5. RESEARCH DESIGN AND METHODS

The methods to be used in the proposed feeding study will be described in sections 5.1 to 5.4, followed by related laboratory analysis methods in section 5.5. The continuing methods used to assess AREE will be briefly described in Section 5.6. Statistical and data management methods for data and specimens derived under both the ongoing and proposed project phases (Aim 2.2) will be described in sections 5.7 and 5.8, followed by the project timeline and a discussion of proposal strengths and limitations in 5.9, and a mention of future directions in 5.10.

5.1 Feeding Study Participant Selection and Recruitment

Feeding study participants will be 150 women from the Seattle WHI clinic who were part of either the DM-comparison or the OS (about 75 from each group). Potential participants must have consented to participate in the WHI Extension Study (82.4 % of eligible DM trial women and 70.6% of eligible OS women in Seattle did so). To ensure the relevance of biomarkers that emerge from this feeding study to WHI cohorts, we will oversample women who were younger (odds ratio (OR) of 3 for women 50-54; OR of 2 for women 55-59), who were underweight (OR of 10 if BMI<18.5), or who were obese (OR of 2 if BMI$\geq$30) at WHI enrollment, as in the current NPAAS phase. All eligible Black and Hispanic women in the relevant Seattle cohorts (DM-comparison and OS) will also be invited to enroll. The upper age limit for participation in the feeding study will be current age $\leq$80 years in view of the upper age limit for WHI recruitees and since women older than 80 years may find it more challenging to complete the protocol. Since the current NPAAS protocol, which obtained measures of total and resting energy expenditure, urinary nitrogen and self-reported diet and physical activity, will be repeated in the current study, the targeted 150 enrollees for this phase will also be able to augment the biomarker subsamples used in NBS and the first phase of NPAAS to add precision to the disease-association studies in WHI cohorts.

Women will be recruited from zip codes in King and surrounding counties**,** where nearly all of the Seattle WHI women reside. In order to enhance ease for women to travel to the Center, women will be given priority if they live in close proximity to the Hutchinson Center (travel time in the middle of the day less than 1 hour). Eligible women will not have medical conditions that would preclude successful completion of the protocol (including but not limited to diabetes mellitus, kidney disease, bladder incontinence requiring the use of special garments or medications, routine use of supplemental oxygen) Women previously participating in the WHI NBS or NPAAS will be eligible The interest survey described above, and the 20.6% recruitment rate among eligibles in the current NPAAS phase, suggests that about 400 Seattle Field Center women will be eligible for, and could be recruited to, this project. Hence there will be some latitude to select women who do not have transportation difficulties, and to implement the oversampling features described above.

Seattle WHI site staff will use lists of potential participants provided by the Clinical Coordinating Center using a logistic regression selection model, as in the first NPAAS phase, to sequence participants in a manner that acknowledges the selection criteria noted above, other than zip code. The lists will be organized by residential zip code, to facilitate recruitment mailings. Zip-codes will be selected via cross-referencing with the Washington Department of Ecology GIS Technical Services County Zip Code list (www.ecy.wa.gov/services/gis/maps/county/zipcode/zipco17.pdf). Potential participants will receive a letter of approach describing the study and indicating that study staff will call to discuss interest and eligibility. Those who agree to participate will be scheduled for an information meeting. Before their scheduled meeting date, they will be sent a confirmation letter, driving directions and a copy of the study consent to review. In the unlikely event that insufficient numbers of WHI OS and DM-comparison participants will volunteer for the study, we will open recruitment to WHI women who participated in the WHI hormone trial, but were not simultaneously enrolled in the DM trial.

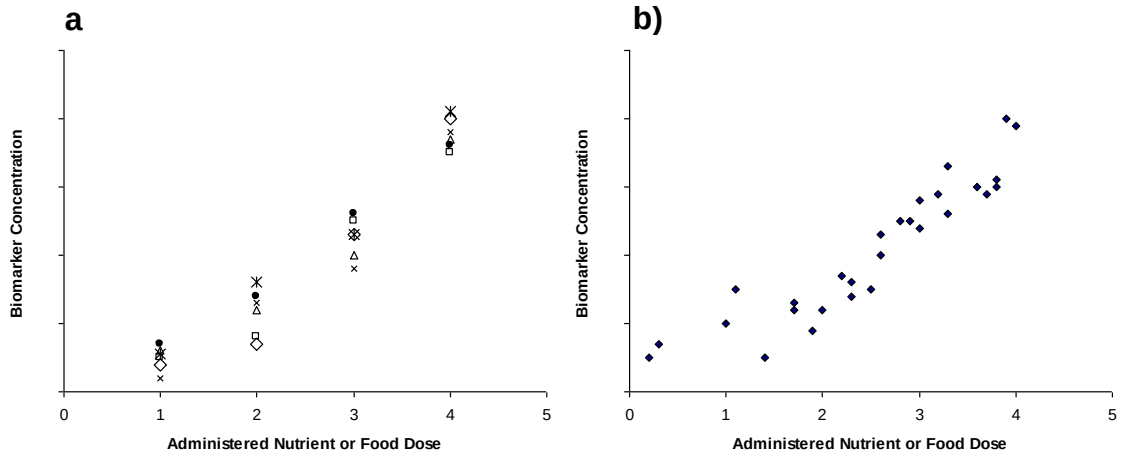
5.2 Feeding Study Design

In this proposed study, we will provide controlled diets that are highly individualized and will replicate the participant's usual eating habits. The rationale for this individualized, but highly controlled, feeding study approach is that it will allow us to evaluate accurately the relationship between intake of specific nutrients or specified quantities of foods and the nutritional measures of interest. A similar approach was used effectively by our collaborators, Drs. Sheila Bingham, and Nataša Tasevska in an initial

study to validate urinary sucrose and fructose as biomarkers for sugar intake.[21] Generally, feeding studies are designed such that all participants receive identical, standardized meals in a randomized crossover design (Figure 5.1a). Such a design is expensive, does not allow for evaluation of nutrient intakes over a dose range or in the context of habitual diet patterns, and would present serious participant burden to this population of older post-menopausal women. Further, it requires that each feeding period be long enough to allow the concentration biomarker to equilibrate and reach a steady-state at the nutrient dose fed, and it limits the evaluation of exposure and biomarker to the doses of nutrient that are chosen to be fed. Less controlled approaches, such as self-reported diet or allowing women to self-select their diets according to a designed menu, do not provide the level of control necessary to meet our goal of developing calibrating equations for fruits, vegetables, meats, grains and sugars. In addition, feeding study diets that closely approximate participants' habitual diets can be expected to substantially maintain the usual diet intake distributions for the study population.

By approximating each person's usual diet, we will minimize deviation from steady-state, which is important for evaluating concentration biomarkers, where our assumptions and modeling are based on an individual's typical circulating concentrations of the biomarkers (Figure 5.1b). Unlike recovery biomarkers that have a known quantitative time-associated relation between dietary intake and recovery (i.e., excretion) in human waste, concentration biomarkers are modestly influenced by an individual's age, BMI, race/ethnicity among other variables.[19,20,75-77] These concentration biomarkers, such as serum carotenoids and serum folate, generally display a linear relationship with dietary intake, but most predictive models at best explain about 25% of the variance of reported intake. We expect that with a controlled feeding study, where 'known' quantities of nutrients are ingested over a two-week period, we will be able to explain a somewhat larger fraction of the variation in short-term provided intake, especially when blood or urine measures of other nutrients/ foods, and various study subject characteristics are included among the 'predictor' variables. In addition to the controlled feeding study, participants will complete other activities similar to the current funding period, including the doubly labeled water protocol to assess total energy expenditure, indirect calorimetry to assess resting energy expenditure and a 24-hour urine collection to measure protein.

Figure 5.1 Theoretical association between administered nutrient or food dose and a concentration biomarker of dietary exposure in a) a randomized, crossover controlled feeding study of 4 nutrient doses and b) a controlled feeding study with doses administered at the levels habitually consumed by the participants.



5.3 Feeding Study Data Collection and Clinic Procedures

Interested and eligible participants will attend an informational meeting with a project coordinator/study dietitian at the FHCRC Human Nutrition Laboratory (HNL) to learn more details about the study. Drs. Lampe and Neuhouser use this approach in an on-going feeding study (U54 CA116847); it has been extremely effective to ensure that participants understand the commitment required and it improves retention. The project coordinator will carefully explain the study, offer a "study-at- a glance", and obtain written, informed consent. Participants will then receive detailed instructions about completing a 4-day food record. As part of this instruction, they will watch the WHI

video “Keeping Track of What You Eat”. Participants will record all their foods and beverages for 4 days. Participants will be counseled to cook and eat as they normally do during the food recording period and to maintain their usual physical activities.

