WHI Nutritional Biomarkers Study
Protocol- Version 3

I. INTRODUCTION

Dietary and nutritional factors likely play a major role in determining the risk of major chronic diseases such as cardiovascular disease and cancer. However, the dietary assessment instruments used in epidemiological investigations of diet and disease are subject to random measurement errors leading to bias in estimates of the relative risk of disease in relation to nutritional factors. Moreover, certain population subgroups, such as obese individuals, may systematically underreport dietary intake. Both types of measurement errors can seriously affect study findings (usually by attenuating relative risk estimates) where diet is an important exposure variable (*1-3*). Regression calibration is a statistical method that can correct for these biases in measurement error, provided suitable biomarkers of nutrient consumption are measured, and thus provide more reliable diet-disease analyses (*4*) (*3,5*). Therefore, the overall objective of this study is to collect objective (i.e., blood and urine) measures of dietary intake to use in regression calibration models in the Women's Health Initiative.

This protocol describes the procedures for a study of nutritional biomarkers among a subset of women enrolled in the Dietary Modification component of the Women's Health Initiative (WHI). This study will provide critical data needed for the interpretation of results from this large clinical trial of diet and health.

II. OBJECTIVES

The objective of this study is to collect biological markers of dietary intake for use in regression calibration models that will correct the random and systematic bias of dietary self-report. The calibrated nutrient intake data will then be used for a broad range of nutrient-disease analyses from the Women's Health Initiative.

The specific aims are:

1) To determine the association of self-reported measures of dietary intake from a food frequency questionnaire (FFQ) with actual intake, with the help of objective measures (biomarkers) of macronutrient intake (energy, fat, protein).
2) To determine the association of self-reported measures of dietary intake from a food frequency questionnaire (FFQ) with actual intake, with the help of biomarkers of micronutrient intake (tocopherols, retinol, folate, carotenoids, B-vitamins, selenium, potassium. sodium).

III. BACKGROUND AND SIGNIFICANCE

Ecological and migration studies support an association between diet and cancer risk, with strong correlations, for example, between dietary fat intake and increased risk for cancer of the breast, prostate, and colon (*6,7*) and inverse associations of plant foods

with cancer risk (*8-10*). Fat may act as a cancer initiator or promoter, while other nutrients such as carotenoids and antioxidant vitamins may decrease cancer risk by protecting cellular constituents from oxidative damage. This biological rationale is supported by both *in vitro* and animal model studies (*9*). However, data are less consistent from analytic epidemiologic studies of diet and cancer risk with many studies unable to detect hypothesized associations nutrients with cancer risk. Clearly, these inconsistencies pose a considerable challenge when making dietary public health recommendations for chronic disease prevention.

Observational investigations of diet and cancer risk typically rely on self-report of diet by using standard dietary assessment tools such as food diaries, 24-hour dietary recalls or food frequency questionnaires. Many of these studies have failed to provide consistent information on the relationships between dietary factors (particularly fat intake) and disease. A potential reason for this failure is the lack of reliability of self-reported dietary intakes (*4*). Most notable is the fact that underreporting of dietary energy intake appears to be a common phenomenon and that the extent of underreporting likely varies by participant characteristics, such as obesity (*11,12*).

These measurement error issues are important for the interpretation of the Women's Health Initiative Dietary Modification (DM) Clinical Trial. The principal results from this large study will contrast intervention and control groups with respect to breast cancer, colorectal cancer, coronary heart disease, and a range of other secondary outcomes. These contrasts do not rely on nutrient and food consumption data for their validity, but the interpretation of the results depends on the assessment of the magnitude of the difference in dietary habits between DM intervention and control groups, and on the Control-Intervention differences for specific nutrients. Aside from blood concentrations for selected vitamins in a 6% Clinical Trial subsample (4.3% in the DM cohort), we have only dietary self-report data from which to "estimate" individual consumption of specific nutrients. Food frequency questionnaires (FFQs), the primary dietary assessment tool in WHI, are available at baseline, 1 year, and approximately every third year thereafter for all DM women. However, the measurement properties of FFQs, as well as food records and recalls, in populations like the WHI DM cohort are substantially unknown, in part due to the typical absence of adequate objective marker substudies in nutritional epidemiologic studies to date (*13*). As noted above these measurement error issues may strongly influence the primary results of nutritional epidemiology studies, and the interpretation of the WHI Dietary Modification trial. For example, a recent nutrient biomarker study conducted by the National Cancer Institute and a substudy in the European Prospective Investigation of Cancer suggest systematic biases in nutritional self-report data of a magnitude that could greatly affect both the validity of observational studies and the interpretation of intervention trials in the nutritional epidemiology areas (*7,14*). It is therefore critical to collect objectives measures of dietary intake whose sources of error are independent of the sources of error in self-reported measures of diet. But since it is neither economically feasible nor practical to collect certain key biological markers of diet from all participants in large studies, these measures should be collected on a subset of participants and the objective data used to "calibrate" the self-reported data from the entire sample (*5*).

