DELTA

Dietary Effects on Lipoproteins and Thrombogenic Activity

DIET PROTOCOL 1

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Chapter 1: PROTOCOL INTRODUCTION AND RATIONALE

The classic studies of Hegsted et al. (1) and Keys and colleagues (2) provided us with expected changes in plasma cholesterol associated with changes in dietary fat and cholesterol content, but those studies were carried out in narrowly defined populations and measured only total cholesterol. Although numerous publications demonstrating that reductions in dietary saturated fat and cholesterol intake are associated with lowering of plasma total and LDL cholesterol have appeared since the work of Hegsted and Keys, most of these studies have compared diets with extreme differences in these constituents (3). Thus although Hegsted recently reported (4) that a large compilation of more recent investigations supported the earlier work of his group and that of Keys and his colleagues, we are still forced to extrapolate from those regression coefficients when we make decisions relevant to diet and health. In particular, we do not have adequate information about the efficacy of the Step 1 diet (5-10), which is advocated by the American Heart Association, the National Cholesterol Education Program and the American Diabetes Association, compared to an average American diet. Investigations directly addressing this issue have, for the most part, used adult Caucasian males. Although survey data are available concerning nutrient intake in females, other racial groups, children and the elderly (11-14), few controlled studies have been performed (15,16). The lack of information concerning these issues highlighted the need for larger studies; a need that could be satisfied only by a multicenter collaborative study. In the proposed protocol, sample size will be large enough to detect differences between the responses to the Step 1 diet in males and females. In addition, the protocol has been designed to allow us to determine if Caucasians and African-Americans of each gender respond to the change in saturated fat content between an average American diet and the Step 1 diet.

Even as the American public has changed its dietary habits and has begun to approach the goals set for intake of saturated fat and cholesterol (17), many individuals and groups have begun to set new goals, with even lower intakes of total as well as saturated fat (18). In particular, because of some data relating fat intake to cancer incidence, it has been suggested that a total fat intake of 20% or less be advocated. Some scientists, however, concerned with the effects of replacing dietary fat with carbohydrate, have called for low saturated fat but high total fat diets. Liberal use of monounsaturated fatty acids has been proposed by these individuals (19,20). In our proposed study, we will be able to carefully compare outcomes associated with consumption of the Step 1 diet and with consumption of a diet that is further reduced in total and saturated fats. This information will be important in planning subsequent studies designed to examine the effects of individual fatty acids on outcomes of interest. These data in normal men and women will lay the foundation for studies planned in future years in which we compare these diets in subjects with insulin resistance and dyslipidemia.

CHAPTER 1

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Although effects of dietary fats on platelet function have been under investigation for some time (21-23), interest in the relationship of diet to other aspects of hemostasis has developed in parallel with increasing awareness of the role of hemostasis in CHD (24-28). In particular, studies of the effects of dietary fats on fibrinogen (29), factor VII (30,31), and plasminogen activator inhibitor 1 (PAI-1) (32,33), the levels of which have been associated with risk for CHD, are clearly needed. The effect of diet on plasma Lp(a) concentrations is also uncertain, and requires careful study. The proposed study will generate much needed information concerning diet and hemostasis. In addition, in this first DELTA protocol, we will obtain crucial data related to the length of time needed to see changes in hemostatic factors, and possibly to determine if their are differences between males and females.

Recent data from several epidemiologic studies (34,35) and intervention trials (36) have suggested that consumption of fish reduces the risk of CHD mortality. These results appear to complement biochemical studies indicating that replacement of omega-6 fatty acids with omega-3 fatty acids may have effects on platelet function and eicosanoid metabolism (21-23) that would inhibit thrombosis. Although the link between fish consumption and effects of omega-3 fatty acids remains to be fully elucidated, studies of the effects of replacing omega-6 fatty acids with members of the omega-3 class seem warranted. In addition, the effects of diets supplemented with omega-3 fatty acids on plasma lipids and lipoproteins have been inconsistent, possibly due to the varying quantities or types of omega-3 fatty acids used (37). The study proposed for the later part of next year will allow a direct comparison between two diets with physiologic quantities of these two types of PF.

A review of the proposed studies outlined above should make it clear that the first DELTA protocol has been developed to meet the goals of the National Heart, Lung and Blood Institute as described in the RFA for this project. In particular, we have focused on gender and race, insuring adequate sample size to allow evaluation of the response to step-wise reductions in dietary total and saturated fat in men and women, and in Caucasians and African-Americans. We have also developed a protocol to study, in a detailed manner, the effects of these dietary modifications on several hemostatic factors.

Finally, DELTA will be the first multicenter, collaborative study of the effects of dietary nutrients in which laboratory and diet preparation are carefully and continually standardized. This is a unique opportunity to determine if even larger diet intervention trials will be feasible. The multicenter approach also affords the possibility to study subgroups of individuals which would not be large enough if the studies were conducted by a single center.

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CHAPTER 2

Chapter 2: ORGANIZATION AND ADMINISTRATION

The organizational structure of this study will include the following main components: the Field Centers, the Coordinating Center, the Steering Committee, and an external Protocol Review Committee to provide oversight and advice to the NHLBI at various stages of the study. The structure and function of these study components are described below.