After 7 days, participants will return to the Hutchinson Center with their completed food record. At this visit, study staff will review the record with the participant, schedule the feeding study dates, measure their height and weight, complete the supplement questionnaires, and make the first clinic visit appointment. The initiation of the feeding study will be within 2 weeks after completing the 4-day food record; this gives sufficient time for the project coordinator to work with HNL staff to plan feeding study meals comparable to those in a food record. This 2-week or shorter lag time between food recording and feeding study initiation is short enough that it is not likely to affect seasonal variation in produce availability. The feeding study will be a 4-day cycle menu that replicates the participant’s individual 4-day food record 3-4 times over a 2-week period (See Section 5.4). The menu will use a corrected estimate of total energy consumption, as determined from food record calibration equations developed from current-phase NPAAS data. The activities and data collection during the approximately 5 week study period (two weeks on feeding study) are outlined in Table 5.1. Specific details on the protocols for doubly labeled water (DLW), resting energy expenditure, and laboratory analysis of blood and urine samples are presented in Section 5.5 and 5.6.

Table 5.1. Data Collection and Study Activities for NPAAS Feeding Study

	Activities	**Data Collection**
Instruction/orientation		
Information meeting: Baseline data collection	Informed consent and training Record all food and drink for 4 days Review food record and discuss feeding study menus with staff	Nutrient and food intake from food record
Food record documentation meeting	Review food record and discuss feeding study menus with participant. Complete supplement questionnaires, measure height and weight, set feeding study dates and first clinic visit.	
Feeding study		
Day 1	Clinic visit: blood draw, height, weight, begin doubly labeled water protocol; complete FFQ and AAFQ and other questionnaires; put on activity monitor, begin controlled diet	Serum folate, tocopherols, carotenoids, Phospholipid fatty acids
Days 2-13	Continue controlled diet Wear activity monitor during all waking hours Pick up food every 2 days Weight measured on food pick-up days.	Monitor adherence to diet with MDIR* Measure physical acitviity
Day 14	Start 24-hurine collection; collect first morning void, then for next 24 hours	
Day 15	Clinic Visit: blood draw; weight ; bring 24-h urine collection and first morning void specimen; complete doubly-labeled water protocol; indirect calorimetry	Serum folate, tocopherols, carotenoids, Phospholipid fatty acids 24 h urinary excretion of sucrose, fructose, alkylresorcinol metabolites, nitrogen, 3-methyl histidine. First morning void comparison vs. 24 hr urinary nitrogen Total energy expenditure Resting energy expenditure

*Monitored Daily Intake Record

On Day 1 of the feeding study, the participant arrives at the FHCRC Prevention Center after a 12-hour fast. A baseline spot urine collection is obtained followed by a loading dose of doubly labeled water (see Section 5.5). The participant stays in the clinic for 4-5 hours to collect 3 more spot urine samples and one additional 10 ml blood draw. After the initial spot urine and dose of DLW, study staff will draw 30 ml of blood from the antecubital vein in the arm, measure participant’s height and weight, have them place the activity monitor around their waist and provide food for Days 1 and 2 of the feeding study.

During the feeding period, the participant will come to the Prevention Center every other day (except on weekends). On days that she comes to the Center, we will measure her weight and she will eat lunch (or other meal as requested) in the HNL dining room. Staff will review check-off forms and collect and record any food not consumed each time the participants comes to the HNL. On the last food pick-up day (Day 12 or 13), the participant will receive instructions on 24-hour urine collections and will receive urine collection kit (urine recording sheet, PABA tablets, toilet hat, funnel, urine collection bottles). She will start the 24-hour urine collection on Day 14, taking 3 PABA tablets (one with each meal or about 6 hours apart). On Day 15, the participant arrives (with the collected urine and activity monitor) at FHCRC after the 12-hour fast, has blood drawn (30 ml), gives the final two spot urine collections for the doubly labeled water protocol, and completes indirect calorimetry measures. Staff measure participant weight and give participant breakfast.

Participants will receive $300.00 upon completion of the study protocol. This is a standard amount that is typically offered in feeding studies to reimburse for time and travel expenses. In addition, for those women who do not have access to transportation, we will arrange car or taxi service to and from the Center.

5.4 Controlled Experimental Diets: Design, Preparation, and Feeding

Each participant's 4-day food record will be turned into a 4-day cycle menu, to be consumed 3-4 times (2 weeks) by the participant. As outlined above (see Section 5.2), our goal is to minimize perturbations to an individual's urine and blood measures by designing the controlled feeding to approximate her usual diet, while using foods with well-established analytic nutrient values available in our food and nutrient database. We will primarily rely on analytic values of foods derived from the National Food and Nutrient Analysis Program (NFNAP), which is directed by the USDA's Nutrient Data Laboratory (www.ars.usda.gov). In this program, the most commonly consumed foods in the US (identified by national survey data) are systematically identified, sampled and analyzed under carefully constructed laboratory protocols. The resulting nutrient data from these assays are provided to the USDA National Nutrient Databank, which forms the backbone of the University of Minnesota's Nutrition Data System for Research food and nutrient database. To the extent possible, we will rely on these analytic values in the database (termed "key foods") and we will avoid foods with imputed values. Given the demographic characteristics and food habits of the WHI participants, we believe that there will be very few, if any, foods in their habitual diets with imputed nutrient values.

The project coordinator/dietitian will work closely with each participant to review the food records and identify specific foods that were consumed, with particular detail to brand names, cooking procedures, and recipes. The food record will be entered into ProNutra® 3.3 dietary analysis software (VIOCARE® Technologies, Princeton NJ), a metabolic diet study management system that is used for all feeding studies at FHCRC. Pronutra® is a nutrient calculation and food management software system that was developed specifically to support metabolic research at the request of NIH research dietitians. It has been refined with input from professional research dietitians and system managers for the NIH General Clinical Research Centers and the USDA Human Nutrition Research Centers. Importantly for our proposed work, Pronutra® uses USDA analytic nutrient values. Foods recorded as volumes by participants will be converted into gram weights to ensure consistency in serving sizes. Recipes based on each individual's record will be developed. Non-processed foods (e.g., fruits, vegetables) will be purchased through the FHCRC's food service vendors. Processed or prepared foods (e.g., cereals, bread and other baked goods, salad dressings, frozen entrees, etc.) will be purchased from local retailers.

All food will be precooked and refrigerated/frozen until the time of service or packing for take-home. Items requiring reheating will be packed in microwave/oven safe containers with reheating directions. Each food item will be weighed, packaged individually, and labeled with ID number, first name, day, meal, and item. The individually packaged food items will be assembled into meals with each take-home meal packed into its own separate labeled paper bag. Since lunch was the meal of choice in the survey of Seattle women, participants will come to the HNL to eat lunch thrice weekly (M, W and F), taking home that evening's dinner and snacks and all meals for the days until the next HNL dining visit. On Fridays, they will take home dinner, snacks, all their food for the weekend, and Monday breakfast. Each participant will be provided a cooler on wheels for safe transport of the meals from the HNL to the participant's home.

5.4.1 Monitoring Adherence to Controlled Diets

Participants will be "free-living" and consume the provided diets as determined by their habitual diet patterns, with 3 meals per week served at our feeding center under supervision of the HNL staff. This is a protocol with which we have extensive experience,[55,56,73] and is consistent with the design of many other feeding studies. We will instruct participants to eat or drink only the food and beverages we provide to them, except for drinking water, coffee, tea, alcohol and other non-caloric beverages. We will encourage them, should they deviate from the prescribed diet, to report changes. We have found that having participants complete daily food check-off forms helps with the diet monitoring. Participants will fill out a food check-off form, Menu Checklist, daily during each feeding period and will also report their daily physical activity on the form. We will use the Menu Checklist to track daily food intake of the provided diet and any deviations from the diet. We will also use these forms and their associated recipes to calculate daily nutrient intake for each participant during the feeding period using the Nutrition Data Systems software (NDS-R, version 2010, Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN). This system of monitoring participant food intake has been used in previous studies at FHCRC with great success. Further, we expect compliance to the prescribed diets to be excellent, given that the foods being fed reflect the participants' habitual diets and involve rather little deviation from usual food intakes.

5.5 Laboratory Analyses of Nutritional Biomarkers

This section provides details on the assay methods for nutrition-based concentration and recovery biomarkers to be utilized in this study.

5.5.1 Biomarker Definitions

Recovery Biomarkers. Recovery biomarkers are those that have a known quantitative time-associated relationship between dietary intake and excretion or recovery in human waste products such as urine or feces.[12,78] Recovery biomarkers are uniquely valuable for understanding the measurement properties of dietary assessment tools because they allow calculation of absolute intake in the same units generated from records of food intake (i.e., grams/day or kcal/day). In addition, recovery biomarkers are particularly important in relation to the specific aims of this study because the sources of random error associated with their collection and measure are plausibly independent of the random error associated with self-reported intake of the nutrients, and these errors are unlikely to depend on participant characteristics. The urinary recovery measures to be collected and evaluated in this study include: total energy expenditure, and urinary nitrogen, urinary alkylresorcinol metabolite, 1- and 3-methylhistidine urinary sucrose and fructose (with correction using para-amino-benzoic acid).