This protocol describes a study of nutrient biomarkers in a subset of about 550 women (275 intervention, 275 control) who are enrolled in the Dietary Modification arm of WHI. This study will be conducted in 12 WHI Clinical Centers (CCs), ten of which will each enroll 50 women, half DM-intervention and half DM-control. The other two CCs will restrict their recruitment to minority women in order to have a final sample whose minority composition is reasonably representative of the entire WHI DM cohort. These CCs are each expected to each enroll about 25 women but are permitted to recruit up to 50 participants if able to do so. The principal nutritional biomarkers to be obtained in this study are: 1) energy expenditure; 2) nitrogen (as a measure of protein intake); 3) fatty acids; and 4) micronutrients (i.e., vitamins and minerals). To complete the protocol, participants will attend two visits to their local WHI Clinical Center where they are enrolled as WHI participants; the visits will be two weeks apart. The first visit will last approximately five hours The second visit will last two hours (or three hours for the Madison, Pittsburgh and Chicago-Northwest for women who chose to do the indirect calorimetry) and will be conducted two weeks after the first visit. Women may elect to do the visits in conjunction with their WHI Annual Visit, but this is not necessary. The biomarkers will be obtained from blood and urine specimens collected at the two clinic visits as well as from a urine collection completed in the participant's home and brought to the clinic.

Measure of total energy expenditure: doubly labeled water

Briefly, total energy expenditure is the amount of energy that an individual uses throughout the day. In weight stable individuals, energy expenditure is approximately equivalent to energy intake such that if we can measure the former with some degree of precision, it gives an unbiased estimate of a person's caloric intake. The method of choice to measure energy expenditure is the doubly labeled water (DLW) technique, which measures energy expenditure over a period of approximately two weeks (*15,16*). The principle is that after a loading dose of water labeled with deuterium (a stable isotope of hydrogen) and the stable isotope ^{18}O, these tracers rapidly equilibrate in body water. The deuterium is eliminated from the body as water and the elimination rate is thus proportional to water turnover. The ^{18}O is eliminated as water and carbon dioxide and thus its elimination is proportional to the sum of water and carbon dioxide production. The difference between these two elimination rates is proportional to carbon dioxide production, which is the end product of energy metabolism (*15*).

The actual measurement involves administration of the labeled water and collection of physiologic specimens (urine and blood) on the day of administration and two weeks later. The participant arrives at the clinic after a four hour fast, provides a baseline urine specimen, is weighed and takes the DLW in a single dose of 10 atom percent ^{18}O labeled water and 0.12 grams of 99.9% deuterium labeled water per kilogram body weight. Participants remain in the clinic for at least four hours post-dosing and provide spot urine specimens at 2, 3, and 4 hours after administration of the isotopes (*14,16*). Women receive a meal replacement beverage one hour after ingesting the DLW and additional fluids if they have difficulty producing a urine specimen. Women aged 60

years and above will provide a blood sample three hours after consuming the DLW to compensate for post-void urine retention that is often experienced by older women (*17*). This will be collected in a standard 10 ml vacutainer. A complementary meal or snack is provided after collection of the 4 hour post DLW dosing void. Women return to the clinic for a shorter visit on about Day 15, at which time they will return a 24-hour urine collection (see below; this 24-hour urine specimen will be used to measure urinary nitrogen as an estimate of protein intake) and their weight will be measured. Two urine specimens, collected one hour apart will be obtained. A complementary meal will be provided to all participants after collection of the final urine void.

The first spot urine, which is collected prior to the DLW dosing, provides data on the participant's natural isotopic abundance. The subsequent spot urines collected during the first visit are necessary to determine the equilibration of the tracer with body water. The two specimens collected at visit 2 give a reliable estimate of isotope turnover.

The isotopes are measured in the biological specimens by mass spectrometry and total energy expenditure is then calculated from carbon dioxide production using standard equations (*14,15*). The isotopes, deuterium and ^{18}O are nontoxic and safe. They are naturally occurring isotopes. The DLW solution is tasteless, odorless and colorless. DLW has been used in studies with babies as small as four pounds (Dr. Dale Schoeller, personal communication). About 1% of all participants experience transient vertigo after drinking the DLW solution that lasts only few minutes (*16*). While the vertigo is rare, as a precaution all participants remain seated for at least 15 minutes after drinking the DLW.

Measurement of Resting Energy Expenditure: Indirect Calorimetry

A subset of the WHI Clinics participating in the Nutritional Biomarkers Study will also participate in studies of Resting Energy Expenditure using Indirect Calorimetry. The resting energy expenditure measurements will serve multiple purposes:

- Identify the physiologic determinants of total energy expenditure
- Use in conjunction with total energy expenditure to provide a measure of physical activity by difference

Resting Energy Expenditure Measurement

Resting metabolic rate will be measured with a Deltatrac II Respiratory Gas Analyzer, a Sensormedics 29 or similar instrument. These are semi-portable units that measure the concentrations of oxygen and carbon dioxide in air streams entering and exiting a clear plastic hood placed over the participant's head. Oxygen consumption and carbon dioxide production are calculated from the change in concentration and flow rate. The measurement must be made under standard conditions and requires about 30-40 minutes to complete. A 30-minute rest period is required prior to the start of the indirect calorimetry test to ensure that resting energy expenditure is measured (*18*).

