mixture were injected on to a reverse-phase high-performance liquid chromatography (HPLC) column (Partisil 10 µm ODS-2, 4.5×250 mm; Whatman, Clifton, NJ) and eluted isocratically with 80% methanol/20% water (v/v) at a flow rate of 2 ml min^{-1}. The cyclocondensation product peak, 1,3-benzodithiole-2-thione, was eluted at *c.* 5.0 min, and its area was integrated at 365 nm using a Waters Photodiode Array detector (Waters Millenium Software®, Version 2.15.01).

Three sets of controls were included with each analytical run: (1) purified cyclocondensation product (200 µl of 2.5, 5.0 and 10.0 µM solutions) was injected to assess the validity of the standard curve; (2) a reaction mixture containing only 1,2-benzenedithiol was included to ensure that no peak is given by 1,2-benzenedithiol alone; and (3) three concentrations (2.5, 5.0 and 10.0 µM) of the *N*-acetylcysteine derivative of allyl isothiocyanate were analysed with and without 1,2-benzenedithiol to ensure that the cyclocondensation reaction went to completion. Standard curves, assay reproducibility, linearity of response and storage characteristics of urine samples were all as detailed in Shapiro *et al.*[11]. Urinary ITC concentration (µmol ml^{-1}) was multiplied by the volume of urine collected during the 24-hour period (ml), to give µmol daily ITC excretion.

Urinary estrogen metabolites

Urinary 2-hydroxyestrone (2HE) and 16α-hydroxyestrone (16HE) were measured using a solid-phase enzyme immunoassay kit from Immuna Care Corporation (Bethlehem, PA)[32,36]. All assays were performed on samples in triplicate, in random order, within one batch, and by a single technician who was masked as to the sequence of the sample collection. The intra-assay coefficients of variation (CV) for 2HE and 16HE were each 4.0% and inter-assay CVs were 10.0% and 9.9%, respectively. Standard urine samples were obtained from women of a similar age and estrogen level as the study participants.

Statistical analysis

A descriptive analysis of dietary *Brassica* intake and urinary ITC excretion is presented at all four measurement

Table 1 Isothiocyanate (ITC) excretion or self-reported *Brassica* intake

Study phase	ITC (μmol/24 h)*					*Brassica* (g/24 h)†				
	Min	Q1	Median	Q3	Max	Min	Q1	Median	Q3	Max
Baseline-1	0 (5)	0.58	1.96	5.21	11.34	0 (15)	0	3.9	20.3	52.6
Baseline-2	0 (12)	0	0.51	4.12	38.97	0 (20)	0	0	10.4	75.6
Intervention	0.92	9.46	16.90	37.69	145.83	53.6	149.8	180.9	245.4	371.5
Postintervention	0 (1)	5.79	12.49	25.98	84.09	0 (3)	50.7	105.0	166.3	241.8

Min: minimum value; Q1: 25th percentile; Q3: 75th percentile; Max: maximum value.
( ): Number of participants with no detectable ITC or reporting 0 g *Brassica* intake.
* ITC excretion measured in 24-hour urine samples collected during each phase of the study.
† *Brassica* consumption measured with three 24-hour recalls (24HR) during the same week that a 24-hour urine sample was collected.

times within the intervention (i.e. Baseline-1, Baseline-2, Intervention and Postintervention). The analysis focused on the association between *Brassica* intake and ITC excretion during the Intervention and Postintervention study phases because few participants consumed *Brassica* at Baseline, and there was insufficient variability in the dietary and urinary data to perform the detailed analysis within this study phase. The isothiocyanate data were natural log (ln) transformed to better meet assumptions of the statistical analysis. *Brassica* vegetable intake data during the Intervention and Postintervention study phases approximated a normal distribution. Pearson correlation coefficients and linear regression coefficients were used to assess the cross-sectional associations. Regression coefficients reflect the amount of urinary ITC excretion (ln(μmol day^{-1})) due to each unit change (e.g. g day^{-1}) in the dietary parameter. Vegetable-specific associations with urinary ITC excretion were identified using partial correlation coefficients, or by simultaneously including each vegetable type in a linear regression model that predicts ITC excretion. Similarly, the amounts of *Brassica* intake consumed as cooked or raw were simultaneously included in a linear regression model.

We evaluated the ability of urinary ITC levels to track individual changes in *Brassica* intake or to induce a change in estrogen metabolism. Measurements across the two baseline time points were not significantly different, and each subject's two baseline values were averaged together for calculation of the change scores, in order to provide the most stable baseline estimate. Change scores for *Brassica*, ITC levels or the relative amounts of estrogen metabolites (2HE/16HE ratio) were computed

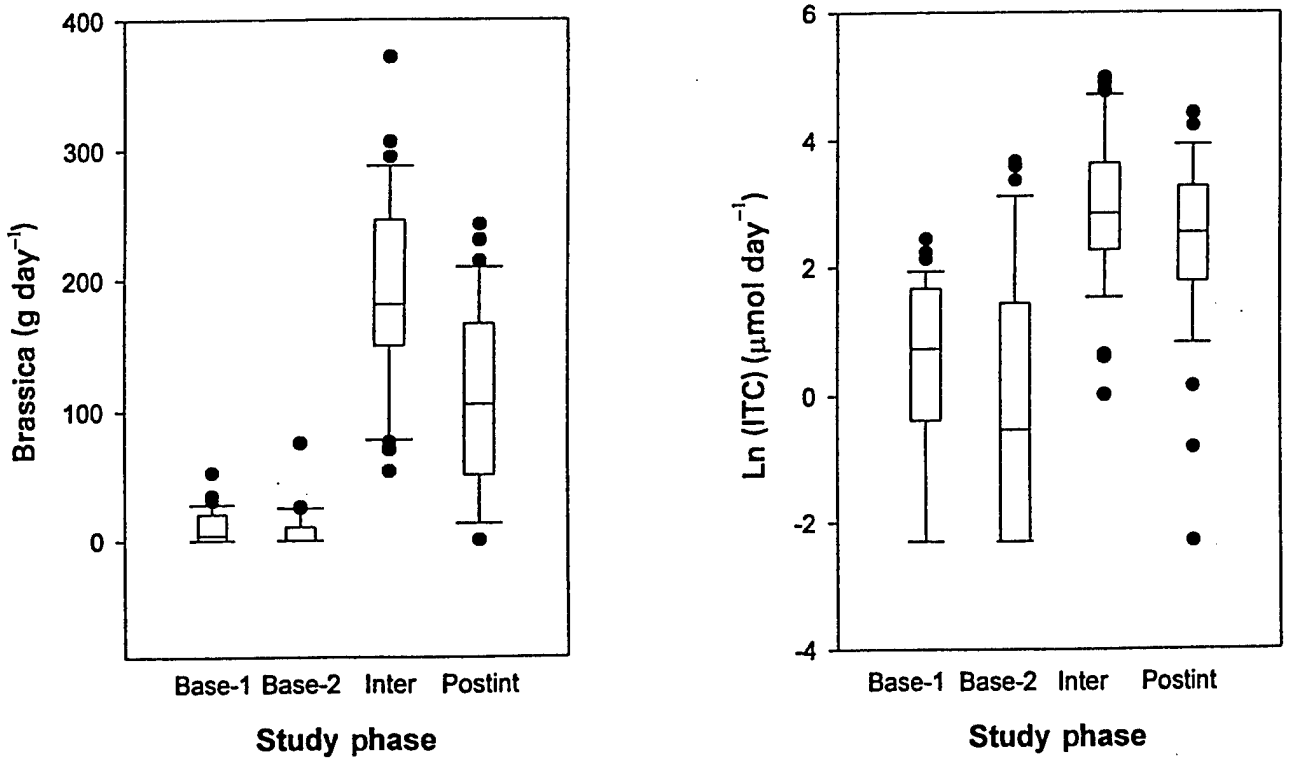

Fig. 2 The distribution of *Brassica* vegetable consumption and urinary isothiocyanate (ITC) excretion across phases of the study (Base-1, Baseline-1; Base-2, Baseline-2; Inter, Intervention; Postint, Postintervention). Horizontal lines at 5th, 25th, 50th, 75th and 95th percentiles

by subtraction of the Baseline value from either the Intervention or the Postintervention value. Paired *t*-tests were used to compare changes in diet or urinary measures between any two time points. Correlation coefficients and regression coefficients were adjusted for baseline (log-transformed) ITC values, in order to reduce the influence of an extreme baseline value on the change score (i.e. regression to the mean).

Results

Reported *Brassica* vegetable consumption and urinary ITC excretion are summarised in Table 1 and Fig. 2. At Baseline, participants' *Brassica* intake was similar across the two baseline measures (P=0.37), and averaged about 9 g of vegetable per day. *Brassica* consumption increased during the intervention to 193 g day^{-1} ($P < 0.001$, Average Baseline value vs. Intervention), and all participants reported greater *Brassica* consumption during the intervention. At Postintervention, average *Brassica* consumption decreased by 84 g day^{-1} ($P < 0.001$ for Intervention vs. Postintervention). Broccoli, cabbage and Brussels sprouts were most commonly consumed (Intervention: 50.5, 42.5 and 75.7 g day^{-1}; Postintervention: 27.0, 14.8 and 43.1 g day^{-1}, respectively).

Urinary ITC levels were lowest at the two Baseline time points, and these Baseline ITC levels were not significantly different ($P = 0.24$). ITC was non-detectable in 17 urine samples at Baseline, whereas all urine samples obtained during the Intervention phase of the study contained detectable ITC. Group-average ITC excretion levels followed the trend of *Brassica* intake, with significant increases from Baseline to Intervention ($P < 0.01$), and a decrease from the Intervention to Postintervention phase of the study ($P < 0.01$).

The association between individual-level ITC excretion and reported *Brassica* intake was evaluated within each measurement period of the study (Table 2, Fig. 3). The scatter plots of Fig. 3 illustrate the unstable association between *Brassica* intake and ITC excretion during the two Baseline collection periods, when *Brassica* consumption was very low and sporadic. Any linear association was due to a few highly influential values, and therefore we restrict further analyses to the Intervention and Postintervention study phases. During the Intervention period, where intake was highest, there was only a weak and non-significant association between urinary ITC level and total *Brassica* intake or vegetable specific intake. During the Postintervention, where intake was moderate, there was a significant association between ITC excretion and *Brassica* intake ($r = 0.58$, $P < 0.01$), at which time each g day^{-1} of *Brassica* led to an increase of 0.24 log units (log(μmol day^{-1})) in urinary ITC levels. Adjustment for macronutrient intake (protein g day^{-1}, fat g day^{-1}, energy kcal day^{-1}, carbohydrate g day^{-1}) did not affect the association between *Brassica* intake and ITC excretion.

The commonly consumed vegetables (i.e. broccoli, cabbage and Brussels sprouts) appeared to contribute proportionally equivalent amounts of ITC to the content of urine at Postintervention, but there was greater disparity during the Intervention. Brussels sprouts intake was highest during the Intervention, and certain glucosinolates common in Brussels sprouts might be less likely to contribute to ITC in urine. When Brussels sprouts consumption was removed from the total amount of *Brassica* consumed, the associations improved slightly during the Intervention ($r = 0.21$, $P = 0.23$; $b = 0.06$, 95% CI (0.04, 0.17)), but regression coefficients at Postintervention decreased slightly ($r = 0.46$, $P = 0.006$; $b = 0.23$, 95% CI (0.07, 0.39)). Raw vegetable intake tended to be more strongly associated with ITC levels as compared with cooked vegetable intake.