*Concentration Biomarkers***.** Blood concentrations may be strongly influenced by diet, but because they represent a quantity per unit of blood (i.e., mg/ml) they cannot be used to estimate absolute intake without the type of development proposed here. In addition, some concentration biomarkers are influenced by host factors, such as BMI, smoking, and age.[18,20] We have carefully selected concentration biomarkers of nutrients and food components (folate, carotenoids, fatty acids) that will reflect dietary intake during the two-week feeding period, but which are not strongly affected by between-person differences in absorption and metabolism. These concentration measures are well-suited for potential use in dietary assessment research.

5.5.2 Urine-Based Recovery Biomarkers

Total Energy Expenditure: Doubly Labeled Water. Total energy expenditure (TEE) is the amount of energy (kcal) 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 a suitable estimate of a person's caloric intake. The method of choice to measure energy expenditure is the doubly labeled water (DLW) technique, which measures TEE over a period of approximately 2 weeks.[9,54] 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.[54]

The protocol involves administration of the labeled water and collection of physiologic specimens (urine and blood) on the day of administration and 2 weeks later. The participant arrives at the clinic

after a 4-hour fast (but can be longer depending on the rest of the particular study protocol, such as fasting blood collections), 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. About 1% of all participants experience transient vertigo after drinking the DLW solution that lasts only few minutes.[9] While the vertigo is rare, as a precaution all participants remain seated for at least 15 minutes after drinking the DLW. Participants remain in the clinic for at least 4 hours post-dosing and provide spot urine specimens at 2, 3, and 4 hours after administration of the isotopes.[9,11,16] Participants will receive a meal replacement beverage 1 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 3 hours after consuming the DLW to compensate for post-void urine retention that is often experienced by older women.[79] This will be collected in a standard 10 ml vacutainer. The first feeding study lunch is provided after collection of the 4-hour post DLW dosing void. Participants return to the clinic 2 weeks later to complete the protocol (in this study, this will coincide with the final day of the feeding study). Clinic staff will measure their weight and 2 spot urine specimens, collected 1 hour apart, will be obtained. We have had excellent success using this protocol in 450 WHI participants in the current funding period and 544 WHI women during the NBS. 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 2 specimens collected at Visit 2 give a reliable estimate of isotope turnover. The spot urine samples with doubly labeled water (assesses total energy expenditure) will be sent to the University of Wisconsin Metabolic Laboratory and will be assayed under the direction of Dr. Dale Schoeller. 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.[9,11,16] The isotopes, deuterium and ^{18}O are nontoxic, safe naturally occurring isotopes. The DLW solution is tasteless, odorless, and colorless. DLW has been used in studies with babies as small as 4 pounds.

Urinary Total 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.[79] More recently, urinary nitrogen has been used as a biomarker of protein intake in epidemiologic studies.[11,16,17,80]

Urinary total nitrogen is an excellent biomarker of absolute protein intake since, in a steady state of metabolism that is neither catabolic nor anabolic, over 80% of the nitrogen in dietary protein is recovered in the urine.[11,16,81,82] Approximately 16% of protein is nitrogen and thus:

$$(\text{Nitrogen}/0.8) \times 6.25 = \text{Estimated Protein Intake}^{83}$$

We will use a microKjeldahl method to measure the urinary total nitrogen[84] and the data will be compared to protein intake from the feeding study. The nitrogen analyses will be performed at Diagnostic Center for Population and Animal Health at Michigan State University. We have been conducting on-going pilot studies with MSU and the CV% for blinded duplicates is 1.58% (range 1.06% to 1.94%).

Urinary PABA. In order to assess completeness of the 24-hour urine collections, participants will take 3 80 mg PABACheck (100% para amino benzoic acid; PABA) tablets, one at each main meal of the day. This B-vitamin, PABA, is nearly completely recovered in the urine and its measurement functions as an excellent measure of compliance with the urine collection protocol.[11,79,85] This is important because incomplete urine collections would result in a miscalculation of the urinary nitrogen, alkylresorcinol, fructose, sucrose, and 1- and 3-methylhistidine measures. We will obtain the PABA Check tablets from a reliable source and the dosage and solubility will be verified by an independent lab. The PABA concentrations in 24h urines will be measured in the laboratory of Dr. Johanna Lampe, using an established HPLC method,[86] with minor modifications. In past studies, we have used a colorimetric method; however, this over-estimates PABA recovery in participants who use common drugs such as acetaminophen.[86] Briefly, 500 µl 8M NaOH/7 mM *m*-hydroxybenzoic acid (mHBA; internal standard) is added to each urine sample (500 µl) and the solution is incubated

at 120°C for 2 h to hydrolyze PABA conjugates. P-aminohippuric acid (PAHA) is run in parallel to check for completion of hydrolysis. Hydrolyzed samples are cooled, acidified with phosphoric acid, and chromatographic separation is carried out using acetonitrile and 0.02M potassium dihydrogen orthophosphate as mobile phase on a Phenomenex Luna 5µ C18, 240 x 4.6 mm column attached to an Agilent 1100 HPLC system. PABA/ mHBA ratios are compared to PABA/ mHBA standard ratios using linear regression to give mg PABA/l and %recovery is calculated from the 24h urine volume. Samples with less than 70% PABA recovery will be considered incomplete and not included in analyses. However, we expect incomplete collections to be rare, as we observed excellent adherence rates to the urine collection protocol in the NBS with only 5% of collections being incomplete as assessed by PABA.

Urinary Alkylresorcinol Metabolite DHPPA. The urinary alkylresorcinol (AR) metabolite 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA) will be assayed by Dr. Herman Adlercreutz (Institute for Preventive Medicine, University of Helsinki, Finland). Dr. Lampe has had a long standing collaboration with Dr. Adlercreutz and he has agreed to collaborate on this project (see attached letter). Drs. Lampe and Adlercreutz showed recently that urinary DHPPA is useful as a marker of whole-grain intake in a U.S. population.[22] Urine (25 µL) will be treated as published[22]: urine is enzymatically hydrolyzed overnight at 37°C, extracted twice with 300 µL ethyl acetate. The combined organic phase is evaporated, reconstituted with 500 µL methanol (MeOH), and further purified using DEAE-Sephadex ion-exchange chromatography in the free base form. The sample is applied to the column with 500 µl MeOH. Neutral steroids are eluted with 6 mL MeOH and discarded. Two alkylresorsinol metabolites, 3,5-dihydroxybenzoic acid (DHBA) and DHPPA are eluted with 8 mL 0.5 mol/L formic acid in MeOH. The fraction is evaporated and reconstituted with 50 µL MeOH. One hundred µL HPLC mobile phase 80%A (50 mmol/L phosphate buffer, pH 2.3; methanol; 90/10 by volume) and 20%B (50 mmol/L phosphate buffer, pH 2.3; methanol; acetonitrile; 40/40/20 by volume) is added and the sample is analyzed for the two alkylresorsinol metabolites, DHPPA and DHBA, by high performance liquid chromatography (HPLC) with coulometric electrode array detection (ESA Biosciences). Given that DHBA co-elutes with acetaminophen and that DHBA and DHPPA are highly correlated,[22] we will only use DHPPA for our biomarker measure.

Urinary Sucrose and Fructose. Urinary excretion of sucrose and fructose will be assayed in the laboratory of Dr. Johanna Lampe in consultation with Dr. Arthur Schatzkin and colleagues at the National Cancer Institute (see attached letter of support). Urinary sucrose and D-fructose will be measured spectrophotometrically (Cecil CE 2041 2000 Series Spectrophotometer) from a kit designed to assay sucrose, D-glucose, and D-fructose (Biochemica Mannheim, R-Biopharm, Roche).[21] For all analytes, the level of quantitation in urine is 2 mg/l and coefficients of variation (%CV) are ***<10%***.

Urinary 1- and 3-methylhistidine (1,3-MH). Urinary 1- and 3-methylhistidine will be assayed in the laboratory of Dr. Johanna Lampe in consultation with Dr. Arthur Schatzkin and colleagues at the National Cancer Institute (see attached letter of support). 1-methylhistidine and 3-methylhistidine will be measured by an ion exchange chromatography technique involving protein precipitation with sulfosalicylic acid, separation of the amino acids on a cation-exchange column under acidic conditions, followed by treatment with ninhydrin, which reacts with the primary or secondary amino groups to form colored derivatives that are detected colorimetrically at 440/570 nm.[87] Assay variation (%CV) for 1-and 3 MH are both <10.0%.

5.5.3 Blood-Based Concentration Biomarkers

The blood concentration biomarkers (folate, carotenoids, tocopherols, phospholipid fatty acids) will be conducted in the FHCRC Biomarker Laboratory under the direction of Dr. Xiaoling Song, who is an NPAAS co-investigator and is currently assaying NPAAS specimens.