Measure of protein intake: urinary nitrogen:

Urinary nitrogen has been used for decades to assess whether or not patients in the clinical setting (i.e., trauma, burn and surgical patients) are in nitrogen balance. Controlled experimental studies show that the correlation between controlled protein intake and 24-hour urinary nitrogen is 0.8-0.9 (*19*). More recently, urinary nitrogen has been used as a biomarker in epidemiologic studies (*14,20*).

Urinary nitrogen is an excellent biomarker of protein intake because over 80% of the nitrogen in protein is recovered in the urine. Because approximately 16% of protein is nitrogen:

Nitrogen/0.8 x 6.25 = Estimated Protein Intake (*19*)

In this WHI substudy participants will be asked to collect all of their urine for a 24-hour period and bring the specimens to the clinic when they return on Day 15 to complete doubly labeled water part of the protocol (see above). A collection kit and instructions will be provided. Participants will be instructed to discard the first void on the day of collection and begin the collection with the next void. The 24-hour period ends with the first void of the next day.

Some women will also take a B- vitamin (Para amino benzoic acid) the day of their 24-hour urine collection. In order to assess completeness of the 24-hour urine collections, participants will take 80 mg PABA (para amino benzoic acid tablets, which is a B-vitamin and is available for purchase over the counter) at each main meal on the day of the urine collections. This is standard protocol for research studies collecting 24-hour urine specimens (*14*). Because this B-vitamin, PABA, is nearly completely recovered in the urine, its measurement functions as an excellent measure of compliance. This is important because incomplete urine collections would result in a miscalculation of the urinary nitrogen measure. A bottle of the vitamins will be placed in the urine kit as they become available from the manufacturer (they will be purchase as a single lot from a single manufacturer. The single lot may not be available right away; the vitamin bottles will be offered to women as they become available**.**

There are no known risks to collection of urine specimens. We will use a modified Kjeldahl method to measure the urinary nitrogen. Urine remaining after the urinary nitrogen is estimated will be used to assess urinary calcium, sodium and potassium, nutrients for which consumption cannot be validly measured using blood specimens.

<u>Measure of fatty acids and micronutrients:</u>

A fasting blood specimen will be collected at the second visit on Day 15 to conduct laboratory measures of fatty acids and micronutrients. We will collect four 7-ml vacutainers of serum and one 10-ml vacutainer of plasma from all participants. Bloods will be processed and aliquoted at the Clinical Centers and all specimens shipped to the WHI Central Specimen Repository at McKesson BioServices in Rockville, MD. All specimens will be stored at –80 º C until analysis. Details about specimen handling, processing and shipment will be identical to standard WHI procedures, which are

specified in the WHI manuals, Volume 2, Section 11 (Blood and urine collection, processing and shipment).

Plasma phospholipid fatty acids will be analyzed by gas-liquid chromotography (*21*). Total, HDL and LDL cholesterol will be measured by an enzymatic method (necessary for statistical adjustments of the fat- soluble nutrients.) Retinol, tocopherols and carotenoids will be analyzed using high performance liquid chromatography (*22*). Folate and vitamin B_{12} will be measured by radioimmunoassay (*23*). Selenium and zinc will be measured by atomic absorption spectrometry (*22*).

Other Measures:

The following standard WHI forms will be completed as part of this study: Form 60 (food frequency questionnaire), Form 45 (dietary supplement use), and Form 35 (personal habits update). We will also measure height and weight. WHI participants are familiar with these measures as they are part of routine data collection in WHI and are approved as part of the primary IRB approval (see IR File # 3467). However, it is important to point out that while this Nutritional Biomarkers Study may coincide with WHI Annual Visits, not all of these forms are routinely collected at each Annual Visit. Because the FFQ, the physical measures, dietary supplement use and physical activity are crucial to the statistical modeling planned for this study, these measures will be completed by all women who participate in this study.

Other Details:

A sample size of 500 will be sufficient to accommodate selected sources of systematic bias in this calibration procedure. However, we will include about an additional 50 women from racial/ethnic minorities to ensure adequate minority representation. DM participants from the 12 selected WHI CCs will be invited to participate in this substudy via a personal letter and follow-up phone call to participate in conjunction with the regularly scheduled clinic visit. The invitation to participate will come from the CC where they complete their Annual Visits. Willing participants will be consented at the clinic visit and complete all the procedures described above. For a subset of about 110 women (a "reliability sample"), the protocol will be repeated in conjunction with a woman's WHI close-out visit, an average of 6 months later. These 110 women will also provide a 24-hour dietary recall in conjunction with each application of the protocol (one about 3 weeks after the first two clinic visit and one at the second application of the protocol six months later). The procedures for the 24-hour dietary recall will be identical to the procedures already in use in the DM trial for the subsample (1%) of DM trial women who complete unannounced dietary recalls each year. These recalls are interviewer administered over the telephone by the Nutritional Assessment Shared Resource of the Fred Hutchinson Cancer Research Center (see IR file 3467). Women who become part of the "reliability sample" will be mailed a portion size booklet, which is used as a guide during the dietary recall interview.