Dietary interventions and metabolic (in-patient) studies often analyse changes in a biochemical measure as an individual-level marker of exposure change. As described

Table 2 Association between isothiocyanate excretion and total *Brassica* intake, and by vegetable type or vegetable preparation

	Intervention				Postintervention			
Brassica	*r*	*P*	*b*	95% CI	*r*	*P*	*b*	95% CI
Model 1*								
Total	0.14	0.45	0.04	−0.06, 0.15	0.58	<0.01	0.24	0.12, 0.36
Model 2†								
Broccoli	0.12	0.51	0.06	−0.12, 0.25	0.51	<0.01	0.38	0.14, 0.63
Cabbage	−0.05	0.76	−0.03	−0.23, 0.17	0.32	0.08	0.31	−0.03, 0.66
Brussels sprouts	−0.05	0.78	−0.02	−0.18, 0.14	0.50	<0.01	0.34	0.11, 0.57
Other	0.32	0.08	0.23	−0.02, 0.49	0.08	0.67	0.05	−0.20, 0.31
Model 3‡								
Cooked	0.07	0.68	0.02	−0.09, 0.14	0.47	<0.01	0.20	0.06, 0.34
Raw	0.23	0.18	0.12	−0.06, 0.31	0.44	0.01	0.40	0.10, 0.70

Isothiocyanate (ITC) measured from 24-hour urine samples collected during each phase of the study. *Brassica* consumption measured by three 24-hour recalls during each week in which a urine sample was collected for isothiocyanate measurement.
* Model 1: log(ITC)=Total *Brassica* (20 g day^{-1}).
† Model 2: log(ITC)=Broccoli (20 g day^{-1})+Cabbage (20 g day^{-1})+Brussels sprouts (20 g day^{-1})+Other (20 g day^{-1}).
‡ Model 3: log(ITC)=Cooked *Brassica* (20 g day^{-1})+Raw *Brassica* (20 g day^{-1}).
Partial Pearson correlation coefficients adjusted for other variables in model.

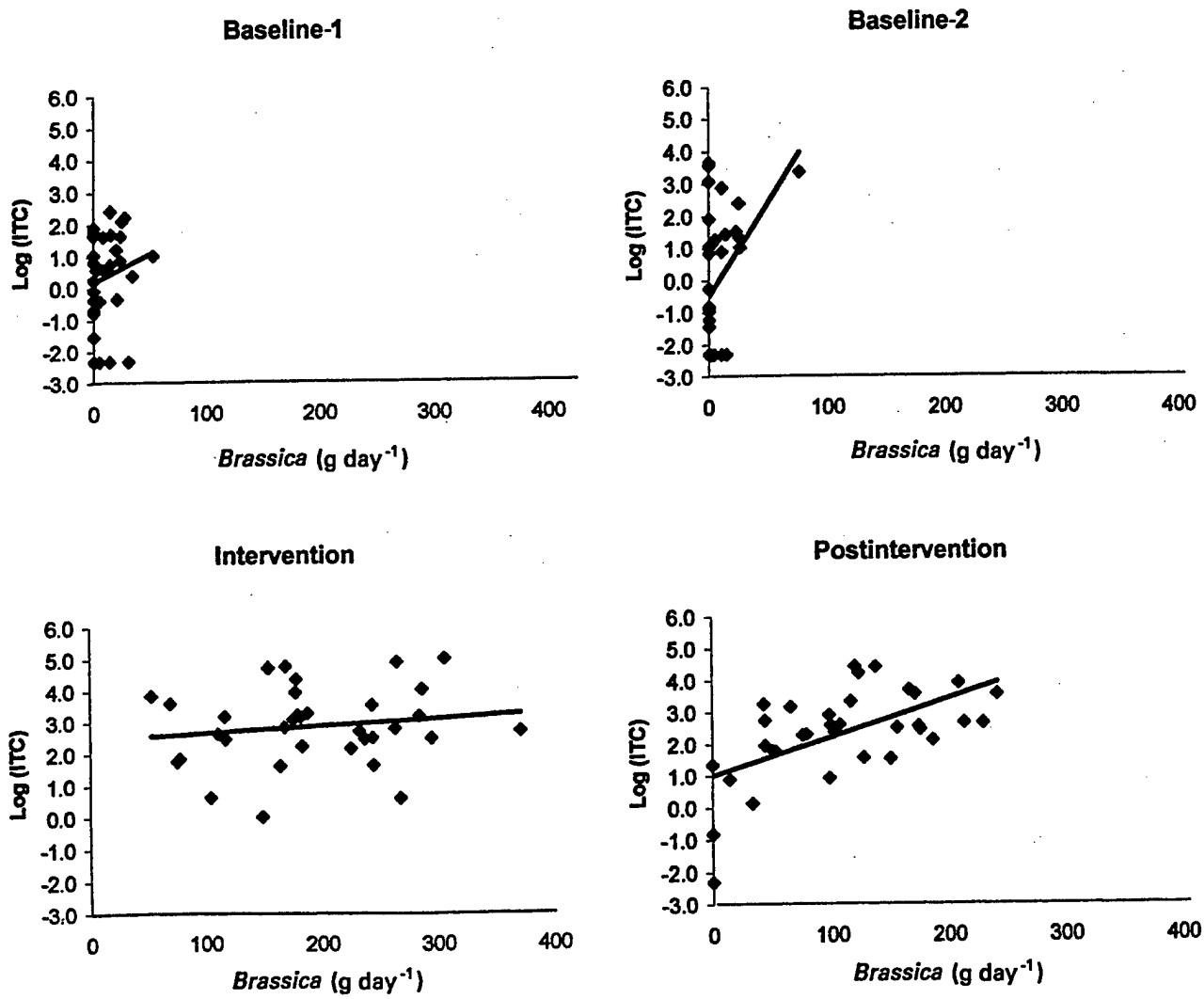

Fig. 3 *Brassica* intake and isothiocyanate excretion within the same participants consuming different levels of *Brassica*

in the Methods section, changes in *Brassica* or urinary ITC levels were calculated by subtracting the average Baseline values from values at either the Intervention or Postervention study phase. Moderate change in *Brassica* intake (Postintervention−Baseline) was associated with a significant change in urinary ITC levels, where each 20 g day^{-1} increase in *Brassica* intake led to a 0.24 log-unit increase in urinary ITC excretion (Table 3). In contrast, larger change in *Brassica* intake (Intervention−Baseline) was associated with almost no change in ITC levels.

In order to explore the consistency between urinary ITC levels and a physiological response to *Brassica* vegetable intake, the urinary estrogen metabolite ratio 2-hydroxyestrone: 16α-hydroxyestrone (2HE/16HE) was regressed on urinary ITC excretion levels. Greater urinary ITC levels were not significantly associated with a higher urinary 2HE/16HE ratio within either the Intervention or

Table 3 Association between change in grams of *Brassica* vegetable intake per day, and change in isothiocyanate (ITC) excretion

	Intervention−Baseline				Postintervention−Baseline			
Brassica	*r*	*P*	*b*	95% CI	*r*	*P*	*b*	95% CI
Total (20 g day^{-1})	0.18	0.31	0.06	−0.05, 0.17	0.57	<0.01	0.24	0.11, 0.36

Ln (Isothiocyanate (ITC)) measured from 24-hour urine samples collected during each phase of the study. *Brassica* consumption measured by three 24-hour recalls during each week in which a urine sample was collected for isothiocyanate measurement.
Change scores calculated by subtracting the average of the two Baseline measurements for dietary intake or log(ITC) levels from values at Intervention or Postintervention.
Model: Δ log(ITC)=Δ *Brassica*+log($ITC_{Baseline}$).

Table 4 Association between urinary isothiocyanate excretion level and the 2HE/16HE estrogen metabolite ratio

	r	*P*	*b*	95% CI	
Cross-sectional*					
Intervention	0.13	0.45	0.12	−0.21,	0.45
Postintervention	−0.04	0.83	−0.05	−0.51,	0.41
Change†					
Intervention−Baseline	−0.18	0.29	−0.20	−0.61,	0.18
Postintervention−Baseline	−0.49	<0.01	−0.74	−1.21,	−0.26

* Cross-sectional model: 2/16=log(ITC).
† Change model: $\Delta 2/16=\Delta(\log ITC)+ITC_{Baseline}$.

Postintervention study phase (Table 4). Adjustment for the previously described dietary factors led to a stronger association during the Intervention ($b = 0.28$, 95% CI(−0.14, 0.69)), but had no impact on the Postintervention association ($b = -0.04$, 95% CI (−0.34, 0.26)). Unexpectedly, increased ITC excretion from Baseline to Postintervention was significantly associated with lower urinary 2HE/16HE levels ($r = -0.49$, $P < 0.01$). No outliers or highly influential data points were evident. Statistical adjustment for changes in macronutrient intake during these time intervals did not change the interpreted results (Intervention−Baseline: $b = -0.24$, 95% CI (−0.53, 0.03); Postintervention−Baseline: $b = -0.41$, 95% CI (−0.62, −0.19)).

Discussion

Nutritional epidemiology often evaluates the association between the macro- or micronutrient components of the diet and disease risk. There is growing evidence that the non-nutrient components of the diet could impact cancer risk. Examples include genestein derived from soy-foods, enterolactone from grain-foods, and isothiocyanates from *Brassica* vegetables. However, it is difficult to measure exposure to these food components accurately. Typical FFQs do not query an exhaustive listing of these vegetables or factors that may modify the phytochemical content. Urinary markers of dietary intake are less susceptible to reporting bias and might provide a chemical-specific exposure level. We created a model dietary intervention developed in part to design and evaluate dietary assessment strategies for functional food intake. In this intervention, the same participants consumed very high or moderate levels of *Brassica* vegetables, providing the unique opportunity to evaluate the performance of the biomarker across a range of intakes within the same individuals. The 24HR is a dietary assessment method that measures the current diet without resorting to standardised comparison portions in order to estimate the quantity of food consumed. This method provides the least biased approach to estimating dietary intake within an intervention[37,38].

We found that ITC excretion was a better predictor of *Brassica* intake when the group consumed a moderate level of *Brassica*. Previously, Seow and colleagues found a greater discrepancy between estimated *Brassica* intake and ITC excretion among those participants who consumed more *Brassica*, and by *GSTT1* gene expression[23]. The metabolism of ITC may vary across individuals according to the expression and activity of GST and other metabolic enzymes, such that the urinary biomarker no longer represents dietary intake beyond a certain level of intake. In this study of US women, that threshold appears to be between 100 g day^{-1} and 200 g day^{-1}, on average. There was a dose–response pattern during the Postintervention study phase, when intake was moderate. The linear trend was lost with higher average intake. Additionally, there was a marginally significant association between changes in ITC excretion levels and changes in *Brassica* intake among free-living participants at Postintervention.

The amount of *Brassica* consumed at Postintervention is a better representation of the amounts consumed in many Asian regions. For example, Seow and colleagues found that Cruciferous intake averaged about 40 g day^{-1} in Singapore[23]. Variance measures were not provided, but there was likely a wide distribution reaching into the ranges observed during the Postintervention phases of this study. According to food-disappearance data, the Japanese consume far more cabbage (19 g day^{-1}), Chinese cabbage (22 g day^{-1}) and other *Brassica* vegetables which are rarely consumed in Western cultures[39]. This study indicates that populations which routinely consume *Brassica* do not appear to do so to such an extent that the urinary ITC marker would be unreliable. Consumption of *Brassica* vegetables in the United States has been estimated at 11 g day^{-1} in an analysis of food production data[30]; or about 2 servings per week in a large dietary survey[40]. These intake levels are similar to our Baseline measures, indicating that urinary ITC levels may be an unreliable estimate of *Brassica* intake in North American study populations.

Different *Brassica* vegetables have different glucosinolate concentrations, and the spectrum of glucosinolates differs from vegetable to vegetable. Although Brussels sprouts are a rich source of glucosinolates, the predominant glucosinolates include progoitrin, a β-hydroxyalkenyl glucosinolate that is hydrolysed by the enzyme myrosinase to produce nitriles, epithionitriles and oxazolidine-2-thiones, and not isothiocyanates. Progoitrin metabolites do not react to produce ITC in the human body, and these progoitrin metabolites do not react in the cyclocondensation reaction[22]. Brussel sprouts consumption was very high during the Intervention phase. However, these results might suggest only the slightest variation in ITC excretion due to variation in the patterns of consumed *Brassica*.