Tocopherols and Carotenoids. The method used to analyze serum tocopherols and carotenoids were developed in the FHCRC PHS Core Laboratory. This method generates a complete profile of carotenoids and tocopherols in a single HPLC run. Briefly, a hexane extract of serum is injected onto a 3-µm C-18 Spherisorb ODS-2 HPLC column and eluted with an isocratic solvent consisting of 73% acetonitrile, 12% tetrahydrofuran, 8% methanol, 7% water, 0.025% ammonium acetate, 0.05% diethylamine (v/v) at the flow rate of 1.2 ml/ minute. Lutein, zeaxanthin, cryptoxanthin and lycopene are detected at 476 nm, α-carotene and β-carotene are detected at 452 nm, and α-tocopherol and γ-tocopherol are detected at 292 nm. The HPLC is a fully automated Hewlett-Packard (Avondale, PA)

1050 HPLC system equipped with a quaternary pumps, electronic degasser, insulated column housing, automatic sampler, diode array detector and software to run the system and to perform data analysis and management. The coefficient of variation (CV%) for the pooled quality control samples is ≤10%.

Phospholipid Fatty Acids. We have selected plasma phospholipid fatty acids for this study.[88,89] Methods to analyze phospholipid fatty acids were developed by scientists in the FHCRC PHS Core Laboratory and have been used in many published reports. In brief, total lipids are extracted from serum by the method of Folch[90] whereby phospholipids are separated from neutral lipids by one dimensional thin layer chromatography (TLC) using 250 micron Silica Gel G plates and a 67.5:15:0.75 hexane/ether/acetic acid (+0.005% BHT) development solvent. The total lipid extract is evaporated under nitrogen and then reconstituted in 50 µl of chloroform. In a nitrogen saturated enclosure, the samples are spotted as a streak onto individual lanes of the TLC plates and then dried for 1 hour under nitrogen. The phospholipid fractions, which remain at the origin, are scraped off with a razor onto weighing paper and transferred to clean tubes. Fatty acid methyl esters (FAME) samples are prepared by direct transesterification using the method of LePage.[91] Quantitative precision and identification are evaluated using model mixtures of known FAMEs and an established control pool. Fatty acids are expressed as relative weight percentages. Interassay coefficient of variations (CV's) are on the average 3.5% or lower for most of the fatty acids that are present at levels of 1% or higher.

Serum Folate. Folate is an essential nutrient found in fruit and vegetables, particularly dark, green leafy vegetables and orange juice. A synthetic form of the vitamin, folic acid, is added to enriched grains and cereal products in the United States. In this study, folate will serve primarily as a biomarker of green leafy vegetable and fruit intake. We recognize that serum folate concentrations are influence by synthetic folic acid as well. One particular strength of conducting a controlled feeding study for this biomarker is that we will have known quantities of intake of folate and folic acid. Serum folate will be determined using well-established radioassay methods (SimulTRAC Radioassay Kit Folate[362] MP Biomedicals, Orangeburg, NY). This assay is routinely performed in Dr. Song's laboratory where the intra and inter-assay coefficients of variation range from 1% to 6.4%. We will also explore the feasibility of assaying unmetabolizable folic acid (using separate funds), which may be a biomarker of synthetic folic acid found in enriched grains and cereals.[92]

Other blood-based biomarkers under development by NPAAS investigators include the use of stable isotopes to quantitatively assess dietary intake of animal protein, vegetable protein and simple sugars (Dale Schoeller, PhD, personal communication). While we expect that funding to assay these biomarkers using NPAAS specimens would be sought under a separate mechanism, we mention them here to note the breadth of our research program, which seeks to develop new biomarkers of dietary intake.

5.5.4 Laboratory Quality Control

Each laboratory used in the study follows strict quality control and quality assurance procedures. Blinded duplicates (10%) will be included in each batch for all laboratory analyses and we will calculate inter- and intra-batch coefficients of variation. Sample aliquot numbers and storage location will be recorded for each specimen collected in this study. This will greatly facilitate retrieval of the specimens for transport to the various laboratories. Cryovials will be labeled with the participant's study ID only. Laboratory personnel will not have access to the tracking systems or databases contain identifying information.

5.6 Objective Measures of Activity-Related Energy Expenditure

5.6.1 Resting Energy Expenditure

All participants will undergo indirect calorimetry on the last day of the feeding study, Day 15. The objectives of this part of the protocol are to obtain resting energy expenditure (REE), which is an important component of total energy expenditure; to use the REE measures in conjunction with the total energy expenditure measures to provide an objective measure of physical activity energy expenditure (by difference and using a constant 9% adjustment for the thermic effect of food); and to obtain measures of the respiratory quotient (RQ). The RQ is the ratio of VCO_2 produced/VO_2 consumed and provides information about substrate utilization. The average RQ for a mixed diet is 0.82-0.85 and estimates of 1.0, 0.7, 0.7, and 0.8 are generally accepted values for oxidation of carbohydrate, fat, alcohol, and protein, respectively. While these RQ estimates do not necessarily directly correspond to ingested macronutrients, known values for the caloric equivalents of CO_2 produced and O_2 consumed can provide estimates of the sources of calories (both endogenous and exogenous).[93-95]

Indirect calorimetry will be conducted by a trained technician at the FHCRC Prevention Center using a Medical Graphics System, Inc. metabolic cart. This semi-portable unit measures the concentrations of oxygen and carbon dioxide in air streams entering and exiting a clear face tent placed over the participant's face. The participant breathes room air at normal respiration frequency. Oxygen consumption and carbon dioxide production are calculated from the change in concentrations and flow rate.

Participants will arrive at the Prevention Center on the last day of the feeding study after a 12-hour fast and will rest for 30 minutes. The rest period ensures a closer approximation to true "resting energy expenditure" by accounting for any energy expended in activities of preparation for the day and transportation to the clinic. The indirect calorimetry must be conducted in a thermal neutral room and, before beginning the test, clinic staff will confirm the room temperature and comfort level of the participant. Clinic staff will check the settings of the equipment, perform the necessary machine calibrations, place the small, ventilated plastic face tent over the participant's nose and mouth and begin the test. For this investigation, all participants will undergo a 30 minutes test; the first 10 minutes of data will not be included in data analysis as this is the period of time needed to achieve steady-state.[96] The participant will remain at rest, without speaking, for the duration of the test, but must not sleep. At the end of the 30 minutes, clinic staff will check the data display to ensure a stable reading, then remove the hood from the participant's head and help her to stand. Excessive movement, agitation of any kind or coughing may require repeating the test. At the end of the procedure participants will be offered their REE information (kcal/day at rest). This protocol has been successfully used in NPAAS during the current funding period.

5.6.2 Accelerometer Biomarker of Physical Activity

All participants will wear the Actigraph GT3X (ActiGraph, LLC, Pensacola, FL) accelerometer for the 14 days of the study, between Clinic Visits 1 and 2. The Actigraph GT3X is a small (3.8 x 3.7 x 1.8 cm), light (27 g) and highly sensitive instrument that records acceleration between 0.05 and 2.5 Gs using a tri-acial accelerometer. This information is digitized by a 12-bit A/D converter 30 times per second (30 Hz), filtered to capture normal human movement (i.e., 0.25 to 2.5 Hz), and saved as an activity count in used defined intervals (epochs). Activity counts (ct) provide an indication of the duration and intensity of bodily movement. The device will be programmed to record in 1 second epochs, providing up to 86,400 observations of behavior per day. The recent addition of memory to the instrument enables data collection over 7 days of observation of 3-dimensional activity counts, inclinometer function, and steps taken each day. Participants will receive the monitor at the end of Clinic Visit 1, along with instructions. They will be instructed to wear the monitor on their waist during all waking hours for the two weeks of the study. Participants will remove the monitors during sleep and since the monitors are not waterproof, participants will be asked to take them off while bathing or swimming. Additionally, they will be asked to record, on their daily menu checklist, the time they put on and took off the monitor each day. Participants will bring the accelerometer to Clinic Visit 2; study staff will download the data, clear the device of any personal identifying information, and ensure that the device is properly calibrated for the next participant.

At present, we do not have the funds to analyze these data, but will seek additional funding to cover this data analysis in the future.

5.6.3 Activity-Related Energy Expenditure.

The REE data obtained via indirect calorimetry data have provided useful information regarding activity related energy expenditure (see preliminary data, section 4.3) and together with the total energy expenditure data yield an objective measure of activity related energy expenditure as [AREE = ***0.9***TEE – REE], as in the first phase of NPAAS.

5.7 Data Management

The study investigators have considerable expertise managing and tracking data and specimens and their established procedures will continue for this competing renewal. This project will generate three kinds of data: laboratory data, questionnaires and food intake data. Overall data management will be the responsibility of the WHI Clinical Coordinating Center (CCC) Data Unit. The WHI CCC has considerable experience and expertise in managing numerous study data and biospecimens. To-date, 378 studies ancillary to the WHI have been

processed through the CCC; currently in progress are 119 studies. WHI biospecimens, tracked through a sophisticated database at the CCC, include blood serum, plasma (citrate and EDTA), DNA, red blood cells; and urine from the 3 WHI clinical center sites that collected bone density. To date, over 523,000 WHI biospecimens have been shipped from the Fisher Bioservices WHI biospecimen repository to 94 analytic laboratories in 6 international countries and 20 states in the U.S. All WHI participants have a computer-generated ID that has been used on all study forms and materials since the inception of WHI. A separate "draw ID" will be generated for each participant and used for all aliquots and cryovials. A password protected file available only to persons at the WHI Clinical Coordinating Center Data Unit links the WHI Study ID with the NPAAS draw ID. All cryovials with plasma, serum or urine specimens for the NPAAS renewal will be labeled with freezer proof draw ID labels and will be devoid of any personal identifiers. All specimens will be archived at the WHI biospecimen repository (Fisher Bioservices) where they will be shipped once every three months from Seattle and stored at - 70° C until analysis at the analytic laboratories. We will utilize WHI's electronic specimen tracking system (including storage box maps) for NPAAS. This system facilitates identification of the exact storage location of each specimen. When cryovials are removed from a freezer box, a data-control technician will use the electronic tracking system to enter the number of the aliquot removed and the destination laboratory. Pull lists will be created for each laboratory that will include the participant ID, aliquot number, specimen type (i.e., serum or plasma), volume and the assay to be performed. Each laboratory will confirm receipt of specimens and will report results using a spreadsheet, which will be verified and uploaded to the primary database at the WHI Clinical Coordinating Center.