All participants who complete the 'entire' protocol will receive $100.00 to compensate for time and travel. The entire protocol includes: the DLW procedures, completion of Forms 35, 45, 60 and 80, fasting blood draw and 24-hour urine collection. Participants who are willing to attend both clinic visits, but are unable to produce sufficient urine or for whom the trained clinic phelebotomists are unable to obtain a sufficient quantity of blood will receive the $100.00. Participants who do not return for the second visit will not receive the $100.00. Women who become part of the reliability sample and repeat the protocol six months after the first completion of the protocol will receive an additional $100.00. All participants will receive a complementary meal at the end of each clinic visit.

This study will be conducted between May 2004 and September 2004. The 110 women in the reliability sample who will repeat the protocol will complete the procedures between October 2004 and March 2005.

IV. STUDY PROCEDURES

A. Recruitment

Twelve WHI Clinical Centers (CCs) have been selected to participate in this substudy (Buffalo, Seattle, Oakland, Pittsburgh, Pawtucket, Stanford, Chicago-Northwest, Worcester, Madison, Chapel Hill, Arizona and Memphis). These CCs were selected based on their overall performance in the Dietary Modification Trial and their stated willingness and ability to complete this protocol. Each Clinical Center will recruit approximately 50 participants, except the two minority recruitment Centers, which are expected to recruit about 25 participants. Each Clinical Center will enroll about 20% of these participants in the reliability sample.

Chicago-Northwestern, Madison and Pittsburgh will also conduct the indirect calorimetry tests. They will ask women to complete these tests at NBS Visit # 2. No additional women will be recruited exclusively for the indirect calorimetry. Women from these three clinics are not obligated to participate in the indirect calorimetry. They may participate in the rest of the Nutritional Biomarkers Study without completing the indirect calorimetry. No additional funds to compensate for time and travel expenses will be offered to those women who participate in the indirect calorimetry, but these women will be offered their resting energy expenditure data (the number of calories their body burns at rest). The indirect calorimetry procedures will be explained at Visit # 1 and those who wish to participate will do so at Visit # 2. All women participating in NBS at the Pittsburgh, Madison and Chicago-Northwestern Clinical Centers will be invited. Up to 150 women from all three Clinical Centers will complete the indirect calorimetry procedures.

IRB approval will be secured at the Fred Hutchinson Cancer Research Center (for the Coordinating Center) and at each institution having a participating CC.

A.1. Participant Eligibility and Exclusionary Criteria

A.1.1. Participants will be recruited from WHI women who are enrolled the Dietary Modification Trial. The study will include participants who are scheduled for an Annual follow-up Visit between May and September 2004 and who are not scheduled for a blood draw at their upcoming Annual Visit (AV). The reason for exclusion of the women who are already scheduled for a blood draw is to minimize any discomfort to the participant that may result from additional blood being drawn and to maximize the efficiency for specimen tracking and data management. The coordination with the AV is intended to minimize the number of times that a woman needs to attend the clinic, but it should also be emphasized that this protocol will not interfere with the AV procedures. Training will be provided by the Clinical Coordinating Center to ensure that all AV procedures as well as procedures for this study will be completed. If clinics desire, they may also invite women to participate who are not scheduled for an Annual Visit. In this case, women would be asked to come to the WHI Clinical Center for the express purpose of completing the Nutritional Biomarkers Study.

A.1.2. The total number of participants will be up to about 275 from the intervention arm and about 275 from the control arm. All 550 women will complete the protocol once and about 110 women will repeat the protocol six months later to obtain measures of reliability.

A.1.3. Exclusion criteria:
Women will be excluded who

1. Do not have a FFQ (Food Frequency Questionnaire) at baseline, year 1, and at least one additional time point.
2. Are not participating in Annual Visits at the full follow-up level.
3. Take insulin or oral hypoglycemic agents to manage diabetes.*
4. Require supplemental oxygen.*
5. Have had blood transfusions, administration of blood products or administration of intravenous fluids in excess of 500 mL in the week prior to the first clinic visit for this study or an expectation of same during the period between visits 1 and 2.*
6. Travel more than 200 miles from home during the week prior to the study and throughout the two week study period.*
7. Have lost or gained more than 10% of their body weight in the previous month.*
8. Have bladder control problems that would make collection of a 24-hour urine specimen difficult.
9. Women who are scheduled for a blood draw as part of their WHI Annual Visit (see above – 4.3% of the sample falls in this group).