As a generalisation, the United States' population typically consumes *Brassica* vegetables after cooking. Glucosinolates are water-soluble and leach into the

cooking water with vegetable boiling, decreasing the glucosinolate concentration within the vegetable[15,28,41–43]. When thoroughly cooked *Brassica* vegetables are administered to subjects, plant myrosinase is inactivated, and essentially all of the glucosinolates/isothiocyanates are presented to the subject's digestive tract in glucosinolate form[11], and additional metabolic/enzymatic steps are required to release the ITC component from the glucosinolate. Alternatively, it could be possible that very light cooking releases myrosinase without enzyme inactivation, leading to increased glucosinolate metabolism. Requiring that participants consume only raw *Brassica* would so highly control the study that results would not be applicable to free-living women, and such a restriction would likely decrease compliance to the intervention. Study participants were instructed during the intervention classes to prepare *Brassica* vegetables using techniques that prevent glucosinolates from leaching or degrading in the vegetable, with the goal of maintaining the glucosinolate content of the vegetables at the highest possible level. We found that both raw and cooked *Brassica* consumption contributed to urinary ITC levels, with raw consumption appearing to contribute a little more ITC to urine, suggesting that ITC excretion might be sensitive to the simplest of food preparation methods.

To further explore the use of ITC, we compared urinary ITC excretion levels to the 2HE/16HE estrogen metabolite ratio. The indole glucosinolates derived from *Brassica* vegetables are converted in the body to aryl hydrocarbon receptor agonists[44], and the activated receptor is able to induce the specific enzyme responsible for hydroxylation of estrone on the second carbon (CYP1A1, CYP1A2), producing 2-hydroxyestrone rather than the highly estrogenic and genotoxic 16α-hydroxyestrone. The ratio of these metabolites, 2HE/16HE, is currently under evaluation as an endocrine biomarker for breast cancer risk[45–50]. We have found that *Brassica* consumption increases the 2HE/16HE ratio in this study population, consistent with a reduced risk of breast cancer[32]. In contrast, urinary ITC levels, derived from *Brassica* vegetables, were not associated with the 2HE/16HE ratio. The common non-isothiocyanate metabolites (e.g. nitriles, thiocarbamates, epithionitriles, oxazolidine-2-thiones and various indole derivatives such as indole-3-carbinol and indole-3-acetonitrile) are not detected by this assay. Therefore, the urinary ITC index may be less informative as a biomarker for *Brassica* when the hypothesised disease mechanism involves indole glucosinolates.

To our knowledge, this is the first attempt to adjust for other dietary constituents. Nutrient intake is able to affect drug metabolism[51] and phytoestrogen excretion, either through induction or inhibition of Phase 1 or Phase 2 enzymes responsible for metabolism, or through increasing or decreasing the likelihood of faecal excretion over urinary excretion of the agent. Log-transformed urinary ITC excretion levels were not associated with dietary macronutrient intake, and adjustment for these nutrients did not affect the associations between ITC levels and either *Brassica* intake or urinary 2HE/16HE values. We did not explore the effects of fibre intake on ITC excretion because increased *Brassica* consumption leads directly to both greater fibre intake and ITC excretion, limiting our ability to identify this independent association in this study.

It is possible that these motivated participants misreported their diet; however, there is little evidence to suggest that these study results are due to dietary misreport. It is conceivable that participants over-reported *Brassica* intake in order to achieve the appearance of compliance. The psychological scales 'social desirability' and 'social approval' have been previously evaluated for their effect on dietary self-report[21]. Social desirability describes a defensive mechanism where one is likely to present oneself in a fashion consistent with social norms and to avoid criticism, while social approval scores are associated with an intrinsic need to seek a positive response to a testing situation[21,34,35]. Consistent with the theoretical construct of the scales, the social approval scale was associated with *Brassica* intake during the Intervention phase ($r = 0.33$, $P = 0.05$). Study personnel motivated participants to consume *Brassica* and provided feedback and problem-solving techniques, thus potentially creating a testing situation. During the Postintervention phase there was no contact with study personnel, thus reducing the 'pressure' to attain a specific level of *Brassica* intake. However, it is difficult to determine if participants with higher social approval scores met the testing challenge by consuming more *Brassica* or by reporting more *Brassica*. Social approval scores were not significantly correlated with ITC excretion ($r = 0.06$, $P = 0.74$), providing some support to the hypothesis that greater social approval scores were associated with misreport. However, adjustment with social approval scores had no effect on the regression coefficients during either the Intervention or Postintervention study phase. While there is some question as to the relationship between *Brassica* intake and social approval scores, misreport – if any – was small, and adjustment for social approval in the analysis did not alter the fundamental interpretation of the results.

It is unlikely that the differences in results over time are due to a training effect among participants. The duplicated baseline measurements were included to provide time for training. We have observed among women receiving repeated 24HR that all training occurs during the first 24HR, and that the effect is very small relative to the overall variability in their diet. The 24HRs administered during the Intervention phase were the seventh, eighth and ninth calls received by these participants.

Other sources of error in the correspondence between

urinary ITC levels and dietary *Brassica* consumption might be possible. Urinary ITC levels peak within several hours after consuming *Brassica*, but require one to three days to be completely excreted[11]. The 24HR assessment protocol contacted participants on three random days within the week of urine collection, and was designed to capture a representative sample of the habitual/regular *Brassica* intake during the week for that participant. It is possible that *Brassica* consumption during a day not queried by 24HR might have contributed to urinary ITC levels. This error may be greatest when *Brassica* consumption is relatively rare and sporadic, such as at Baseline, and less important with consistent and daily (or near daily) *Brassica* consumption (Intervention and Postintervention). None of the participants used tobacco, but it may be possible that environmental tobacco smoke contributed to urinary ITC levels[11]. Participants were instructed not to eat mustard or horseradish during the urine collection week, but eating foods prepared by other people may lead to condiment consumption without participant knowledge.

Improper urine sample handling or refrigeration by study participants may have led to microbial contamination, thus resulting in degradation of glucosinolate/isothiocyanate in the urine and decreasing the association between ITC and self-reported *Brassica* intake. However, there was no difference in the time between reported completion of the urine collection and urine storage across the different phases of the study, making it unlikely that urine sample handling alone could explain the variable associations across the study. The vast majority of urine samples were returned to the research institution within hours of the completed urine collection. Further work is planned to identify a simple and effective ITC-compatible preservation protocol for epidemiological research.

In summary, categories of *Brassica* intake follow the pattern of categories of urinary ITC excretion, and there was a significant correlation between these two measures among participants who consumed an average of about 100 g day^{-1}. The cyclocondensation reaction is important because it provides an overall estimate of glucosinolate exposure across *Brassica* species and food preparation that standard FFQs do not capture. In using ITC as an exposure marker in large epidemiological studies, careful consideration should be given to variability in cooking practices, the amounts consumed, and the theorised biological mechanisms of the disease of interest.

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Appendix 2
Assessment Instruments

2607113527

ID

BRASSICA HEALTH STUDY
Baseline

Date Form Completed

Month / Day / Year

First Initial	Middle Initial	Last name
A B C D E F G H I J K L M N O P Q R S T U V W X Y Z	A B C D E F G H I J K L M N O P Q R S T U V W X Y Z	A B C D E F G H I J K L M N O P Q R S T U V W X Y Z

Contact Information:

E-mail address (Optional): _____ @ _____

First some questions about your personal characteristics.

1. **How tall are you (without shoes)?** ☐ Feet ☐☐ Inches

2. **What is your current weight?** ☐☐☐ Pounds

3. **Are you: (Specify more than one, if applicable)**

- ○ White (Non-Hispanic)
- ○ Black (Non-Hispanic)
- ○ Hispanic/White
- ○ Hispanic/Black
- ○ Asian
- ○ Other (specify):

4. **What is your current marital status? (Select only one.)**

- ○ Married
- ○ Living with a partner
- ○ Widowed
- ○ Divorced
- ○ Separated
- ○ Single, never married and not living with a partner

5. **How would you describe your religion? (Mark ONE only)**

- O Roman Catholic
- O Protestant
- O Jewish
- O Muslim
- O Other (Specify): _____
- O No Religion (go to question #7)

6. **Do you regularly (at least once monthly) attend religious services?**

O Yes
O No

7. **What is the highest year or level of school you have completed? (Select only one.)**

◯ 8th grade or less
◯ More than 8th grade and less than high school
◯ High school completed, no college
◯ High school completed, some college (Associates degree, RN, etc.)
◯ College completed (BS, BA, BSN, etc.)
◯ More than college completed (MA, MS, PhD, etc.)

8. **Are you presently employed? (Select only one.)**

◯ Yes, employed full time
◯ Yes, employed part time
◯ No (go to question #13)

9. **If employed, how do you classify your usual position? (Select only one.)**

◯ Skill or craft
◯ Machine operator
◯ Manual labor
◯ Sales
◯ Scientific/Technical work
◯ Service work
◯ Clerical or office
◯ Professional, managerial or administrative

10. We would like to know about your activity at your job.
(Please fill in one circle for each item)

	Never	Seldom	Sometimes	Often	Always
a. At work, I sit...	○	○	○	○	○
b. At work, I stand...	○	○	○	○	○
c. At work, I walk...	○	○	○	○	○
d. At work, I lift heavy objects...	○	○	○	○	○
e. At work, I am tired...	○	○	○	○	○
f. At work, I sweat...	○	○	○	○	○

11. Did anyone at your workplace smoke cigarettes, cigars, or a pipe in the past week?

○ Yes
○ No (Go to question 13)

12. If YES, please indicate the type of tobacco smoke and amount of time you were exposed to tobacco smoke at the workplace in the past week.

Smoke Source	Amount of time in last week that you were in the same room or car as a coworker that smoked tobacco
Cigarette	
Cigar	
Pipe	

13. Were you exposed to cigarette, cigar, or pipe smoke anywhere outside the workplace (for example: at home, at a friend's house, or eating out) in the past week?

O Yes
O No (Go to question 15)

14. If YES, please indicate the type of tobacco smoke and amount of time you were exposed to this smoke in the past week.

Smoke Source	Amount of time in last week that you were exposed to smoke, not at work
Cigarette	
Cigar	
Pipe	

15. Do you ever drink any alcoholic beverages (i.e. beer, wine, spirits/liquor)?

O Yes
O No (Go to question 17)

16. If yes, then please indicate how much of each beverage you drink in a typical week.

Beverage		*Typical # drinks in a week*	
a) Beer	O Yes O No	☐☐	bottles or cans (12 oz.)
b) Wine	O Yes O No	☐☐	glasses (6 oz.)
c) Spirits	O Yes O No	☐☐	drinks (1.5 oz. liquor)

Next, some questions about your pregnancy history and children.

17. Have you ever been pregnant?

O Yes
O No (Go to question 22)

18. Have you ever had a pregnancy that lasted beyond the first trimester (in other words, past the first three months)?

O Yes
O No (Go to question 20)

19. Please list dates of your pregnancies that lasted beyond the first trimester, the result of the pregnancy, and the sex of the child.