The following forms and questionnaires will be key-entered by NPAAS study staff: body measurements, biological specimen collection, (including the timing of blood draws and dose of the doubly labeled water and collection of spot urine specimens). The 4-Day Food Records will be coded and data-entered by trained and certified staff at the FHCRC Nutrition Assessment Shared Resource. Food data from the feeding study will be data-entered by dietetic technicians at the HNL using ProNutra® 3.3 dietary analysis software (VIOCARE® Technologies, Princeton NJ). FFQs are scanned and SAS (SAS Institute, Cary, NC) datasets are created directly from the scanned output. Indirect calorimetry data are directly uploadable; the metabolic cart produces an electronic file of minute-by-minute measures of oxygen consumption and $C0_2$ production in addition to detailed information on the gas calibration of the instrument. These electronic files will be directly uploaded to the WHI database. Data range checks and logic checks will be established for all variables and procedures will be established for handling of missing data. Strict quality control procedures for data entry and verification have been part of WHI since its inception.

5.8 Statistical Modeling, Design, and Analysis

5.8.1 Measurement Error Modeling

Let W denote the logarithm of the DLW assessment of daily energy consumption, Z the corresponding logarithm of average daily energy consumption over a longer term (e.g., 6 months), and Q the logarithm of average daily energy consumption as assessed by a food frequency, record, or recall procedure. We continue to use our 2002 measurement model[97] for the (unmeasured) Z, given by

$$W = Z + u \qquad (A)$$
$$Q = Z^* + e$$

where the 'target' Z* of the self-report assessment can be written

$$Z^* = a_0 + a_1 Z + a_2^T V + a_3^T V Z + c$$

In these expressions $a_0, \ldots, a_3$ are coefficients to be estimated, V is a vector of numerically-coded study subject characteristics (e.g., body mass index, age, …) that may influence the self-report measurement error, c is a mean zero person-specific bias that allows the measurement errors for multiple self-reports on a study subject to be correlated, and u and e are mean zero 'noise' variables. All random variables on the right side of (A) are assumed to be statistically independent given V.

The measurement error (u) for the biomarker can be thought of as comprised of the sum of variation of the short-term 'consumption' targeted by the biomarker about Z, plus variation of W about the targeted short-term value. The utility of the biomarker is enhanced if the variance of u is small relative to the variance of Z.

An excellent self-report procedure will have a_1 close to unity, and a_0, a_2, a_3 each close to zero, with residual measurement error (c + e) having a small variance. The FFQ log-energy estimate W does not have such strong properties (Table 4.2) with a_1 estimated by 0.062, with a_2 far from zero, and with c + e contributing about half the variation in Z* (from reliability sample correlation of 0.70).[16] Also, even though the DLW method is thought to give a quite accurate estimate of short-term energy consumption, about 50% of the variance in log-DLW energy in (A) derives from actual variation in Z in our study cohort (from reliability sample correlation of 0.72), and 50% from measurement 'error' u,[16] the latter presumably mostly due to temporal variation in the energy consumption of individual women.

The measurement model (A) will provide the framework for the biomarker development, for the self-report consumption calibration, and for the nutritional and physical activity association study goals of this project: To study measurement error properties of a self-report method using an established biomarker W, linear regression of W on Q and V, under (A) along with a joint normal distribution assumption for (Z, c + e) given V, produces a calibrated estimate, $\hat{Z}$, of Z from Q and V (as illustrated in Tables 4.2 and 4.4 above). The coefficients of these 'calibration equations' provide direct insight into the measurement properties of the self-report, and competing self-report procedures can typically be compared by contrasting corresponding calibration equation coefficients. Also, calibrated consumption estimates $\hat{Z}$ will be calculated from corresponding Q and V values throughout the larger WHI cohorts, and suitable methodology is available[98] to relate actual consumption (Z) to disease risk under model (A), as illustrated above (Figure 4.1). Note that a crucial feature of (A) is the independence of the biomarker and self-report measurement errors 'u' and 'c + e'. It is departure from this assumption that generally precludes the use of another self-report assessment in place of W, to calibrate the self-report Q that may be available for a study cohort.

The feeding study biomarker development goals of this project, in terms of the measurement model (A), involve the identification of biomarker measurements W that adhere to the classical measurement model in (A), with an error variance for 'u' that is of tolerable magnitude relative to the variance of the underlying actual (longer-term average) consumption Z.

5.8.2 Statistical Aspects of Biomarker Development and Evaluation

Let Z_0 be the logarithm of a short-term nutrient consumption variable for which we wish to develop a biomarker estimate. During the 2 weeks of the proposed feeding study we expect to be able to estimate Z_0 with good precision by a corresponding variable, W_0, based on the amounts of provided food and drink and the nutrient composition thereof. The 7-day food record collected prior to the feeding period will allow an individualized diet that approximates a participating woman's usual diet, to be calculated. The principal purpose of such an individualized approach is to allow rapid stabilization of urine- and blood-based nutritional variables that will be used to produce candidate nutritional biomarkers. Secondarily, the individualized approach preserves the inherent variation in consumption patterns in the study cohort, a useful feature for biomarker evaluation. Note, however, that these 7-day food record estimates of nutrient intake will not be assumed to be accurate, nor will they otherwise be used in biomarker evaluation.

Biomarker development will be based on the linear regression model

$$W_0 = b_0 + b_1^T X + b_2^T V + e_0 \qquad (B)$$

with predictor variables X that arise from (log-transformed) urine- or blood-based nutritional measures, V again denotes a vector of study subject characteristics, and e_0 is a mean zero error term that is independent of the variates on the right side of (A), given V. For example, X in (B) may include, X_1, the logarithm of a urine- or blood-based nutrient measure for which a biomarker is sought, along with other urine or blood nutrient measures, X_2, that may influence X_1, and thereby help to explain variation in the provided log-nutrient consumption W_0. Similarly, body mass index or other study subject characteristics in V may also affect the urine or blood concentrations for the nutrient of interest, and their inclusion in (B) also may help to explain provided nutrient consumption variation. The feeding study data will be used to build a regression model using (B), and to produce estimates of the regression coefficients b_{0*}, b_{1*}, b_{2*} from which candidate biomarker values $W_* = b_{0*} + b_{1*}X + b_{2*}V$ can be calculated from the pertinent set of urine/blood concentration measures and study subject characteristics.

The essential criterion for determining whether W_* can serve as a biomarker in (A) for nutritional epidemiology purposes is adherence of W_* to the classic measurement model for W in (A). This implies that the model (B) must be rather complete, in the sense that major factors that determine W_0 are either included in the

regression model or can reasonably be included in the independent error e_0 (e.g., most genetic factors). The power of association studies will be enhanced if the regression variables in (B) are able to 'explain' a sizeable fraction of the variance among study subjects in W_0 values, which also will tend to support the comprehensiveness of the regression model development. We propose to use the ability to explain 50% or more of the variance in W_0 as one criterion for assessing the suitability of W_* as a biomarker for Z. Factors that will tend to reduce the % of variation in W_0 explained by (X, V) include:

(i) 'noise' in the estimation of nutrient/food consumed over the feeding period because of inaccurate food composition data, or consumption departures from food and drink provided;

(ii) blood and urine measures (X) that are influenced by factors other than nutrient/food consumption in the preceding two weeks or are not sensitive to some aspect of the recent consumption (Z_0);

(iii) the omission of some important 'predictive' characteristic from V.

As reviewers of our original submission noted, it may be difficult to explain as much as 50% of the variation in estimated log short-term consumption. While we expect log-DLW to be able to explain the vast majority of log short-term energy consumption, especially if the calorie content of provided foods is known with some precision, this is not expected to be the case for most blood measures, or even for urine measures that recover a small fraction of the nutrient consumed. To enhance the % of W_0 variation explained, we will give priority to foods having analytic (i.e., not imputed) nutrient composition data in developing feeding period diets, and will supplement the blood/urine nutrient measure with other correlated blood/urine measures (in X) and with accurately measured study subject characteristics (in V). Note, however, that there may be some trade-off between the complexity of model (B) and the utility of the potential biomarker in other populations. Hence, potential biomarker models for W_* will be as parsimonious as practical, while simultaneously trying to explain a sizeable fraction of the variation in W_0. Potential biomarkers that explain a smaller fraction of W_0-variation may still be useful for calibration equation development and association analyses, even though association study power will be reduced as this %-variation explained becomes smaller. As noted above, the essential requirement for validity of association analyses based on model (A), however, is adherence of W_* to a classical measurement model $W_* = Z + u_*$, as could be the case even if W_* explains only a small fraction of short-term log-consumption. Also, it is perhaps worth reiterating that a W_* biomarker capable of explaining a sizeable fraction of short-term daily consumption, may only explain a small fraction of longer-term (e.g., 6-month) daily consumption, depending on the magnitude of the temporal consumption variation for a study subject, compared to that across the study population.