* These factors interfere with the doubly labeled water measurements of energy expenditure (Dr. Dale Schoeller, personal communication).

A.1.3.a.
In addition to the exclusion criteria noted above, women will be excluded from the Indirect Calorimetry procedures who have self-reported claustrophobia, which would cause the participant to become anxious when the plastic RMR hood is placed over their head. These procedures require that the participant lie quietly for 30 minutes and anxiety caused by claustrophobia would produce inaccurate test results.

A.2. Recruitment

A.2.1. The 12 participating WHI Clinical Centers will receive a list of eligible participants based on exclusions #1, #2, # 3 and # 9 (information that is in the WHI database) one month prior to the woman's scheduled AV. These lists will be provided by the WHI Clinical Coordinating Center at the Fred Hutchinson Cancer Research Center.

A.2.2. These lists of participants will be generated to include women across the age and race/ethnicity distribution of the WHI DM. The lists for the two minority recruitment CCs will be comprised of minority women only.

A.2.3. CCs will mail letters of invitation to potential participants no less than two weeks prior to scheduled AV. For women who are being invited to participate not in conjunction with their AV, there are no date parameters around mailing the letter of invitation.

A.2.4. CCs will telephone potential participants within one week of mailing the letter. CC staff will conduct telephone screening and recruitment script. During this telephone screening, staff will determine eligibility based on exclusions # 4-8 to assess current status in relation to eligibility. Up to five attempts will be made to contact potential participants by telephone. Because this study must be completed in a short amount of time (prior to the end of WHI) in the event that clinic staff are unable to reach participant after five attempts, the participant will be classified as "ineligible/unreachable" and no further inquiries will be made by phone. If the participant inquires about the NBS study participation when they arrive at the clinic for their Annual Visit and they meet the eligibility criteria, they are permitted to be enrolled and scheduled for the study procedures. Disposition of the telephone screening call will be noted on Form 74.

Clinic staff may refer to the "Frequently Asked Questions" document when answering questions about the DLW protocol during the telephone screening.

A.2.5. CCs will schedule eligible participants. Every effort should be made to avoid scheduling women from the intervention and control arms on the same day. Up to this time every effort has been made to avoid "contamination" of the WHI DM control group. Scheduling the intervention and control women on different days helps to maintain that principle.

B. Study Procedures

B.1 Pre-examination procedures – one day prior to visit 1.

B.1.1. Telephone participant to confirm appointment and remind participant to refrain from eating any food or drink any beverages for four hours prior to the appointment time. If participant is scheduled for Monday, the call may be made on the preceding Friday. Remind participant to bring dietary supplements to the clinic for the Form 45 (current supplements) completion.

B.1.2. Confirm that all supplies are available for the participant visit.

B.2.1 Day 1 (May or may not be day of Regular AV visit)

1. Greet participant
2. Complete questions on NBS Eligibility Worksheet (Visit 1 version).
3. Complete informed consent (The Frequently Asked Questions may also be used here) and give participant "Study at a Glance"
4. Obtain participant height and weight and record on Visit Form 75 (Visit 1 version).
5. Obtain baseline urine specimen. Place hat over toilet and ask participant to void into hat. Record time of void. Fill 5 mL corning cryotube with 4.0 mL urine specimen, label with participant ID and date and time of collection and store in freezer. Discard remaining urine. Rinse and dry hat. (<u>It is critical that the hat be thoroughly dried</u>.)
6. Select the appropriate doubly labeled water (DLW) bottle appropriate for the participant's weight:

$\leq$ 65 kg – Bottle A
66 to $\leq$ 80 kg – Bottle B
81 to $\leq$ 105 kg – Bottle C
$\geq$ 106 kg – Bottle D

The DLW will be a dose of approximately 2 g of 10 atom percent ^{18}O labeled water and 0.12 g of 99.9 atom percent deuterium labeled water per kilogram of measured body weight. The total volume to drink is about ¼ cup.

7. Ask participant to drink all of the DLW from the pre-weighed bottle. Participant should remain seated for 15 minutes to minimize the small chance for vertigo that may occur upon drinking the loading dose.
8. If any DLW water spills, mop up with the pre-weighed tissue in the ziplock bag. Return to the bag and seal. Note any spillage**.**
9. Fill the DLW dose bottle with 50 mL of tap water. Replace the cap and mix gently. Ask the participant to drink the rinse water. Record the time.
11. One hour post-dose, provide participant with 8 oz of Sustacal or other meal replacement beverage and allow them to drink it completely. Record the time and volume consumed.