Pregnancy No.	*Date Pregnancy Ended (mm/dd/year)*	*Result*	*Sex (M/F)*
1	☐☐ / ☐☐ / ☐☐☐☐	O Live Birth O Still Birth O Other Loss	O Male O Female
2	☐☐ / ☐☐ / ☐☐☐☐	O Live Birth O Still Birth O Other Loss	O Male O Female
3	☐☐ / ☐☐ / ☐☐☐☐	O Live Birth O Still Birth O Other Loss	O Male O Female
4	☐☐ / ☐☐ / ☐☐☐☐	O Live Birth O Still Birth O Other Loss	O Male O Female
5	☐☐ / ☐☐ / ☐☐☐☐	O Live Birth O Still Birth O Other Loss	O Male O Female

Pregnancy No.	*Date Pregnancy Ended (mm/dd/year)*	*Result*	*Sex (M/F)*
6	☐☐ / ☐☐ / ☐☐☐☐	O Live Birth O Still Birth O Other Loss	O Male O Female
7	☐☐ / ☐☐ / ☐☐☐☐	O Live Birth O Still Birth O Other Loss	O Male O Female

20. Have you ever had a first trimester miscarriage or abortion?

O Yes
O No (Go to question 22)

21. If so, please provide the number of first trimester miscarriages or abortions: ☐☐

22. Do you have children?

O Yes
O No (Go to question 26)

23. How many children do you have?

☐☐ Biological children

☐☐ Adopted children

☐☐ Step children

☐☐ Total number of children

24. How many children currently live with you? ☐☐ Children

25. What is the age of the youngest child living with you? ☐☐ Years Old

26. How old were you when your periods or menstrual cycle started? ☐☐ Years Old
(Please be as accurate as possible.)

27. Have you ever had menstrual problems?

O Yes
O No (Go to question 29)

28. If yes, which of the following problems have you experienced?
(Please indicate all that apply to you.)

☐ **Cramps**
☐ **Irregular periods**
☐ **Heavy bleeding**
☐ **Other (please describe):** _____

_____

_____

29. Have you had a period in the last 12 months?

O Yes (Go to question 32)
O No

30. How old were you when your periods stopped? ☐☐ Years Old
(Please be as accurate as possible.)

31. Why did your periods stop?
(Please indicate all that apply to you.)

O **They stopped naturally.**
O **They stopped as a result of a hysterectomy.**
O **They stopped as a result of another surgical procedure.**
O **They stopped as a result of a medicine or therapy**
O **They stopped due to other reasons. (please describe):** _____

_____

_____

32. Have you ever had a breast biopsy, where a doctor removed some breast tissue either surgically or with a needle?

O Yes
O No (Go to question 35)

33. If YES, how many surgical breast biopsies have been performed? ☐☐ Surgical biopsies

34. How many needle breast biopsies have been performed? ☐☐ Needle biopsies

---

Now, some questions about the activities you did in your home last week.

35. Did you do the light household work (dusting, washing dishes, mending/sewing)?

O Never
O Sometimes (only when partner or help not available)
O Mostly (sometimes assisted by partner)
O Always (alone or with partner)

36. Did you do the heavy household work (washing floors, windows, carry trash, etc.) ?

O Never
O Sometimes (only when partner or help not available)
O Mostly (sometimes assisted by partner)
O Always (alone or with partner)

37. For how many people did you keep house including yourself? ☐☐ Person(s)
(Fill in '0' if you answered 'never' to Questions 35 and 36)

38. How many rooms did you keep clean, including kitchen, bedroom, garage, cellar, bathroom, attic, etc.?

O Never do housekeeping (Go to question 40)
O 1 to 6 rooms
O 7 to 9 rooms
O 10 or more rooms

39. If any rooms, on how many floors in your home are these rooms? ☐☐ Floor(s)

40. Did you prepare warm meals yourself, or did your assist in preparing?

- O Never
- O Sometimes (1 to 2 times a week)
- O Mostly (3 to 5 times a week)
- O Always (more than 5 times a week)

41. How many flights of stairs did you walk on a typical day (one flight of stairs is 10 steps)?

- O I never walk stairs
- O 1 to 5 flights of stairs
- O 6 to 10 flights of stairs
- O More than 10 flights of stairs

42. If you went somewhere in your hometown, what kind of transportation did you use?

- O I never go out
- O Car
- O Public transportation
- O Bicycle
- O Walking

43. How often do you go out for shopping?

- O Never or less than once a week (Go to question 20)
- O Once a week
- O Twice a week
- O Every day

44. If you went out for shopping, what kind of transportation did you use?

- O Car
- O Public transportation
- O Bicycle
- O Walking

45. Did you play a sport last week?

O Yes
O No (Go to question 47)

46. Please indicate the type of sport and the number of hours you played last week.

Type of Sport	Hours per Week

47. Did you have other physically active activities last week?

O Yes
O No (Go to question 49)

48. Please indicate the type of activity and the number of hours.

Type of Activity	Hours per Week

49. Did you watch more than 30 minutes of television a day?

O Yes
O No (Go to question 51)

50. If yes, how many hours a day did you usually watch television? ☐☐ Hour(s)

Next, some questions on diet.

51. Are you on any special diet for health reasons, such as a low salt diet or a low sugar diet?

O Yes
O No

52. Have you ever tried to make dietary changes?

O Yes
O No (Go to question 55)

53. Which of the following changes have you tried to make? Also, which changes DID you <u>make and maintain</u> for a period of 6 months or longer? *(Check ALL that apply)*

	Asked to Make	Did Make
Eat less red meat	☐	☐
Cut down on fat intake	☐	☐
Cut down on calories	☐	☐
Cut down on cholesterol	☐	☐
Lose weight	☐	☐
Eat more fruits and vegetables	☐	☐
Eat fewer dairy products	☐	☐
Other (please describe below):	☐	☐

_____

_____

54. In general, how difficult was it for you to make the dietary changes you noted?

Very Easy	Easy	Not Easy or Difficult	Difficult	Very Difficult
O	O	O	O	O

55. How much do you agree with the following statements?
(Please fill in one circle for each item)

	Strongly Disagree	Disagree	Partially agree and disagree	Agree	Strongly Agree
a. What I eat is important to the way I feel.	○	○	○	○	○
b. What I eat is important to my health.	○	○	○	○	○
c. Breast cancer is a very important health issue.	○	○	○	○	○
d. Heart disease is a very important health issue.	○	○	○	○	○
e. Osteoporosis is a very important health issue.	○	○	○	○	○
f. Lung cancer is a very important health issue.	○	○	○	○	○

56. Have you seen a dietician or attended nutrition classes in the last 3 months?

○ Yes
○ No (Go to question 58)

57. If yes, why? _____

_____

_____

58. Do you have any food allergies?

○ Yes
○ No (Go to question 60)

59. If yes, please identify these foods. _____

_____

_____

60. **How much has your diet changed from when you were growing up?**

Not at all	Very little	Don't Know	Quite a bit	Very Much
○	○	○	○	○

61. **Aside from this study, were you planning to make any of the following changes in your eating habits in the next 6 months?** *(Check ALL that apply)*

- ☐ Cut down on calories
- ☐ Cut down on fats
- ☐ Eat more fiber
- ☐ Cut down on cholesterol
- ☐ Eat less red meat
- ☐ Eat more fruits and vegetables
- ☐ Lose weight
- ☐ None of these. (Go to question 63)

62. **How positive do you feel that you can stay focused on your dietary goals during the next 6 months?**

Not at all Confident	A little	Don't Know	Quite a bit	Very Confident
○	○	○	○	○

63. **How important do you feel other peoples' support is in helping you change your diet?**

Not at all Important	A little	Don't Know	Quite a bit	Very Important
○	○	○	○	○

64. **How much support would you get for eating more vegetables....**
(Please fill in one circle for each item)

	Never	Rarely	Sometimes	Quite Often	Always
a. **from people at work?** *(Skip this question if not employed)*	○	○	○	○	○
b. **from close friends?**	○	○	○	○	○
c. **from your spouse or family?**	○	○	○	○	○

65. How important are the following in deciding which foods you eat?
(Please fill in one circle for each item)

	Never Important	Rarely Important	Sometimes Important	Usually Important	Always Important
Convenience	○	○	○	○	○
Taste	○	○	○	○	○
Appearance	○	○	○	○	○
Smell	○	○	○	○	○
Cost	○	○	○	○	○
Health	○	○	○	○	○
Ethics eg. Animal Rights	○	○	○	○	○
Religion	○	○	○	○	○
Social Concerns	○	○	○	○	○

66. In a typical week, how often do you eat meals or snacks?
(Please fill in one circle for each item)

Days Per Week

	0 to 1	2 to 3	4 to 5	6 to 7
Breakfast	○	○	○	○
Lunch	○	○	○	○
Dinner	○	○	○	○
SNACKS:				
Morning	○	○	○	○
Afternoon	○	○	○	○
Evening	○	○	○	○

67. In a typical week, how often do you eat the following meals <u>with other people</u> (family, friends, co-workers)? *(Please fill in one circle for each item)*

	Days Per Week			
	0 to 1	2 to 3	4 to 5	6 to 7
Breakfast	○	○	○	○
Lunch	○	○	○	○
Dinner	○	○	○	○
<u>SNACKS:</u>				
Morning	○	○	○	○
Afternoon	○	○	○	○
Evening	○	○	○	○

68. In a typical week, how often do you eat the following meals OUTSIDE of your home (cafeteria, fast foods, vending machines, restaurant, at someone else's home)?
(Please fill in one circle for each item)

	Days Per Week			
	0 to 1	2 to 3	4 to 5	6 to 7
Breakfast	○	○	○	○
Lunch	○	○	○	○
Dinner	○	○	○	○
<u>SNACKS:</u>				
Morning	○	○	○	○
Afternoon	○	○	○	○
Evening	○	○	○	○

69. How much do you agree with the following statements?

	Strongly Disagree	Disagree	Partially agree/disagree	Agree	Strongly Agree
a. I am open to trying foods that I never tried before.	○	○	○	○	○
b. Low-fat foods can taste good.	○	○	○	○	○
c. I enjoy the taste of most vegetables.	○	○	○	○	○

70. How confident are you that you can make healthy food choices when you....

	Almost never Confident	Rarely	Sometimes	Quite Often	Almost always Confident
a. are anxious (or nervous)?	○	○	○	○	○
b. feel physically run down?	○	○	○	○	○
c. are depressed (or down)?	○	○	○	○	○
d. are angry (or irritable)?	○	○	○	○	○
e. are bored or have nothing to do?	○	○	○	○	○
f. are pressured by others to eat?	○	○	○	○	○
g. have experienced failure?	○	○	○	○	○
h. think others will be upset if you don't eat?	○	○	○	○	○
i. have to go out of your way to eat a healthy meal?	○	○	○	○	○
j. are ill or not feeling well?	○	○	○	○	○
k. are offered unhealthy but tasty foods?	○	○	○	○	○
l. are very hungry?	○	○	○	○	○
m. have limited time to plan your meal?	○	○	○	○	○
n. have many available unhealthy foods?	○	○	○	○	○
o. others offer you less healthy foods?	○	○	○	○	○
p. eat out (at restaurants, friends' homes, etc.)?	○	○	○	○	○
q. during holidays or special occasions?	○	○	○	○	○
r. are socializing with friends?	○	○	○	○	○

71. How do you feel about each of the following foods?
(Please fill in one circle for each item)

	I would never eat this food	I would eat this food if I had to	I sometimes like this food	I always like this food
Carrots	○	○	○	○
Cabbage	○	○	○	○
Broccoli	○	○	○	○
Asparagus	○	○	○	○
Brussels Sprouts	○	○	○	○
Green Peas	○	○	○	○
Radish	○	○	○	○
Kidney Beans	○	○	○	○
Cauliflower	○	○	○	○
Zucchini	○	○	○	○

72. Do you eat beef or chicken less than four times in a week?

○ Yes
○ No

73. Are there any foods you dislike so much that you would never eat them?

○ Yes
○ No (Go to question 75)

74. If yes, please identify these foods. _____

_____

_____

75. Compared to most of your friends, family, and co-workers, how do you rank your present diet according to amount of ... *(Please fill in one circle for each item)*

	Lower in Fat		Average in Fat		Higher in Fat
a. Fat?	○	○	○	○	○

	Lower in Vegetables		Average in Vegetables		Higher in Vegetables
b. Vegetables?	○	○	○	○	○

76. What is the recommended highest percent of fat in the diet? (Check ONE only)

○ 10% ○ 20% ○ 30% ○ 40% ○ 50%

77. I am more likely to get heart disease, cancer or another serious disease if I ...

	Strongly Disagree	Disagree	Partially agree/disagree	Agree	Strongly Agree
a. eat a lot of high-fat foods	○	○	○	○	○
b. eat a lot of vegetables	○	○	○	○	○
c. eat a lot of fiber/roughage	○	○	○	○	○