A biomarker W_* meeting the type of criteria just discussed for a study nutrient can be calculated for the 450 women in the current NPAAS phase, using urine/blood measures obtained from stored specimens in these women. These nutrient biomarker values can be used in (A) to study self-report consumption measurement properties for food frequencies, 4-day food records, and 24-hour recalls (all collected in the current phase), and to produce calibrated consumption estimates from FFQ and 4DFR consumption estimates in WHI cohorts, for use in nutrient-disease association studies.

5.8.3 Feeding Study Biomarker Analysis and Sample Size Estimates

The biomarker development model (B) can be considered for a broad range of nutrients, or dietary components, for which there are pertinent urine or blood measures. Specific Aim 2.1.2 highlights a few nutritional factors where dietary consumption may lead to much of the variation in a corresponding urine or blood measure. The data and stored specimens assembled under this proposal will also provide a resource for evaluation of additional potential biomarkers (such as the stable isotope measures of protein and sugars mentioned earlier in the application), and as may subsequently be proposed by persons in the nutrition and population science communities.

Consider a special case of (B) with W normally distributed, univariate X and $b_2 = 0$, to examine necessary feeding study sample size. The variance (using the moment generating function) of $b_{1*}^2 \sigma_x^2 \sigma_{w0}^{-2}$ is $4b_1^2 \sigma_x^2 \sigma_{w0}^{-2} n^{-1} + 2n^{-2}$, where σ_x^2 and σ_{w0}^{-2} are variances for X and W_0 in the study population and n is the feeding study sample size. Hence, if $b_1^2 \sigma_x^2 \sigma_{w0}^{-2} = 0.5$, so that X explains 50% of the variation in W_0, then the standard deviation of the observed percent of variation explained is about the square root of $4(0.5)n^{-1} + 2n^{-2}$. For example, this standard deviation takes values 0.14, 0.12, and 0.10 at feeding study sample sizes of 100, 150, and 200 respectively. Hence, rather large sample sizes are needed to estimate the fraction of variation in W_0 explained by the regression variables in (B) with precision. A sample size of n=150 will provide reasonably precise estimates of the ability of the biomarker to explain short-term consumption variation.

Another aspect of feeding study sample size is the magnitude of variance in the potential biomarker W_* as an estimate of W_0, as compared to the variance in W_0 in the study cohort. Under the assumptions of the preceding paragraph, the variance of W_* for a study subject having $X = \mu_x + k\sigma_x$ is $(1 + k^2)/n$ times the variance of W_0, where μ_x is the mean of X. Hence if X is within k=2.5 standard deviations of the mean, a sample size of n=150 will imply that the variance of W_* is at most 4.8% of the variance of W_0. The inclusion of additional predictor variables in (B) will add to the variance of W_*, but a sample size of 150 would seem sufficient from the perspective of precision of the biomarker estimates that emerge from (B), relative to variation in the study cohort.

To ensure the relevance of biomarkers that emerge from this feeding study to WHI cohorts, we will oversample women who were younger (odds ratio (OR) of 3 for women 50-54; OR of 2 for women 55-59), who were underweight (OR of 10 if BMI<18.5), or who were obese (OR of 2 if BMI≥30) at WHI enrollment, as in the current NPAAS phase. All eligible Black and Hispanic women in the relevant Seattle cohorts (dietary modification comparison group; observational study) will also be invited to enroll. In fact, the current NPAAS protocol will be substantially incorporated in the current study so that the targeted 150 enrollees will also be able to augment the biomarker subsamples used in NBS and the first phase of NPAAS to add precision to association studies in WHI cohorts.

We expect to see a broad range of nutrient intake among the 150 participants as suggested by the existing self-report data from the WHI DM comparison and OS cohorts (Table 5.2). We recognize that dietary self-report data (FFQ, food record, or recall) distributions are artificially widened by the noise aspect of measurement error, and may also be distorted by systematic bias, and thus the data are provided solely as illustrative approximations.

Table 5.2. Nutrient Intake Variation per Approximation of Baseline WHI Food Frequency Questionnaire (self-report)

Nutrients/day	**DM Comparison**			**OS**		
	N	Geometric Mean	SD	N	Geometric Mean	SD
Dietary Total Carbohydrate (g)	26120	178	68	79537	175	70
Dietary Protein (g)	26120	62	25	79537	60	25
Dietary Total Fat (g)	26120	58	26	79537	48	24
Dietary Total Carotenoids (mcg)	26120	4645	2762	79537	5144	3283
Dietary Animal Protein (g)	26120	43	20	79537	40	21
Red meat (med serv)	26050	0.6	0.5	79537	0.4	0.4
Dietary Total Sugars (g)	26120	84	38	79537	85	40
Combined Fruits / Veggies (med serv)	26120	3.4	1.9	79537	3.8	2.2

5.8.4 Feeding Study Data Analysis and Novel Biomarker Application

Evaluation of potential new biomarkers listed in Aim 2.1.2 will be based on a linear regression of log-provided nutrient (or food) component on the logarithm of the pertinent urine or blood measure. Other available (log-transformed) urine/blood measures that may respond to consumption of the nutrient in question, or that may affect the magnitude of the primary urine/blood response to such consumption, will also be considered for inclusion in this model (p=0.10 criterion), as will study subject characteristics (age, BMI) that may help to 'explain' variation in provided nutrient. The total number of regression variables entertained for a given nutritional factor will be restricted to be fairly small (≤10) to avoid overfitting influences. The fraction of variation explained by the fitted model will be used to help decide whether the biomarker is suitable for application to the 450 women in the ongoing NPAAS phase as will any available external knowledge concerning the sensitivity and specificity of the urine/blood measures to consumption variations. Funding, separate from the current request, will be sought for determining biomarkers that emerge other than those listed in 2.1.2. As suitable biomarkers emerge, they will be used to assess the measurement properties of self-report approaches for the nutrients/foods in question (frequencies, records, and recalls), and for association study applications in WHI cohorts.

5.8.5 Activity-Related Energy Expenditure

The 150 women enrolled in the feeding study will complete a DLW assessment of short-term total energy expenditure (TEE), and an indirect calorimetry assessment of resting energy expenditure (REE) to yield the biomarker ***0.9***TEE-REE of activity-related energy expenditure (AREE) as in the ongoing study phase.

5.8.6 Continuing Analysis of NPAAS and NBS Data

The laboratory assays from the ongoing phase of NPAAS are expected to be complete by Fall 2009, after which an intensive period of analysis and reporting will follow, and can be expected to continue into the early years of the renewal period, until feeding study data become available for analysis.

One aspect of these analyses will compare coefficients in (A) for FFQ, 4DFR, and 24HR self-reports of energy, protein, and percent energy from protein consumption, and will compare coefficients in (A) for AAFQ, 7dPAR, and PHQ self-reports of activity-related energy expenditure. These coefficients have been compared based on data from most of the 450 NPAAS women along with NBS data, and differences are already apparent ($p<0.05$) between 4DFR and FFQ coefficients for energy and protein, and between FFQ and 24HR for protein, but no significant differences are yet evident among the three dietary assessments for % of energy from protein. These analyses will also explore the use of the respiratory quotient (RQ) from indirect calorimetry, in conjunction with total energy (T) and protein biomarkers (P) to develop biomarkers of fat (plus alcohol) and carbohydrate. This type of exploration is also underway, using a measurement model for (logT, logP, logRQ) to compute individual estimates of fat and carbohydrate for NPAAS women.[41] We will consider the application of these fat and carbohydrate biomarkers to study measurement properties of the FFQ, 4DFR, and 24HR for these nutrients and for corresponding nutrient densities, but more definitive evaluation of these potential biomarkers will need to await an evaluation in the context of the feeding study proposed here, using the criteria described above.

Another aspect of the analyses of current-phase NPAAS data will be application of calibrated energy, protein, and % of energy from protein from FFQ and 4DFR measures, and application of calibrated AREE from the PHQ in association studies of cancer, cardiovascular disease, diabetes, and other outcomes under model (A) as was illustrated in Section 4.2 using NBS data. In fact, for the FFQ analyses, it will be advantageous to combine NBS and NPAAS data to add precision to calibration equation coefficients, and to hazard ratio parameter estimates. Additional analyses will explore combinations of frequencies, records, and recalls for more precise estimates of calibrated consumption or calibrated AREE, for use in association analyses. Along these same lines, the feeding study has been designed so that data from the 150 participating women can be combined with first phase NPAAS and NBS data to enhance the efficiency of association analyses. This is particularly useful for analyses based on 4DFRs or AREE since these were not included in NBS, and the present proposal will increase the biomarker sample size from 450 to 600 women.