12. Give participant Form 60 (Food Questionnaire), Form 35 (Personal Habits Update) and Form 45 (Dietary supplement use) to complete. Complete AV tasks if necessary.
13. Two hours post-DLW dose obtain a urine specimen. Place hat over toilet and ask participant to void into hat. Record time of void. Fill 5 mL corning cryotube with 4.0 mL urine specimen, label with participant ID and date and time of collection and store in freezer. Discard remaining urine. Rinse and dry hat.
14. Three hours post DLW dose, obtain a urine specimen. Place hat over toilet and ask participant to void into hat. Record time of void. Fill 5 mL corning cryotube with 4.0 mL urine specimen, label with participant ID and date and time of collection and store in freezer. Discard remaining urine. Rinse and dry hat. <u>If participant is over 60 years of age, draw 1 Lavender EDTA tube of blood.</u> Use the standard WHI blood handling and processing procedures in WHI manual Volume 2, section11. See also NBS Visit Form 75 (visit 1 version) Label specimen with ppt ID and date of collection and place in freezer.
15. Four hours post DLW dose, obtain a urine specimen. Place hat over toilet and ask participant to void into hat. Record time of void. Fill 5 mL corning cryotube with 4.0 mL urine specimen, label with participant ID and date and time of collection and store in freezer. Discard remaining urine. Rinse and dry hat.
16. After collection of the fourth urine specimen, provide participant with meal/snack.
17. Collect completed Form 60 (FFQ), Form 35 (Personal Habits Update) and Form 45 (Dietary Supplements).
18. Instruct participant on 24-hour urine collection procedures. Give participant 24-hour urine collection kit. Answer questions participant may have.
18. Schedule/confirm appointment for visit # 2 (two weeks later).
19. Complete any remaining tasks.
20. Thank participant.

<u>Note:</u> 250 mL of beverages may be provided between hours 2-4 of the DLW protocol. All volumes and time of consumption must be recorded.

<u>Protect urine specimens from all sources of water during the collection and storage procedures. If hat is rinsed between urine collections, it MUST be dried prior to re-use.</u>

C.2.2. Day 13.

1. Telephone participant to remind her to collect all of her urine the next day in the specially provided urine collection kit. , Remind her to take the PABA pill (the B-vitamin) at each main meal of the day if her urine collection kit contained the vitamin bottles.
2. Remind participant of her appointment at the clinic two days later (Day 15). Remind participant to fast for six hours prior to appointment (or 12 hours if completing the indirect calorimetry - Madison, Pittsburgh and Chicago-Northwestern only. A 12 hour fast is required prior to measuring resting energy expenditure because some energy is expended in digestion and metabolism of food (the "thermic effect of food") 12 hours of fasting ensure that we are measuring resting energy expenditure ONLY and not any

residual thermic effect of food from the last meal). If clinic visit is scheduled for a Monday, call may be made the preceding Friday.

C.2.3. Day 15.

1. Greet participant.
2. Collect 24-hour urine specimens.
3. Obtain participant weight and record on NBS Visit Form 76 (Visit 2 version).
4. Complete NBS Participant Update worksheet (Visit 2 version).
10. Obtain blood specimen. Draw 3 Royal Blue and 1 Lavender (EDTA) tubes of blood. Use the standard WHI blood handling and processing procedures in WHI manual Volume 2, section11. See also NBS Visit Forms 75 and 76. Label specimens with participant ID label. Place in freezer.
5. Obtain urine specimen. Place hat over toilet and ask participant to void into hat. Record time of void. Fill 5 mL corning cryotube with 4.0 mL urine specimen, label with participant ID and date and time of collection and store in freezer. Discard remaining urine. Rinse and dry hat.
6. One hour after the first urine collection, obtain a second and final urine specimen. Place hat over toilet and ask participant to void into hat. Record time of void. Fill 5 mL corning cryotube with 4.0 mL urine specimen, label with participant ID and date and time of collection and store in freezer. Discard remaining urine. Rinse and dry hat.
7. Provide participant with meal/snack.
8. Thank participant for completing the protocol and give the $100.00.
9. Inquire about interest in returning in six months to repeat all procedures by asking her to read and sign the "consent for future contact form." If participant is eligible and willing to be a member of the returning group, inform her that she will be called to obtain a 24 hr dietary recall within the next three weeks. Schedule the six month return appointment.
10. If participant will repeat study in six months, notify the Clinical Coordinating Center (CCC) so they can send the portion size booklet and schedule 24-hour recall.

C.2.4. Indirect Calorimetry

On the workday prior to the visit for measurement of resting metabolic rate, the participant should be contacted and reminded that she should not eat after 8 PM the night before the scheduled clinic visit for measurement of resting energy expenditure (12 hour fast).

Script: "You are scheduled for a clinic visit at (time and date). During this visit, you will undergo a test to measure the number of calories that you burn at rest. In order to obtain accurate test results, you must not eat after 8 PM the night before the visit or on the morning before the visit. You may not drink any calorie containing beverages on the morning of your visit. It is also important that you do not smoke or take any nicotine products (chew, nicotine gums or patches), any caffeine containing products (coffee or tea other than decaf, colas or caffeinated soft drinks, or drugs to stay awake) for 2 hours before the visit."