78. Choose the vegetable with the most nutritional value. (Circle ONE answer only)

○ Iceberg Lettuce ○ Broccoli ○ They are the same ○ Don't know

79. Choose the vegetable with the most nutritional value. (Circle ONE answer only)

○ Spinach ○ Zucchini ○ They are the same ○ Don't know

80. Choose the vegetable with the most nutritional value. (Circle ONE answer only)

○ Summer Squash ○ Cauliflower ○ They are the same ○ Don't know

81. According to government recommendations, how frequently should fruits or vegetables be eaten?

- O 0 to 1 servings per day
- O 2 to 3 servings per day
- O 3 to 4 servings per day
- O 5 to 6 servings per day
- O More than 6 servings per day

82. For each food listed below, choose the cooking method that is the healthiest.

	Fried	Steamed	Baked	Boiled	Raw
a. Carrots	O	O	O	O	O
b. Broccoli	O	O	O	O	O
c. Onions	O	O	O	O	O
d. Chicken	O	O	O	O	

83. Which vegetable do you eat more frequently? (Circle ONE answer only)

O Iceberg Lettuce O Broccoli O Same Amount O Don't know

84. Which vegetable do you eat more frequently? (Circle ONE answer only)

O Spinach O Zucchini O Same Amount O Don't know

85. Which vegetable do you eat more frequently? (Circle ONE answer only)

O Summer Squash O Cauliflower O Same Amount O Don't know

86. Typically, how frequently do you eat vegetables?

- O 0 to 1 servings per day
- O 2 to 3 servings per day
- O 3 to 4 servings per day
- O 5 to 6 servings per day
- O More than 6 servings per day

87. For each food listed, circle the cooking method you typically use.

	Fried	Steamed	Baked	Boiled	Raw
a. Carrots	O	O	O	O	O
b. Broccoli	O	O	O	O	O
c. Onions	O	O	O	O	O
d. Chicken	O	O	O	O	

88. We will be contacting you by telephone several times over the study period. What time(s) of the day are you most likely to be home?

Day of the Week	Good Times to Call	Bad Times to Call
Sunday		
Monday		
Tuesday		
Wednesday		
Thursday		
Friday		
Saturday		

Thank you for taking the time to complete this questionnaire!

4320007758

ID

BRASSICA
Vegetable and Food Questionnaire

Date Form Completed
Month / Day / Year

First Initial	Middle Initial	Last name

Please tell us how often you have eaten the specified food item, and the typical portion size in the past seven days, excluding today. All portion sizes refer to cooked size unless otherwise noted. Please write in the number of times that you have consumed the food and check off your usual portion size as compared to the Comparison Portion Size.

For example, if you ate broccoli three times (one cup at one sitting and ¼ cup the other two times:

	Number of Times Eaten	Comparison Portion Size	Your Average: Half this Size	Equal to this Size	Twice this Size
eg. Broccoli	03	½ cup	☐	☐	☐

Food Item	Number of Times Eaten	Comparison Portion Size	*Your Average: Half this Size*	*Equal to this Size*	*Twice this Size*
Broccoli		½ cup	☐	☐	☐
Brussel Sprouts		4 sprouts	☐	☐	☐
Cabbage		½ cup	☐	☐	☐
Cauliflower		½ cup	☐	☐	☐
Chinese Cabbage		½ cup	☐	☐	☐
Collard Greens /Swiss Chard /Kohlrabi		½ cup	☐	☐	☐
Mustard Greens or Turnip Greens		½ cup	☐	☐	☐
Rutabaga /Turnips		½ cup	☐	☐	☐

Food Item	Number of Times Eaten	Comparison Portion Size	Your Average: Half this Size	Your Average: Equal to this Size	Your Average: Twice this Size
Kale	☐☐	½ cup	☐	☐	☐
Spinach	☐☐	4 sprouts	☐	☐	☐
Onions	☐☐	1 sm or ¼ cup	☐	☐	☐
Carrots	☐☐	1 med or ¼ cup	☐	☐	☐
Sweet Potatoes	☐☐	¾ cup	☐	☐	☐
Soybeans - whole	☐☐	1- 8 oz. Glass	☐	☐	☐
Soy milk 8oz. Glass	☐☐	¾ cup	☐	☐	☐
Tofu	☐☐	½ cup	☐	☐	☐
Tempeh	☐☐	½ cup	☐	☐	☐
Broccoli Sprouts	☐☐	½ sprouts	☐	☐	☐
Alfalfa /Clover /Mung Bean /Soy sprouts (raw)	☐☐	½ cup	☐	☐	☐
Pinto Beans /Round Split Pea Pods	☐☐	½ cup	☐	☐	☐
Fresh Green or Mung Beans	☐☐	½ cup	☐	☐	☐
Garbanzo, Kidney Beans, or Black-eyed, Yellow Split or Chinese Peas	☐☐	½ cup	☐	☐	☐
Peas	☐☐	½ cup	☐	☐	☐
Lentils /Dal	☐☐	½ cup	☐	☐	☐
Seaweeds eaten dry (e.g. dulse, purple laver, nori)	☐☐	¼ cup or 7" sq sheet	☐	☐	☐
Seaweeds eaten cooked or soaked (e.g. arame, kombu, kelp)	☐☐	1 Tbsp or 2" sq sheet	☐	☐	☐
Apples	☐☐	1 med or ½ cup	☐	☐	☐
Bananas	☐☐	1 medium	☐	☐	☐
Apricots	☐☐	2 medium	☐	☐	☐
Nectarines	☐☐	1 med or ½ cup	☐	☐	☐
Peaches	☐☐	1 med or ½ cup	☐	☐	☐

Food Item	Number of Times Eaten	Comparison Portion Size	Your Average: Half this Size	Your Average: Equal to this Size	Your Average: Twice this Size
Strawberries	☐☐	½ cup	☐	☐	☐
Grapefruit	☐☐	½ Grapefruit	☐	☐	☐
Lemon, squeezed	☐☐	¼ medium	☐	☐	☐
Orange	☐☐	1 medium	☐	☐	☐
100% Fruit Juice (any type)	☐☐	1- 8 oz. Glass	☐	☐	☐
Other Soy products not listed above.	☐☐	Your Portion Size: _____			

Please Specify: _____

7539051190

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BRASSICA
Dietary Reactions

Date Form Completed
Month / Day / Year

First Initial | Middle Initial | Last name

We are interested in the effects of this intervention. Have you experienced any of the following conditions in the last week

- ☐ dizziness
- ☐ nervousness
- ☐ irritability
- ☐ fatigue
- ☐ tremor
- ☐ diarrhea
- ☐ constipation
- ☐ loss of appetite
- ☐ blurry vision
- ☐ eye irritation
- ☐ weight gain
- ☐ weight loss
- ☐ dry skin
- ☐ yellowish skin
- ☐ loss of hair
- ☐ gas/flatulence
- ☐ difficulty remembering / difficulty thinking clearly
- ☐ muscle pain
- ☐ muscle weakness
- ☐ depression
- ☐ sleep disturbances (including insomnia)
- ☐ swelling

Please indicate any other conditions that you have experienced, *if any*.

_____

_____

○ I have not experienced any of the above reactions this week.

Appendix 3
Recruitment and Consent

Breast Cancer Awareness Month

OCTOBER IS....

Dear

Most women do not realize that once they've conquered breast cancer, there are still things they can do to help themselves. We, here at the University of South Carolina's Norman J. Arnold School of Public Health, are inviting you to take part in a study. It focuses on foods that show some promise for reducing harmful types of estrogen, thus possibly reducing risk of recurrence.

In the next few weeks we will be calling you to ask for your participation in this study. Your involvement would be greatly appreciated. Not only may you benefit but your participation may help other women at risk for this disease. Please remember, without your active support, there is no chance for a cure.

Thank you for your support!

UNIVERSITY OF SOUTH CAROLINA.

«Pt_First_Name» «Pt_Last_Name»
«Pt_Address_Line_1»
«Pt_City», «Pt_State» «Pt_Zip»

[Insert Header]

Dear Ms. «Pt_Last_Name»,

Breast cancer affects millions of people every day in South Carolina. It touches the lives of your friends, your family, and perhaps even you. Research has led to progress against breast cancer – better treatments and improved quality of life.

Your diet is an important part of your life, and the foods you eat may help to prevent disease. We are inviting women to participate in a short-term dietary study called the Brassica Health Study. This study will help to determine whether or not simply eating certain vegetables every day may reduce the risk of developing breast cancer.

We will be calling you in the next few days to answer any questions that you might have at this time. Once we are sure that all your questions are answered and you are willing to participate, we will mail you a brief questionnaire. If you do not wish to be called, please call my assistant, Vickie Smith at 777-7666 and leave a message that you do not wish to be called.

Your participation benefits everyone. You are helping to improve the health and quality of life of your children and grandchildren.

Thank you in advance for your help.

Yours Sincerely,

James R. Hebert, MSPH, ScD
Professor and Chair
Department of Epidemiology and Biostatistics
Norman J. Arnold School of Public Health
University of South Carolina

Palmetto Health Alliance

CONSENT TO PARTICIPATE IN A RESEARCH PROJECT

IRB#: 2000-78

TITLE: Phase I Induction and Estrogen Metabolism in Women With and Without Breast Cancer and in Response to a Dietary Intervention.

PRINCIPAL INVESTIGATOR: James R. Hebert, Sc.D.

RESEARCH SUBJECT'S NAME: _____ **DATE:**_____

SPONSOR: United States Department of Defense

INVITATION TO TAKE PART AND INTRODUCTION:

You are invited to volunteer for a research study. You have been asked to be in this study because you have undergone a screening procedure at the South Carolina Cancer Center within the Palmetto Health Alliance (Columbia, S.C.) to see if you might have breast cancer .

PURPOSE OF THE RESEARCH:

The main purpose of this study is to determine if a 7-session dietary education program can help women incorporate into their diet certain foods that could alter levels of hormones thought to influence the risk of breast cancer. These foods are members of the *Brassica* genus. The most commonly consumed of these vegetables include cabbage, broccoli, cauliflower, and Brussels sprouts. The results of this study will help to develop dietary guidelines directed towards breast cancer prevention and altering the course of disease in women with breast cancer.

YOUR RIGHTS: It is important for you to know that:

- **YOUR PARTICIPATION IS ENTIRELY VOLUNTARY.**
- **YOU MAY DECIDE NOT TO TAKE PART OR DECIDE TO QUIT THE STUDY AT ANY TIME.**
- **YOU WILL BE TOLD ABOUT ANY NEW INFORMATION OR CHANGES IN THE STUDY THAT MIGHT AFFECT YOUR PARTICIPATION.**
- **THE QUALITY OF CARE YOU RECEIVE AT THE HEALTH CENTER WILL NOT BE AFFECTED IN ANY WAY IF YOU DECIDE NOT TO PARTICIPATE OR IF YOU WITHDRAW FROM THE STUDY.**

RANDOMIZATION:

Because it is not known whether changes in diet are effective in breast cancer prevention, not everyone in the study will be assigned to receive the dietary intervention. You will be assigned to one of two groups. One group will receive the dietary intervention, one group will not. This will make it possible for us to judge the effect of eating these vegetables in the fairest, most impartial way possible because the process of randomization ensures that the two groups of people (those receiving and those not receiving the intervention) are similar in other ways. The decision as to whether you receive the dietary intervention or not will be made by chance, like the flip of a coin, not by your doctor or based on your medical condition. You will have a 50% chance of receiving the intervention.

PROCEDURES:

This dietary study will last about three months, and 90 women will participate. If you are assigned to the dietary intervention, you will be asked to first meet with a study dietitian for a one-hour individual session. This session will be followed by 6 two-hour group sessions over a two-month period. These six classes will be held weekly. Approximately fifteen people will attend each class, and classes will be scheduled either on a weekday morning or evening. These sessions will include: 1. classroom presentations during which we will provide information about the vegetables – their chemical properties and their effects on health; 2. a group cooking experience in which you will be asked to learn about preparing the foods; and 3. a chance to eat what you have cooked with other women in the group.