5.9 Project Timeline, Strengths and Limitations

The timeline for this 4-year project is given in Table 5.3. The first 6 months of the study will be devoted to securing IRB approval, staff training, development of procedures, creation of participant recruitment lists and related materials, and ordering all supplies including the stable isotopes for the doubly labeled water. Subsequent to receiving the deuterium and ^{18}O, each dose of doubly labeled water must be carefully weighed, measured, and labeled at the University of Wisconsin Stable Isotope Laboratory to ensure quantitative precision before they are shipped to the FHCRC.

During months 7-36, participants will be recruited and screened for feeding study eligibility on an on-going basis. Months 7-36 will be dedicated to implementing the feeding study protocol, data entry, and on-going quality control procedures. The analysis of the stable isotopes recovered in biological specimens by mass spectrometry is labor intensive; 18 months (Year 3 and part way into Year 4) will be needed to complete the sample analysis from all 150 women. The remaining laboratory analyses (urinary nitrogen and PABA; urinary alkylresorcinols, urinary sugars and 1-and 3-methyhistidine; blood measures of carotenoids, phospholipid fatty acids, tocopherols, and folate) will be also completed between Years 3-4. Data analysis and manuscript preparation will take place throughout the 4-year grant in the early project years, using biomarker data collected in the initial funding period.

Table 5.3. NPAAS Timeline

	Year 1		Year 2		Year 3		Year 4	
Month	**1-6**	**7-12**	**13-18**	**19-24**	**25-30**	**31-36**	**37-42**	**43-48**
Start-up, IRB approval, order supplies								
Participant Recruitment								
Feeding Study Procedures								
Laboratory Analyses								

Data Analysis, Manuscript Preparation								

Strengths

1. This research builds on our successful and productive work in the current funding period, and in the preceding Nutrient Biomarker Study, in which we have collected objective biomarkers of nutrient consumption and physical activity in postmenopausal women. These data will continue to be used to study the measurement properties of prominent assessment instruments, and to produce biomarker-calibrated estimates of nutrient consumption and activity-related energy expenditure for use in disease association studies in the WHI cohorts. Our efforts in this regard are already beginning to be.[16,17,41] We are optimistic that this biomarker-calibration approach will advance the field of nutritional and physical activity epidemiology and improve the ability to reliably assess associations of these important lifestyle exposures with chronic disease risk. These achievements are directly in line with the goals of PAR-09-224.

2. A major strength of this competing renewal is the study design. A controlled feeding study is a strong design to develop and evaluate additional biomarkers for use in regression calibration analytic approaches. To obtain a quantitative, objective measure requires a carefully controlled quantitative estimate of intake that is best done in a controlled setting. The potential biomarkers for meat, fruit, vegetables, sugars, and grains will be used to develop regression-calibrated models of diet and physical activity and disease risk. We have added Dr. Johanna Lampe as the Dual-PI to this phase of the research. Dr. Lampe directs the Hutchinson Center's Human Nutrition Laboratory and brings nearly 20 years of experience in the conduct of controlled feeding studies.

3. This competing renewal is nested within the well-characterized WHI Cohorts. From a logistic and cost perspective this context provides a natural recruitment source of women who are already familiar with the rigors of population research; provides investigators and staff who have collaborated recently on implementing many of the protocol elements through the current funding period; provides larger cohorts from the same population base who have dietary (FFQ and 4DFR) and physical activity (PHQ) assessments available over the preceding decade, along with carefully adjudicated clinical outcome data, standard confounding factor data for major clinical outcomes, and a variety of data items that may relate to systematic bias in nutrient consumption or energy expenditure assessment. These features imply an efficient study, having direct application to a broad range of nutritional and physical activity association studies for an important population (middle-aged and older American women) through application to data from the 161,808 women in the WHI cohorts.

Limitations:

1. The WHI is restricted to postmenopausal women in the United States. The measurement properties of nutritional and physical activity assessment procedures may differ for men, younger women, or possibly even for other populations of postmenopausal women.

2. Even though 150 is quite a large sample size for intensive, controlled feeding studies and studies collecting objective measures of physical activity, it may still may be on the small side (depending on the complexity of the measurement models that prove to be needed) for efficient association analyses to relate disease risk to calibrated nutritional or physical activity exposure estimates.

3. For logistical reasons, the proposed feeding study will be conducted only at the Seattle WHI site, where few of the WHI participants are racial and ethnic minorities. Still, we do not expect biomarker suitability to depend on race/ethnicity, even though self-report measurement properties may do so.

4. We will calculate nutrient content of the controlled diets using NDS-R (version 2008, Nutrition Coordinating Center, University of Minnesota) rather than conducting chemical analysis of duplicate diets for each woman. Although the latter is the gold standard, it is not critical for development of the calibration equations; the majority of foods used in the feeding study will have analytic nutrient values available.

5.10 Future Directions

The scientific team assembled for the competing renewal of this Nutrition and Physical Activity Assessment Study is well-positioned to complete the aims put forth in this proposal. We have had numerous successful collaborations in the past, which we expect to continue during the second funding period and beyond. New collaborations proposed in this competing renewal with Drs. Arthur

Schatzkin and Herman Adlercreutz will further enhance our research program in relation to measuring and using novel biomarkers of nutrient consumption and physical activity and comparing to self-report. As the second funding period progresses, we will seek and use additional biomarkers of nutrient consumption and activity-related energy expenditure as they are developed and become available.

6. INCLUSION ENROLLMENT REPORT

Inclusion Enrollment Report

This report format should NOT be used for data collection from study participants.

Study Title: Women's Health Initiative Nutrition and Physical Activity Assessment Study

Total Enrollment: 450 **Protocol Number:**

Grant Number: 2 R01 CA119171-04

PART A. TOTAL ENROLLMENT REPORT: Number of Subjects Enrolled to Date (Cumulative) by Ethnicity and Race

Ethnic Category	Sex/Gender			
	Females	Males	Unknown or Not Reported	Total
Hispanic or Latino	64	0	0	64 **
Not Hispanic or Latino	381	0	0	381
Unknown (individuals not reporting ethnicity)	5	0	0	5
Ethnic Category: Total of All Subjects*	450	0	0	450 *
Racial Categories				
American Indian/Alaska Native	1	0	0	1
Asian	8	0	0	8
Native Hawaiian or Other Pacific Islander	0	0	0	0
Black or African American	92	0	0	92
White	344	0	0	344
More Than One Race	0	0	0	0
Unknown or Not Reported	5	0	0	5
Racial Categories: Total of All Subjects*	450	0	0	450 *

PART B. HISPANIC ENROLLMENT REPORT: Number of Hispanics or Latinos Enrolled to Date (Cumulative)

Racial Categories	Females	Males	Unknown or Not Reported	Total
American Indian or Alaska Native	0	0	0	0
Asian	0	0	0	0
Native Hawaiian or Other Pacific Islander	0	0	0	0
Black or African American	0	0	0	0
White	0	0	0	0
More Than One Race	0	0	0	0
Unknown or Not Reported	64	0	0	64
Racial Categories: Total of Hispanics or Latinos**	64	0	0	64 **

8. Protection of Human Subjects
8.1. Risks to Human Subjects

Human Subjects Involvement and Characteristics

Participants in the NPAAS feeding study will each complete a 1-week training, a 2-week feeding study, and two in-person clinic visits. During the 1-week training, after having signed an informed consent, participants will come to the Fred Hutchinson Cancer Research Center (FHCRC) Human Nutrition Lab (HNL) to receive training for recording their daily food and beverage intake during the week. At the end of the week, participants will return to the FHCRC HNL to review their records with study staff in preparation for designing individualized customary meal plans for use during the feeding study. During the 2-week feeding study participants will eat all foods and most beverages as provided by the FHCRC HNL and complete a 24-hour urine collection. During the 2-week feeding study period, participants will either come to the HNL three times weekly to pick up their foods and beverages. During the two clinic visits, participants will have a fasting blood drawn, participate in a doubly labeled water protocol and indirect calorimetry (Visit 2), and complete study forms. The first clinic visit will mark the start of the feeding study and will last about 5 hours. The second clinic visit will mark the end of the feeding study and will last about 2 hours.

The NPAAS feeding study will enroll 150 WHI Seattle Field Center participants who had been enrolled in the WHI Dietary Modification Trial comparison arm or Observational Study. The current age range will be 60-80 years. Participants will be of general good health.

Inclusion criteria are the following: (1) being an active participant in the WHI Extension Study at the Seattle Field Center, (2) living within 25 miles one-way of the Seattle FC, and (3) current age ≤80 years. Exclusion criteria are the following: (1) having medical conditions precluding successful completion of the protocol (such as diabetes, kidney disease, bladder incontinence, routine use of oxygen) and (2) not having participated in the WHI Nutritional Biomarkers Study or NPAAS.