C.2.4.a Indirect Calorimetry Measurement Procedures- The Indirect Calorimetry may be conducted either before or after the rest of the NBS Visit # 2 procedures. The participant must remain fasted until the conclusion of the indirect calorimetry.

1. Ensure that the equipment has been properly calibrated at the start of each day.
2. Ensure that the equipment is turned on and warmed up for at least 30 minutes prior to using it with the participant.
3. Ask the participant to lay down and rest quietly for about 30 minutes.
4. If the participant feels cold, offer her a blanket. If the participant feels hot, alter the environment to insure that she does not sweat.
5. Check that the monitor is in canopy mode. Change if needed (This may differ between instruments).
6. Check that the monitor is in the artifact suppression mode with a 10 min start delay (This may differ between instruments).
7. Check that the hoses from the hood to the metabolic monitor are connected and that the unit is turned on.
8. Perform a calibration of the metabolic monitor.
9. After the initial 30 minute rest period, measure resting metabolic rate as per instrument instructions.
10. The printer should be reporting data on a minute by minute basis. Check connections, printer power or see PRINTER SETUP if it is not printing.
11. The measurement will proceed for 30-40 min.
    a. The technician must remain with the participant and monitor gas flow alarms and visually check for labored breathing to insure that gas flow does not fail.
    b. The participant must remain at rest <u>but not sleep</u>.
    c. The participant must not talk, except when necessary to communicate a potential problem. If the participant does talk, lift their arms to scratch an itch, shift their weight to prevent stiffness etc, indicate the time and movement on the printout using a pen or pencil.
    d. Confirm that the participant is still thermally comfortable.
    e. If the participant has to get up because they need use the bathroom, then the measurement can be terminated. The measurement sequence, however, needs to be repeated beginning with the 10 min rest in place of the 30 min called for in the basic protocol.
12. At 30-40 minutes, check the display data printout for a stable reading.
13. End the measurement.
14. Obtain the output data from the metabolic cart.
15. Remove the hood from over the participant's head.
16. Ask the participant to sit upright.
17. Help the participant to their feet and be sure that they steady. Remember that they have fasted and there is a small risk of hypoglycemia.
18. Give the participant their snack.

19. Offer the participant their resting energy expenditure results. Answer any questions.

Note: The primary safety concern is that airflow through the hood is maintained while the hood is in place over the participant's head. Loss of flow due to a rare failure of the fan in the metabolic cart or due to a loose hose will cause discomfort and in an extreme case may cause asphyxiation. Although an alarm will sound if the unit does not detect breathing, the EE technician should remain with the participant throughout the measurement. Care should also be exercised when the participant stands-up after the measurement should dizziness develop secondary to the fast.

The indirect calorimetry procedures will add approximately one to one and one-half hours to the total time for NBS Visit # 2.

C.2.4.b Post-Indirect Calorimetry Quality Check

1. Check that the printout/electronic record is legible. If not, correct problem and reprint.
2. Check that the average RQ is between 0.75 and 0.9. Values outside of this range may indicate that the participant fasted longer than 15 h (<0.75), ate within the last 6 h (>0.93), or hyperventilated during the measurement (>0.93). Other possible explanations are very high fat diets (<0.75), a weight loss diet (<0.75) or very high carbohydrate diets (>0.93). If the participant admits to a recent meal, reschedule the test.
3. Check that the coefficient of variation is less than 10%. Possible explanations are excessive participant movement, irregular breathing pattern, failure to suppress the first 10 min of the measurement, or instrument maintenance problems. If the first 10 min of the measurement were not deleted, manually calculate the average and SD without the first 10 min. If the revised coefficient of variation is less than 10%, record these values. If not, repeat the measurement of resting metabolic rate.

C.2.4.c. Data Management

1. Save all data at the end of each test. All raw data should be sent to the Coordinating Center in an ASCII file format.

Six month visit procedures will be identical to visits 1 and 2, with the exception that women who complete the six month repeat visits will also complete two telephone administered 24-hour dietary recalls.

D. Study Timeline and Tasks

3-4 weeks prior NBS visit	**(Day 1)**	**At home (Day 14)**	**Visit 2 (Day 15)**
Invitation letter	Informed consent	Participant collects 24 hour urine at home	Receive 24 hr urine at CC
Telephone	Height, weight, FFQ,		Weight, fasting

recruitment and screening	DLW protocol, blood draw (if over 60 years of age), physical activity questionnaire, dietary supplements other AV tasks (if completing AV). Give complementary meal.	blood draw, 2 spot urines to complete the DLW protocol. Give $100.00 when protocol completed. Give complimentary meal Indirect Calorimetry (3 Clinical Centers only)
Schedule appointments	Confirm visit # 2, give 24-hour urine collection kit and instructions	Inquire about repeat visit in 6 months Notify CCC to schedule 24-hr recall

E. Supplies

1. Participant recruitment letters, envelopes, postage
2. Participant telephone recruitment script*
3. Informed consent document and HIPPA authorization*
4. "Study at a Glance" Instruction Sheet*
5. Forms:

NBS Visit Forms (Eligibility Worksheets, Participant Update Worksheets and Forms 74-78)*
Form 35 (Personal Habits Update)
Form 45 (Dietary Supplements)
Form 60 (Food Frequency Questionnaire)
24-hour urine collection instructions*
Consent for future contact*
WHI NBS Blood and Urine Processing*

6. Doubly labeled water (supplied by University of Wisconsin) – 1 bottle per participant on each protocol application.
7. Urine collection "hats" – 3 per participant; 1 to use at each clinic visit, 1 to send home with participant for 24 hour urine collection.*
8. Corning cryotubes (5 mL) – 6 per participant*
9. Royal blue vacutainers – 3 per participant
10. Lavender EDTA vacutainers – 2 per participant
11. Syringes, needles, gloves, cryovials needed for blood draws
12. Graduated cylinders to measure total volume of urine from 24 hr urine collection
13. Preweighed tissue (provided by University of Wisconsin) and Ziplock-type storage bags*
14. Sustacal, 8 oz – 1 for each participant*
15. Participant nourishment/meal

16. 24 hour urine collection kits – 1 per participant – hat, urine collection bottles, PABA the B-vitamin pills (if part of the reliability subsample), instructions*
17. $100.00 per participant to for time and travel expenses; $100.00 additional for women who repeat the protocol in six months).
18. Participant ID labels for forms and specimens*
19. Frequently Asked Questions and Answers about DLW Sheet*
20. Clinics who conduct the indirect calorimetry will supply their own metabolic cart and will train their own staff.

* These supplies to be provided by CCC

F. Specimen Handling, Mailing and Storage

1. All specimens will be handled and processed in accordance with WHI Manual Volume 2, Section 11. All specimens will be shipped by Fed Ex to McKesson BioServices in Rockville, MD per the standard WHI procedures on a quarterly basis.

V. Data Management

Data management and quality control will be the responsibility of the WHI Clinical Coordinating Center. Quality control will be achieved by adherence to the WHI Manuals of Operations for procedures that are already used in WHI (i.e., the collection of the body measurements, blood, urine, and self-report data). For aspects of this substudy that are not already part of WHI, such as the Doubly Labeled Water protocol, quality control measures will be provided to all clinic staff by our Doubly Labeled Water collaborator, Dr. Dale Schoeller at the University of Wisconsin. Dr. Schoeller is the nation's leader the in the DLW protocol. Third, all clinic staff will receive training prior to conducting this study and there will be on-going support from the WHI Clinical Coordinating Center. All data will be managed at the WHI CCC.

The Clinical Coordinating Center will provide specimen aliquot labels for all biospecimens. Neither the specimen repository nor the laboratories conducting the assays will have access to any participant identifiers. All specimens will be logged and tracked in the primary WHI database. The Clinical Coordinating Center will be responsible for verifying that all samples are sent to the correct laboratories at the correct time, ensuring security and confidentiality of all data, quality assurance and coordination of necessary data for analysis.

The standard WHI forms (Form 35, Form 45, Form 60) will either be scanned or directly data-entered into the WHI database. Other data, which are unique to this study, such as the time of void for the spot urine collections, will be data entered by clinic staff into the WHI database. The Clinical Coordinating Center will be responsible for daily backups of the WHI database

<u>Quality Control (QC)</u>

Laboratory quality control will be assessed for all blood and urine analyses.

For the nutritional biomarkers from blood analyses (i.e., vitamins and minerals) a 5% blind duplicate testing rate will be added using blood samples from the banked WHI QC reserve.

For the urine analyses (DLW and urinary nitrogen), samples collected at each time point from 5% of the women will be randomly assigned to have one extra aliquot prepared. These 5% samples (for DLW and urinary nitrogen) will receive blinded identification numbers and become the QC samples. We will randomly select any remaining serum aliquots from the 3-hour post dose blood draw for participants $\geq$ 60 years of age as blind duplicates.

VI. DATA ANALYSIS

The DLW measure of total (short term) energy expenditure (TEE) and the urinary urea nitrogen measure of protein expenditure will be used, in conjunction with FFQ and other measures on the participating women to develop calibration equations that adjust FFQ energy and protein assessments in a manner that allows the FFQ measure to incorporate systematic bias as a function of age, ethnicity, body mass, and possibly other characteristics, and that allows the FFQ assessment to include a person-specific bias. These calibration equations will then be used to produce corrected estimates of Total Energy Expenditure (TEE) and protein for each woman participating in the Dietary Modification Trial at baseline and one year from randomization. These corrected estimates, in turn, will be used in conjunction with FFQ or blood measures of percent energy from other nutrients to produce adjusted estimates of nutrient consumption for each DM trial participant. These estimates will play a fundamental role in (Cox regression) analyses that attempt to explain observed intervention versus control group hazard ratio estimates in terms of nutrient consumption changes.

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