You will be asked to add about four commonly known vegetables to your diet during the six weeks of the intervention. We will not be asking you to restrict your diet, or limit the other foods that you eat, in any way. The dietary intervention is not a weight loss program. You may eat anything that you wish to eat, but we ask that you also eat about two or three servings per day of the vegetables promoted in the intervention classes. These classes are designed to help you incorporate these vegetables into your normal meals.

We will ask to schedule two clinic visits with you. The first clinic visit will be scheduled at a time before the intervention starts, and the second visit will occur near the end of the intervention. During each of these clinic visits, a blood sample will be drawn in the usual way, by inserting a needle into a vein in your arm. About 4 teaspoons (20 milliliters) of blood will be collected, and this blood will be used to determine if there are any changes in levels of the hormones that are thought to be important in modifying breast cancer risk. We will measure your weight and the circumference of your hips and waist. We will provide you with a small urine collection container to collect a first-morning urine sample, and this urine sample can be brought to the clinic when you have your blood drawn. It is important that this urine sample be collected before you eat that day. This urine sample will be used to determine if there are changes in the levels of certain female hormones (estrogens) that are excreted from your body in your urine. Additionally, urine samples will be used to determine the levels of chemicals that naturally exist in the foods you will be asked to eat. We also will collect a small number of breast cells. This will be done by a procedure called core biopsy, using a needle similar to that used for drawing blood. The amount of material removed by core biopsy is always very small, less than a one-quarter of a thimble-full. This material will be used to determine levels of enzymes that are important in regulating levels of female hormones (estrogens).

In summary, each of the two clinic visits will include:

- ❑ Collection of a blood sample
- ❑ Delivery of a first-morning urine sample
- ❑ Collection of a small amount of breast material by core biopsy
- ❑ Measurement of your weight, waist, and hips

Finally, you will be asked to complete several questionnaires about your present health, diet, medication use, and the current level of depression and anxiety. These questionnaires will be completed near the time of your clinic visit, and will require about one hour.

After the end of the week of your last class, you will be advised that you may remove the intervention vegetables from your diet.

ALTERNATIVES:

You may choose not to take part in this study. If so, then you would not have to do any of the things listed above. This would in no way affect other aspects of your treatment or medical care.

RISKS AND INCONVENIENCES:

Drawing blood may hurt slightly, and you might have a black and blue mark. Occasionally a person may become dizzy or faint when blood is drawn and there is a slight possibility of infection or temporary nerve damage. There may be pain associated with the core biopsy. This pain is usually short-lived (i.e., less than 12 hours), and well tolerated. Pain medication, for example Tylenol or Advil, can be taken to relieve this pain, and Tylenol capsules will be available at the time of the biopsy. Stronger pain medication may be prescribed if you think it is needed. There may be a small amount of bleeding which would present no health risk. There is a slight possibility of infection. Sterile techniques are used to avoid infection, but antibiotics can be used to treat an infection if this occurs. There is a very slight risk of temporary nerve damage, which should begin to heal within a few days. There should be no risk from answering any of the study questions, or in providing a urine sample.

Sometimes people find a question on a questionnaire sensitive or uncomfortable to answer. While there are reasons why the question is asked, you do not have to answer a particular question if you feel uncomfortable to do so. Please remember, all results will remain confidential. When we do the statistical analyses for the entire study we will not reveal your identity or the identity of anyone else in the study.

Adverse or allergic reactions to the foods promoted by the dietary intervention are rare. Occasionally, individuals have reported that consumption of the intervention foods leads to excess gas or diarrhea. We will ensure that you are in weekly contact with the project nutritionist and other research staff, and we will encourage you to call if you suspect any side effects. If any side effects occur, you may be advised to eat fewer of the vegetables.

Incorporation of a few additional foods to the diet may at times be an inconvenience when dining out or visiting people. There also may be inconvenience when planning or preparing meals for others in your home. The intervention class content and project staff will try to provide as much help as reasonably possible to overcome such inconveniences and to make these changes enjoyable. Through discussion and

conversation, other classmates also may be able to help with these issues.

COMPENSATION IN CASE OF INJURY:

All forms of medical diagnosis, treatment and research, whether routine or experimental, involve some risk of injury. In spite of all precautions, you might develop complications from participation in this study. In the event of any injury resulting directly from the research procedures, neither the study personnel, the University of South Carolina, nor the Palmetto Health Alliance have made any provision for the payment of any financial compensation to you or to provide any financial assistance for medical or other costs.

This study is being funded by the Department of Defense and conducted by the United States Army in conjunction with the University of South Carolina. Army regulations provide that, as a volunteer in a study conducted by the United States Army, you are authorized all necessary medical care for any injury or disease that is a direct result of your participation in the research. The Principal Investigator or his designee will assist you in obtaining appropriate medical treatment under this provision, if it is required. If you have any questions concerning your eligibility for Army-funded medical treatment you should discuss this issue thoroughly with the Principal Investigator or his designee before you enroll in this study. This is not a waiver or release of your legal rights.

BENEFITS:

This study may be of no direct benefit to you. However, we will make study results available to you when the final results are compiled and written. At the end of the study, you may request a summary of all of your own results with a brief description of what they mean. As results from the entire study are published, we will advise you and you may request them as well. Additionally, the knowledge gained from your participation in this research may help to better understand how to prevent or treat breast cancer.

COSTS:

There will be no direct cost to you for participating in the study. The analyses of questionnaires, blood, urine, biopsy material, and the dietary intervention classes will be provided free of charge.

If you are assigned to the dietary intervention, you will receive a supply of vegetables during each class that can be incorporated into the regular diet. This is done as a convenience to you, and the amount of vegetables supplied should be more than enough to meet the intervention objectives. However, such supplies are intended to be eaten by the study participant, and there will not be a sufficient quantity to share with others. In the event that you wish to share the provided vegetables with friends or family members, we would ask that you purchase additional vegetables.

REMOVAL FROM STUDY

You may be taken out of the research study if it appears that you are unable to: keep your appointments, provide blood, urine, two biopsy samples, or do not provide answers on the questionnaires. If this occurs, you will be given a full explanation.

Subject's Initials _____ Witness's Initials _____

CONFIDENTIALITY:

Your research records will be confidential. In all records of the study you will be identified by a code number and your name will be known only to the researchers. Your name will not be used in any reports or publications of this study.

Because this study is funded by the United States Department of Defense it has a special set of requirements known as "Volunteer Registry Data Base Requirements". It is the policy of the U.S. Army Medical Research and Materiel Command, the entity that regulates this research, that data sheets are to be completed on all volunteers participating in research for entry into this Command's Volunteer Registry Data Base. The information to be entered into this confidential database includes your name, address, Social Security number, study name and dates. The intent of the database is two-fold: first, to readily answer questions concerning an individual's participation in research sponsored by USAMRMC; and second, to ensure that the USAMRMC can exercise its obligation to ensure research volunteers are adequately warned (duty to warn) of risks and to provide new information as it becomes available. The information will be stored at USAMRMC for a minimum of 75 years. It should be noted that representatives of the U.S. Army Medical Research and Materiel Command are eligible to review research records as a part of their responsibility to protect human subjects in research.

SAMPLE DONATION:

During this study, you will be asked to provide two breast biopsy samples, two blood samples, and two urine samples. These samples will be used for hormone analysis related to breast cancer research. They also may be used for purposes that are currently unknown. There is a chance that the samples that you are donating under this study may be used in other research studies and may have some commercial value. No commercial value is anticipated at this point. Should your donated sample(s) lead to the development of a commercial product, the University of South Carolina will own it and may take action to patent and license the_product. The University of South Carolina does not intend to provide you with any compensation for your participation in this study nor for any future value that the sample you have given may be found to have. You will not receive any notice of future uses of your sample(s).

PATIENT PROTECTION:

Further information on the research to be performed, or on any risks, benefits or alternative treatments may be obtained from James R. Hebert at 803-777-7666. This study has been approved by the committee to protect human rights for the Palmetto Health Alliance. Information concerning your rights as a research subject can be obtained by contacting the Office of Corporate Counsel at (803) 296-2124.

Consent to Participate in the research project **IRB #2000-78**, entitled:

Phase I Induction and Estrogen Metabolism in Women With and Without Breast Cancer and in Response to a Dietary Intervention

Subject's name: (printed or typewritten)_____
P.I. Name: James R. Hebert, Sc.D.

"The purpose and procedures of this research project and the predictable discomfort, risks, and benefits that might result have been explained to me. I have been told that unforeseen events may occur. I have had an opportunity to discuss this with the investigator and all of my questions have been answered. I agree to participate as a volunteer in this research project. I understand that I may end my participation at any time. I understand that there is a possibility that the blood, tissue, or urine samples, which I am providing under this study, may also be used in other research studies and could potentially have some commercial applicability. I have been given a copy of this consent form."

Person Obtaining Consent: _____

Subject's signature:_____ **Date:**_____

Subject's permanent address: _____

_____

Witness signature: _____ **Date:**_____

_____ _____
Witness' name (printed or typewritten) Relationship to subject

Subject's Initials _____ Witness's Initials _____

PHONE SCRIPT

Hello, may I please speak to _____.
(If participant is not available, ask whether participant lives at this address and when would be a better time to call back.)

My name is _____. We are currently conducting a study on women to see the role of specific foods in reducing the risk of breast cancer. This study is a collaborative effort between the South Carolina Cancer Center, the Palmetto Health Alliance, and the Norman J. Arnold School of Public Health.

We were given permission by Palmetto Richland Memorial Hospital and the University of South Carolina to contact women who have received breast cancer screening or breast health care at Palmetto Richland Memorial Hospital.

Do you have a few minutes for me to tell you a little more about this study to see if you may be interested in taking part?

If no, (either thank the participant for her time or enquire when would be a better time to call her back. Also record in the verification table of the ACCESS database the appropriate response and time to call back)

If yes, then continue:

The findings of this study will be important because very little is known about what a woman can do to affect breast health. Lifestyle choices, especially around eating, may hold the key to reducing the risk of breast cancer. In this study, we are interested in whether eating certain types of vegetables every day may help prevent disease. In our previous work we have seen that diet may play an important role in reducing breast cancer risk ***{Be ready to answer a few questions here- on published study results & role of estrogen in BC}.***

Description of the study:

For intervention group say this:
If you agree to be in the study, we will be asking you to eat certain vegetables during the four weeks of the study. To help you, we will provide you with vegetables, recipe books, and cooking classes. In addition to eating these vegetables, you will be asked to schedule two appointments at our clinic during which we will collect a urine sample and a tissue sample from your breast.

Just to let you know, this procedure would be performed by a trained professional and is simple and less painful than having blood drawn, though we understand that you may have some concerns about how it works. **{Be ready to answer a few questions here:** Using a syringe, a small sample of your tissue will be extracted. As I said before, this is a

simple and less painful process than having blood drawn. The visit shouldn't take more than about an hour in total. } We will also be taking some measurements such as your height, weight, abdominal circumference and percent body fat. We estimate that the entire visit should take approximately one hour.

For non-intervention group say this:
If you agree to be in the study, you will be asked to schedule two appointments at our clinic during which we will collect a urine sample and a tissue sample from your breast. Just to let you know, this procedure is less involved and less painful than having blood drawn. We will also be taking some measurements like height, weight, abdominal circumference and percent body fat. We estimate that the entire visit should take approximately one hour.

Also, if you are interested we will provide you with cooking classes and a recipe book at the end of the study period.

For both groups, say this:
We will also be sending you a questionnaire that you can complete over the next week and return to us. The only way that we can learn more about the effect of lifestyle choices is with your participation. Would you be interested in helping us? **or** Does this seem like something that you would be interested in doing?

[If no, thank the participant for her time:
Thank you for your time and have a nice day.]