There are no special classes or vulnerable populations involved.

All of the human subjects research will be conducted at the Fred Hutchinson Cancer Research Center, which houses the WHI Coordinating Center, the WHI Seattle Field Center, the Prevention Clinic, and the Human Feeding Lab.

Sources of Materials

Participants will be asked to provide blood and urine biospecimens, be assessed for physical measures such as height and weight, complete study information forms, food records, and a physical activity questionnaire,. Demographic and historical subject characteristic data will be obtained from the WHI database with such data usage being agreed to in the signed consent for the WHI Extension Study.

Specific data to be collected includes the following:

(1) telephone screening script collecting data about use of medications for diabetes, receipt of supplemental oxygen, receipt of blood transfusions or intravenous fluids, bladder control, body weight management, travel plans;
(2) multiple day food record for collection of food intake during 4 days;
(3) activity frequency questionnaire collecting data about occupational and non-occupational activities;
(4) monitored daily intake and activity records for use during the 2-week feeding study;

(5) 14 day accelerometer data
(6) 24-hour urine collection asking about date and time of collection, time of PABA pill intake, problems with the urine collection, such as missing collections, away from home, blood or stool contamination, and spillage;
(7) WHI personal habits questionnaire collecting data about physical activity, alcohol use, and smoking;
(8) WHI food frequency questionnaire collecting data about foods and beverages consumed during the previous 3 months.

The only persons having access to the individually identifiable private information about the participants will be the WHI Seattle Field Center principal investigator, lead operations staff, and data entry staff; the WHI Clinical Coordinating Center NPAAS investigators, lead operations staff, data monitoring staff; and the Human Nutrition Lab lead staff who are implementing the NPAAS feeding study.

The biospecimens will be collected at the FHCRC Prevention Center Clinic by staff trained in biospecimen obtainment and handling procedures. Screening data will be collected by the WHI NPAAS lead staff at the WHI Seattle Field Center. The food, activity, and personal habits questionnaires be completed by the participants. The material and data are being collected specifically for the proposed research project.

Potential Risks

Venipuncture from an arm vein may cause discomfort and a bruise at the site of the needle puncture, although this is uncommon. Persons with known PABA sensitivities or allergies may get a slight rash if they take the PABA; they will be queried as to sensitivities and not asked to take the PABA if sensitive or allergic. During the indirect calorimetry, people may feel claustrophobic and if so will not be asked to complete the test. There is also the possibly of loss of air flow due to a rare failure of the fan in the metabolic cart or due to a loose hose which may lead to discomfort and in rare cases, may cause asphyxiation. Potential inconveniences, though not risks, may be writing down all foods and beverages consumed and collecting urine for 24 hours.

This is not a treatment study and thus there are no alternative treatments. Participants may choose not to participate at any time during the study.

8.2. Adequacy of Protection Against Risks

Recruitment and Informed Consent

The Seattle Field Center lead staff for the NPAAS feeding study will use the WHI database to select potential participants with respect to eligibility and living in King County within 25 miles (one-way) of the Seattle Field Center. This subset of potential participants will receive a letter of approach describing the study and indicating that the study staff will call to discuss interest and eligibility. Those who agree to participate will be scheduled for an informational clinic visit to meet with the HNL NPAAS staff member and will receive the consent form by mail. During the informational clinic visit, staff will discuss the consent form with the participant and the participant will have the opportunity to ask questions.

Consent will be discussed by the HNL NPAAS lead staff with the potential participant in a private interview space within the FHCRC HNL. All items in the informed consent will be discussed. These include why the study is being done, the number of people participating, what is involved in the study,

how long the participant will be in the study, monetary compensation for time and travel upon completion of the protocol, risks and benefits of the study, options to participating, confidentiality, costs, rights as a participants, and who to call if the participant has questions. Consent will be documented by data entry in the study database.

Protections Against Risk

Venipuncture will be performed by a trained phlebotomist familiar with working with the age range of the study population to minimize the risk of discomfort or bruising. Persons will be queried about PABA sensitivities or allergies and not asked to take the PABA if sensitive or allergic. Before the indirect calorimetry, participants will be asked about claustrophobia and comfort in small spaces; if uncomfortable, the indirect calorimetry will not proceed, and may be stopped during the procedure. The metabolic carts are equipped with alarms if the unit does not detect normal respirations. We also require that staff stay with a participant throughout the testing period.

All participant study records will be kept in secure locked areas. Computers with access to participant data will be locked when not in use. Only authorized personnel at the FCHRC Prevention Center (Clinical Research Center), WHI Coordinating Center, and the FHCRC Nutrition Assessment Shared Resource will have access to participant data. Personal information may be disclosed if required by law. Laboratory biospecimens will be labeled with a specimen id that has no identifiable participant information. These systems have been used for the WHI since 1993, for the Nutritional Biomarkers Study (part of the WHI), and for the WHI NPAAS ancillary study with no breeches of confidentiality.

Adverse events will be reported. All participants in the WHI are required to have their own health care providers. In the event of a medical situation, their health care providers will be contacted. In the event of an emergency, emergency personnel will be summoned by 911.

8.3. Potential Benefits of the Proposed Research to Human Subjects and Others

It is hoped that the information learned from this study will benefit the WHI Extension Study. The knowledge gained may help scientists learn about blood nutrients and energy needs in postmenopausal women. The risks are minimal on their own and in relation to the potential benefit to a more informed understanding diet and chronic diseases known to affect postmenopausal women, such as cancers, heart disease, and diabetes.

8.4. Importance of the Knowledge to be Gained

The knowledge to be gained is a more reliable assessment of dietary and physical activity patterns in postmenopausal women in relation to reducing risk of chronic diseases, such as cancers, heart disease, and diabetes. The risks are minimal on their own and in relation to the potential benefits of coming to a more informed understanding diet and chronic diseases.

8.5. Data and Safety Monitoring Board

This research does not call for a DSMB. However, as an ancillary study to the WHI, the application has been reviewed and approved by the WHI Observational Safety Monitoring Board (OSMB). The WHI OSMB will review progress and safety annually.

8.6. Clinical Trials.gov Requirements

The WHI Clinical Trials.gov identifier is NCT00000611.

9. INCLUSION OF WOMEN AND MINORITIES

The proposed research is an ancillary study to the Women's Health Initiative, which solely includes postmenopausal women. Thus, no men will be recruited.

The targeted enrollment is 150 postmenopausal participants from the Seattle, WA WHI Field Center. Participants from all race/ethnicity groups are eligible. The Seattle FC population is 1.3% Hispanic or Latino ethnicity and 95.3% White, 2.3% Asian, 2% Black or African American race. All Hispanic, Asian, Black women will be offered the opportunity to participate.

The protocol is being implemented solely at the Seattle WHI Field Center to utilize the skills, experience, and expertise in conducting feeding studies of the investigators and staff at the FHCRC Human Nutrition Laboratory (HNL). The HNL director, Dr. Johanna Lampe, who serves as dual PI (with Dr. Ross Prentice) for the NPAAS feeding study, has led the successful implementation of numerous feeding studies. The HNL staff have worked together since the inception of the HNL five years ago. Consideration of preparing the foods at the FHCRC HNL and shipping them to remote WHI FCs was considered and decided against due to budgetary and logistical reasons.

The subject selection criteria pertain primarily to having an active participation status in the WHI Extension Study and being able to complete the protocol and are not related to ethnicity or race. Specifically, eligible women will (1) currently have consented to the WHI Extension Study, (2) previously have been in the Dietary Modification Trial comparison arm or Observational Study, (3) not have participated previously in the WHI Nutritional Biomarkers Study or Nutrition and Physical Activity Assessment Study, (4) have a deliverable address, (5) be of full follow-up status (excludes proxy, request not to receive mail from the WHI Clinical Coordinating Center, request for phone-only contact, alive, not lost to follow-up, and not having requested no follow-up), (6) have a zip code in King County, and (7) be currently ≤80 years of age, and (8) not have medical conditions that would preclude successful completion of the protocol (including but not limited to diabetes mellitus, kidney disease, bladder incontinence requiring the use of special garments or medications, routine use of supplemental oxygen).

10. TARGETED/PLANNED ENROLLMENT TABLE

Targeted/Planned Enrollment Table

This report format should NOT be used for data collection from study participants.

Study Title: Women's Health Initiative Nutrition and Physical Activity Assessment Study

Total Planned Enrollment: 150

TARGETED/PLANNED ENROLLMENT: Number of Subjects			
Ethnic Category	**Sex/Gender**		
	Females	**Males**	**Total**
Hispanic or Latino	2	0	2
Not Hispanic or Latino	148	0	148
Ethnic Category: Total of All Subjects *	150	0	150
Racial Categories			
American Indian/Alaska Native	1	0	1
Asian	6	0	6
Native Hawaiian or Other Pacific Islander	0	0	0
Black or African American	1	0	1
White	142	0	142
Racial Categories: Total of All Subjects *	150	0	150

*The "Ethnic Category: Total of All Subjects" must be equal to the "Racial Categories: Total of All Subjects."

Previous versions: 11/3/10, 2/14/11