If yes, continue: Thank You! Do you have about 10 minutes to spare so that I can ask you a couple of questions? (open the ACCESS database and record the information in the survey form from now on)

If no then record the best time to call back at weekdays and weekend in the verification part of the ACCESS database.

ASK QUESTIONS FROM THE QUESTIONNAIRE.

We can schedule your first appointment at your convenience during the next week.

When will be the best time to schedule your appointment?
(RECORD APPOINTMENT ON THE SURVEY FORM OF THE ACCESS DATABASE and then consequently transfer at the end of the day on the **CALENDAR)**

The appointment will be in the same clinic where you will be coming for your work-up. A staff member will meet you in the lobby.

We will also be taking waist, hip, and abdominal body measurements so we would like to ask that you refrain from exercising or sitting in a sauna within 8 hours of your appointment. In order for the measurements to be accurate, we also need you to refrain from alcohol for 12 hours prior to your appointment. Before agreeing to take part in the study, you will be asked to sign a consent form during this visit. Do you have any questions at this time?

Well, we've reached the end of the interview. We will be mailing out the questionnaire to you in the next few days. When you receive it, please take the time to read the directions and fill it out as well as you can. If you have any questions, you can call the project coordinator, Mary Modayil, at 777-6217.

On behalf of the entire Breast Health Intervention Study research staff I would like to thank you for participating in this study. Have a nice afternoon.

5347315165

ID

BRASSICA HEALTH STUDY
Phone Eligibility

Date Form Completed

Month / Day / Year

Time : O am O pm

First Initial	Middle Initial	Last name

Name of Interviewer: _____

I have several questions that I would like to ask you to see if you may qualify to take part in this study (it should take about 10 minutes). Is this a convenient time for you?

O **Yes** O **No**

If not, when would be a better time to call you? _____

Personal Characteristics:

O Y O N **Are you over 45 years of age?**

O Y O N **Are you completely past menopause or past the change of life? (i.e. have you had no period in the past 12 months)**

O Y O N **Do you plan to live in South Carolina for the next six months?**

Subjective assessment of interviewer:

O Y O N **English sufficient to understand questions and provide quality data?**

O Y O N **Likely to complete study protocol as described in Consent Form?**

Diet:

O Y O N **Are you on any diet program such as "weight watchers" to reduce or control your weight?**

O Y O N **Are you on any special diet for health reasons such as a low salt diet or a low sugar diet?**

O Y O N **Do you drink more than 3 alcoholic drinks in a day?**

O Y O N **Do you eat beef or chicken at least 3 times in a week?**

Medication Use:

O Y O N **Do you smoke cigarettes or use any other tobacco product?**

O Y O N **Are you currently taking any hormone replacement medications or exogenous estrogens? If so, what kind? (List these "estrace mix" name brands)**

Please list the name of this medication, and the frequency you use it.

Name	*Number of Times per Week*
_____	☐☐
_____	☐☐

O Y O N **Do you take any over-the-counter hormones (e.g. melatonin, DHEA) or herbal remedies? If so, what kind? (List these name brands....such as black-cohoch, saw palmetto, etc.)**

O Y O N **Do you regularly use Tagamet or Pepcid AC for indigestion or heart-burn?**

O Y O N **Are you on any medication for hypertension or diabetes? If so, what kind? (List these "diuretics")**

O Y O N **Are you currently taking any antibiotics? If so, for how long do you expect to take these (i.e. would you not be taking these antibiotics during the study period)?**

Are you currently taking hormone replacement medications or exogenous estrogens?

O Yes (Go to next question)
O No (Skip next question)

Please list the name of this medication, and the frequency you use it.

Name *Number of Times per Week*

_____ ☐☐

_____ ☐☐

Are you currently taking any other diet or nutritional supplement regularly? (i.e. over 3 times per week)

O Yes (Go to next question)
O No (Skip next question)

Please list the name of this supplement, and the frequency you use it.

Name *Number of Times per Week*

_____ ☐☐

_____ ☐☐

Health History:

O Y O N **Have you been diagnosed with any type of cancer or malignancy in the past 5 years (excluding superficial skin lesions)?**

O Y O N **Have you ever had surgery to remove a kidney or adrenal gland removed?**

O Y O N **Have you ever been diagnosed with a liver disease, such as cirrhoses?**

O Y O N **Within the past year, have you been admitted to a psychiatric hospital?**

We will be contacting you by telephone several times over the study period.

Do you have another day-time phone number at which you can be reached (other than this one)? O Yes O No

Alternate day-time phone: ☐☐☐ - ☐☐☐ - ☐☐☐☐

What time(s) of the day are you most likely to be home?

Day of the Week	Good Times to Call	Bad Times to Call
Sunday		
Monday		
Tuesday		
Wednesday		
Thursday		
Friday		
Saturday		

Appendix 4
Collection & Processing

URINE PROTOCOL

Collection:

The sample will be collected on the day of the clinical visit in a standard sterile collection cup. To prevent oxidation of labile products, 100 mg ascorbic acid will be added to each cup prior to the urine collection.

Supply

1. Urine Collection cup.
2. 100 mg of Ascorbic Acid.
3. Plastic bags.
4. Paper bags.

Urine Collection

- Label the container with the participants ID number, initials, and the date.
- Ask participant to provide a urine sample. If she is able to do so at that time, a urine collection cup, a plastic bag and a paper bag will be provided to her.
- Give instructions for urine collection. "Please fill to this line on cup" and mark on cup at 50 ml line.
- Escorted to the ladies room. If she is not able to pass urine she will be asked to drink a glass or two of water and she can collect the sample anytime during the course of the visit.

- Record date and time on the tracking form.
- Check container lid for tightness.
- Store sample in refrigerator or cooler (if at Baptist site) at 4 degrees C until the sample is processed. (Must be placed in a biohazard sample bag).

PROCESSING SAMPLE (can be completed up to 5 hrs after collection).

- Aliquot approximately 1.25 mLs into 4 orange-topped cryovials.
- Place the cryovials in a -70° C freezer for long-term storage.

Blood Processing

- Two (2) vacutainers of blood are collected from each subject
  - Blood drawn from arm, using standard sterile procedures
  - 1 red topped tube without anticoagulants
  - 1 lavender topped tube with EDTA
  - The blood will be centrifuged at 3000 g for 15 minutes
  - Plasma or serum will be removed
  - Stored as five x 1 ml aliquots at -70 °C in labeled cryo-vials.
- Labels:
  - ID
  - S if serum
  - P if plasma
  - B for Baseline
  - F for Follow-up
  - Example : 43PF

BUCCAL PROTOCOL

Buccal cells are appropriate for determining genetic polymorphisms.

Supply for Collection Kits:

- 1.5 oz of mouth wash
- Sterile collection container. Make a mark with black pen on the side of container to indicate 10 ml.
- Plastic bag and freezer-resistant label with ID and name of the participant printed on it.
- Paper Towels.
- Plastic cup with water.

Directions for Mouthwash

1. The participant should be asked not to eat or drink anything other than water till her buccal sample is taken.
2. Collect buccal cells after the BIA measurement
3. Write date, initials, and ID number on the collection container side (not on lid, as lid might be lost).
4. Pour 10 ml mouthwash into collection cup. Give cup to participant.
5. Instruct participant to swish the mouthwash around in her mouth vigorously for 60 seconds. **(This should be timed)**. It is important that the participant

should not shorten the time she swishes the mouthwash, but there is no harm in doing it longer than 60 seconds. The patient may be advised to expectorate early if they begin to gag or if the burning of the mouthwash becomes unbearable.

6. Instruct participant to spit the mouthwash into the container. Replace the cover on the container tightly.

PROCESSING THE SAMPLE (can be done up to 5 hours after collection)

7. Mix specimen thoroughly by gently inverting the wide mouth collection, cup 4-6 times.
8. Using a 10ml. pipet, transfer the specimen into a 15mL, conical tube (large, blue-topped tube).
9. Centrifuge the specimen at 3750 rpms for 10 minutes. Once completed be careful not to jostle the tube. This will re-suspend the DNA
10. Discard the supernatent (liquid part of the tube) by decantation.
11. Resuspend the buccal cell pellet with 3.0 mL of 70% ethanol. Prepare 70% ethanol by adding 70 mL of ethanol to 30 mL depc water. Store excess ethanol and diluted solution at ambient temperature.
12. Using a 5 mL pipet, aliquot equal amounts of specimen into 4 blue-topped sterile cryovials.
13. Place the cryovials in a -70° C freezer for long-term storage.

1. **Clinical appointment Instructions**

- You should not exercise or take a sauna before 8 hours of the study.
- You should refrain from alcohol intake for 12 hours prior to the study.

ANTHROPOMETRIC MEASUREMENT PROTOCOL

This section contains the standardized procedures for the anthropometric measurements (height, weight, and the abdominal, waist, and hip circumferences).

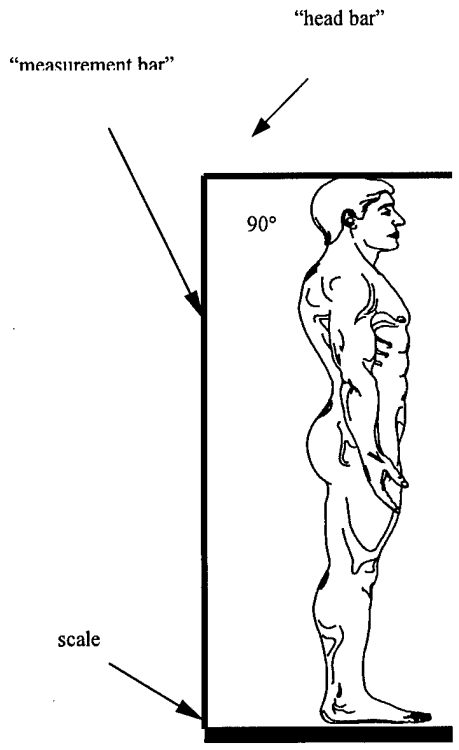
Instruments and Supply:

1. Scale for measuring height in feet and inches. (Qty.: 2)
2. Weighing scale with units in pounds (Qty.: 2)
3. Measuring tape (Gulick II tape measure) with units in centimeters. (Qty.: 2)

In order to approximate, as closely as possible, the participant's size and weight without clothes, all measures should be made **without shoes** and **without extra clothing**, such as sweaters, jackets, or coats.

Height

- Raise the height "measurement bar", extend the "head bar", and have participant step up on the scale facing out into the room
- They should be standing up straight, be looking directly ahead, and have her arms resting comfortably by their side
- To make the measurement, have the participant stand up straight, take a breath and exhale, and then, using two hands, lower the measurement bar down until the head bar rests on the crown of the head.
- The "head bar" should be at a 90° angle to the "measurement bar"
- Have the participant step down off the scale
- View the participant's height at the READ arrow on the



Body Weight

- Before taking individual measurements, be sure the scale(s) properly zeros when the "on" button is pushed. Also be sure that the scale is set to measure in pounds.
- To make a measurement, have the participant step up onto scale. Scale must read "0.0" before the participant steps onto the scale, otherwise the scale will register an error with a blinking display.
- **Record the weight <u>in pounds</u>, to the <u>nearest 0.1 pound</u>**

Circumference Measures

For all measures, be sure that the measuring tape is level (perpendicular to the ground), not twisted, and is pulled firmly around the participant.

Take all of the measures in order (waist, abdomen, and hip and record the values after each measurement. Repeat the measures at each site, again, in order. If two measures differ by **<u>more than 2 cm</u>**, repeat the measurement of that site a third and final time. Please record all measures taken (i.e., 2-3 per site).

To obtain measurements while holding a constant tension on the measuring tape, use the Gulick II tape measure. That is, when making a circumference reading, hold the cylinder end of the tape and pulling the tape tight across the participant. Be sure that the measurement is read from where the 0 mark at the beginning crosses the end of the tape.

Record the circumferences <u>in centimeters</u> (cm); to the <u>nearest 0.1 cm</u>. Participant's with circumferences greater than 150 cm (~ 60 inches) should be measured using a standard tape measure.

WAIST

- Face the participant; locate **the narrowest part of the torso** (or a site between the lower rib and the crest of the hipbone). You will have to poke and prod to find the correct site.
- Once the site is located, place the measuring tape around the torso and pull it snug. Check that the tape is level, and make the measurement.

ABDOMEN

- Have the participant point out her belly button. Lower the measuring tape to this spot, make sure the tape is level, and make the measurement **at the belly button**. If the location of the belly button is not at the greatest expansion of the participant's abdomen, take the measurement at that position.

HIP

- Have the participant turn to the side.
- Make the measurement at the largest expansion of the rear end, making the tape is level

6884012931

ID

BRASSICA
Measurements

First Initial | Middle Initial | Last name

Cycle:

BASELINE

Date Form Completed — Month / Day / Year

Time: : ○ AM ○ PM

Aliquots: Red Top:

Phlebotomist: _____

Lav Top:

Urine: ○ Yes ○ No

Time Collected: : ○ AM ○ PM

First Morning: ○ Yes ○ No

Fasting: ○ Yes ○ No

Aliquots:

Weight: . lbs

Height: ft in

Measurements:	1st	2nd	3rd (if needed)
Waist:	. in	. in	. in
Ab2:	. in	. in	. in
Hip:	. in	. in	. in

- Waist: narrowest part of abdomen, laterally, midway between lowest portion of rib cage and iliac crest, and anteriorly, midway between the ziphoid process of the sternum and the umbilicus.
- Ab2: abdomen2: laterally at the level of the iliac crest and anteriorly at the umbilicus.
- Hip: (buttocks): anteriorly at the level of the symphysis pubic and posteriorly at the maximal protrusion of the gluteal muscles.

POST-INTERVENTION

Date Form Completed Month / Day / Year

Time : O AM O PM

Aliquots: Red Top:

Phlebotomist: _____ Lav Top:

Urine: O Yes O No Time Collected: : O AM O PM

First Morning: O Yes O No

Fasting: O Yes O No

Aliquots:

Weight: . lbs **Height:** ft in

Measurements:	1st	2nd	3rd (if needed)
Waist:	. in	. in	. in
Ab2:	. in	. in	. in
Hip:	. in	. in	. in

- Waist: narrowest part of abdomen, laterally, midway between lowest portion of rib cage and iliac crest, and anteriorly, midway between the ziphoid process of the sternum and the umbilicus.
- Ab2: abdomen2: laterally at the level of the iliac crest and anteriorly at the umbilicus.
- Hip: (buttocks): anteriorly at the level of the symphysis pubic and posteriorly at the maximal protrusion of the gluteal muscles.

FNA (Fine Needle Aspiration)

BASELINE

Date Form Completed Month / Day / Year

Time : O AM O PM

Technician: _____

Breast side: O Left O Right

1. Any adverse reaction within a half-hour? O Yes O No

   Our response: _____
   eg. drugs or call-back

2. Any adverse reaction in evening? O Yes O No

   Our response: _____
   eg. drugs or call-back

POST-INTERVENTION

Date Form Completed Month / Day / Year

Time : O AM O PM

Technician: _____

Breast side: O Left O Right

1. Any adverse reaction within a half-hour? O Yes O No

   Our response: _____
   eg. drugs or call-back

2. Any adverse reaction in evening? O Yes O No

   Our response: _____
   eg. drugs or call-back

Appendix 5
Intervention Materials

DIRECT Study: Class Schedule

Class 1

Goals
- Introductions
- Discussion: Describe major focus of study
- Explain study design, objective, and expectations

Events
- Introductions
- Distribute recipes and other written material
- Introduce study vegetables (snack time)
- Discussion: Overview of Brassica and Breast Cancer
- Diet Diaries

Take Home Messages
- Eat several servings a day of Brassica to reach study goals
- Do not changes other parts of the diet
- Record diet in diaries

Class 2

Goals
- Cooking practicum
- Discussion: Preparation of Brassica
- Summarize Diet Diary

Events
- Comments/Issues of Concern
- Describe active ingredient(s) in Brassica
- Cooking Practicum
- Preparation Techniques

Take Home Messages
- Continue to add Brassica to diet
- Record diet in diaries
- Plan for Pot-luck meal

Class 3

Goals
- Potluck dinner
- Discussion: Overall Health Effects of Brassica

Events
- Potluck dinner - B,K
- Discussion: Brassica and Health
- Distribute urine collection bottles and questionnaires

Take Home Messages
- Continue eating Brassica
- Focus on preparation methods
- Urine and blood collection week

Class 4

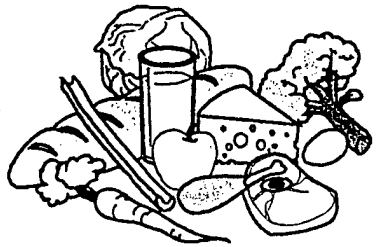
Goals
- Cooking practicum
- Guest speaker

Events
- Cooking practicum
- Guest speaker
- Study summary, discussion, and closing statements

Take Home Messages
- Continue with intervention diet, on your own, until blood drawn and urine collected
- Reminder that final urine/blood/24HR in about 3 weeks

DIRECT

Dietary Intervention to Reduce
the Risk of Breast Cancer



FOOD DIARY

Name: _____

Dates of Diary: _____, _____, _____

1. Record everything you eat or drink in your Food Diary for 3 days.
2. Look up the number of points on last page of diary for each Brassica serving, and record this number in the column labeled BRASSICA PTS.
3. Include comments about cooking and preparation for all Brassica foods.

Abbreviations: M/S = meal/snack, B=breakfast, L=lunch, S=snack, D=dinner

EXAMPLE Day: Wednesday, 4/1/98 (Brassica minimum goal = 10 points)

M/S	Amount	Foods and Beverages	Brassica pts	Preparation/Cooking
B	1	biscuit, 2" diameter	0	
	1 tsp.	jam	0	
	1 cup	coffee	0	
	3 tsp.	whole milk	0	
L	1	tuna sandwich	0	
	1 cup	broccoli	3	chopped, raw
	1 cup	cole slaw /white cabbage	3	chopped
	12 oz	Coca-Cola	0	
	1 oz	cheddar cheese	0	
S	1	Brussels sprouts	2	steamed
	8 oz	whole milk	0	
D	1 cup	beef stew, homemade	0	
	1 cup	tossed salad, (lettuce, tomato, onion, cucumber)	0	
	1/2 cup	savoy cabbage	3.5	chopped, raw
	1 cup	iced tea	0	
		Total Brassica	11.5	

1. Record everything you eat or drink in your Food Diary for 3 days.
2. Look up the number of points for every serving of Brassica, and record this number in the column labeled BRASSICA PTS.
3. Include comments about cooking and preparation for all Brassica foods.

Day 1: _____ (Brassica minimum goal = 10 points)

Abbreviations: M/S = meal/snack, B=breakfast, L=lunch, S=snack, D=dinner

M/S	Amount	Foods and Beverages	Brassica pts	Preparation/Cooking
		Total Brassica		

1. Record everything you eat or drink in your Food Diary for 3 days.
2. Look up the number of points for every serving of Brassica vegetable, and record this number in the column labeled BRASSICA.
3. Include comments about cooking and preparation for all Brassica foods.

Day 2: ____________________ (Brassica minimum goal = 10 points)

Abbreviations: M/S = meal/snack, B=breakfast, L=lunch, S=snack, D=dinner

M/S	Amount	Foods and Beverages	Brassica pts	Preparation/Cooking
		Total Brassica		

1. Record everything you eat or drink in your Food Diary for 3 days.
2. Look up the number of points for every serving of Brassica vegetable, and record this number in the column labeled BRASSICA.
3. Include comments about cooking and preparation for all Brassica foods.

Day 3: _____ (Brassica minimum goal = 10 points)

Abbreviations: M/S = meal/snack, B=breakfast, L=lunch, S=snack, D=dinner

M/S	Amount	Foods and Beverages	Brassica pts	Preparation/Cooking
		Total Brassica		

1. Find the vegetable and serving size that best fits what you ate.
2. If you ate more or less Brassica in a serving than what is listed here, adjust and record the Brassica points accordingly.
   For example, ½ cup of chopped broccoli = 1.5 points. It is not necessary to be more precise.

Minimum Goal = 10 Points

Vegetable	Serving Size	POINTS / SERVING
Brussels Sprouts	1 sprout	2
Savoy Cabbage	1 cup chopped	7
Savoy Cabbage	1 cup shredded	5
Kale	1 cup	4
Red Cabbage	1 cup chopped	3
Red Cabbage	1 cup shredded	3
Broccoli	1 cup chopped	3
Broccoli	1 cup flowerets	3
Broccoli	1 floweret	0
Collards	1 cup chopped	1
White Cabbage	1 cup chopped	3
White Cabbage	1 cup shredded	2
Cauliflower	1 cup chopped	3
Cauliflower	1 cup flowerets	2
Turnip	1 cup-cubed	1
Turnip	1 large	2
Turnip	1 med	1
Turnip	1 small	1
Turnip	1 slice	0

Dietitian Script and Methodology

1. For all interviews, the interviewer introduces him/herself by name, the study with which he/she is affiliated, and that (s)he is going to conduct a 24-hour diet recall. (S)he also will ask if this is a good time to talk. At this point, the dietitian will enter data on age and gender into the Header section of the NDS program.

2. To initiate the recall process, the interviewer will state the following "I would like to know what foods you ate after midnight yesterday, which was (state the day). Please tell me everything you ate or drank, including meals, snacks, beverages, candy, and alcohol. Start with the first thing you ate or drank and progress through the rest of the day. Please indicate approximately what time you had the items, whether it was a snack or a meal, and where you were. I will be entering this information directly onto a computer, so please speak slowly. After you have completed the list, I will be asking you some detailed questions about the recall." The control of the interview is then passed to the subject so (s)he can report food intake. Once the subject has begun to recall food intake, we try to not interrupt his/her train of thought - portion sizes, preparation information, etc. will be gathered in the next step. Some attention and encouragement are appropriate, such as "OK" or "what next." If the subject has difficulty getting started, we ask the subject to recall what (s)he did yesterday, and wait for the subject to start listing the food eaten. We always allow ample time for contemplation. When the subject has completed the 24-hour recall, the interviewer reviews the QUICK LIST and checks for snacks and alcoholic/non-alcoholic beverages and any other forgotten main food items.

3. After the subject has finished providing a description of his/her food intake, we employ standardized probing techniques as directed by the NDS system to assure that the foods are completely described, including detailed food preparation, size of portions eaten, items added to foods, etc. In addition to these on-line instructions we are aware that omissions often occur around snack items and foods taken in situations that are not typically considered eating (for example, using milk to "wash down" bed time medications). When the recall is completed, the interviewer asks the subject to hold the line while she quickly, but carefully, scans/reviews the FOOD REVIEW section of the NDS system to make sure the entries look correct (e.g., accidentally logging in "24 cups" instead of "24 ounces" of a calorie-containing beverage can produce a major difference in calories!). The interviewer should do this verbally, as often the subject may remember additional foods or detect errors as the review is conducted.

4. The interviewer then gathers the information to complete the TRAILER of the program. (S)he then thanks the subject for her/his time and if the situation warrants (i.e., the study requires that the subject be interviewed again) tells her/him that (s)he will be calling them again.

Missed interview protocol

When a call that you have been assigned cannot be completed, we would like you to make a 2nd and, if necessary, a 3rd attempt to contact the subject. First, check the patient information to see if there is revised information on "best time to call". Next, make the follow-up attempt on the first available day that corresponds to type of call day (weekday or weekend day) that was missed. For example, if the missed call is on the weekend, simply try to interview the subject on the next weekend day. If you are not available on a make-up date, please notify the project coordinator who will reschedule the interview.

Keep in mind that study subjects agree to participate and are expected to cooperate to complete the interviews. The project coordinator should be notified of any problems with cooperation. The project coordinator will then contact the site coordinator and enter a note into the study database.