A. Field Centers

There will be four Field Centers responsible for the recruitment, feeding and investigation of study participants. Each Field Center will consist of a team of investigators who will provide the necessary skills and effort to develop and carry out this protocol successfully. The principal investigator and designated key co-investigators from each Field Center will participate in protocol development and in decisions concerning the conduct of the study and the analysis and publication of its results via the Steering Committee (see below) and its subcommittees.

Field Centers and Principal Investigators are as follows:

Columbia University,
Henry W. Ginsberg, M.D.

University of Minnesota,
Patricia Elmer, Ph.D.

Penn State Univerisity,
P.M. Kris-Etherton, Ph.D.

LSU/Pennington Biomedical Research Center
Paul Roheim, M.D.

B. Coordinating Center

The Coordinating Center will have primary responsibility for coordinating the efforts of the study investigators and for editing, storing, and analyzing the data generated by the Clinical Centers and by central laboratories established for key study measurements. Its investigators and staff will have a central role in designing the data collection system and in monitoring data quality.

Food Analysis Laboratory Control Center (FALCC)

FALCC is the central laboratory for food analysis and associated research; it will receive, composite, assay and archive diet samples from the study.

The FALCC will undertake the following tasks:

1. Develop the necessary protocols for diet sampling, diet composition, analysis of and storage of diet composites.

2. Develop, modify and validate the various analytical methods needed for the assay of the diet composite for the food components of concern.

3. Monitor the diets being fed to verify that they have the nutrient content that is planned for the particular experimental feeding trial, and that the diets being fed at the different centers are virtually identical.

The director will be a member of the Diet Subcommittee and will participate as a non-voting member of the Steering Committee.

Nutrient Composition Laboratory (NCL) U. S. Department of Agriculture

The NCL will provide standard reference materials for quality control of the food assays. It will also provide expert consultation on all aspects of the control of diet composition. The director will be a member of the Diet and Laboratory Subcommittees and will participate as a non-voting member of the Steering Committee.

C. Steering Committee

The Steering Committee is primarily responsible for all decisions pertaining to the design and conduct of the studies undertaken in the DELTA project. It will determine the scientific objectives of the studies, design various studies to attain these objectives -- e.g., this protocol -- and oversee the scrupulous implementation of the study protocols. The voting members of the Steering Committee are the principal investigators of each Field Center and the Coordinating Center and the NHLBI Project Officer. Each has a single vote; a simple majority decides. However, approval of the external Protocol Review Committee will be required for any significant changes in protocol, including the initiation of ancillary studies that are recommended by the Steering Committee during the course of the study. The chairman of the Steering Committee will be appointed by the Director, Division of Heart and Vascular Diseases, NHLBI.

The Steering Committee will meet at least 6 times during the first year and quarterly in subsequent years. It will designate the following subcommittees of investigators and/or staff as needed to oversee aspects of the study that require more frequent attention and/or special expertise. Each subcommittee will have appropriate representatives from each field center and the coordinating center:

1. Protocol Subcommittee: will oversee the development of study protocols for implementing study objectives identified by the Steering Committee. The protocol subcommittee will develop study design, sample size calculations, eligibility criteria, data variables and sequencing of measurements. It will review relevant data collection forms, consent forms, and manuals of operation for each study protocol.

2. Diet Subcommittee: will oversee the development and testing of the study diets, identify food composition assays, evaluate nutrient databases, review relevant data collection forms and manuals of operation.

3. Manual(s) of Operation and Forms Subcommittee: will provide editorial assistance and final approval for manual(s) of operation, provide content input for data collection forms, approve design and format of forms, review suggested or required changes to forms and procedures after the start of the study.

4. Laboratory Subcommittee: the Laboratory Subcommittee will advise the Steering Committee on appropriate laboratory measurements to achieve study objectives, and monitor performance of the hemostasis, lipid and lipoprotein laboratories and will oversee training and certification of phlebotomists, and laboratory standardization via reports from the coordinating center. This committee will review relevant manual(s) of operation and data forms.

5. Publications Subcommittee: will advise the Steering Committee on publication policy. It will oversee the preparation and review of abstracts and manuscripts emanating from the study to assure proper distribution of analysis topics and authorship credit among study investigators. It will ensure that collaborative manuscripts represent the study accurately.

6. Conflict of Interest Subcommittee: will draft guidelines regarding outside activities of study investigators that represent potential conflicts of interest and collect annual disclosure statements from investigators regarding relevant activities.

DELTA investigators in cooperation with the NHLBI project office will seek donations of products, equipment and services to support specific activities of the study.

We will follow the guidelines established by the NHLBI for obtaining third party support.

A major effort will be made to get food donations from national companies in order to defray the cost of feeding the diets across clinics. Other potential third

party support would include cholesterol screening machines and various participant incentives.

7. Ancillary Studies Subcommittee: will review and make recommendations regarding the merit and feasibility of ancillary studies that are proposed by study investigators or other interested parties.
The Steering Committee will retain the prerogative to add, combine, delete, or redefine subcommittees as the study evolves and its needs change.

D. **External Oversight**

1. **Protocol Review Committee**: This committee of at least five experts not otherwise affiliated with the study will be appointed by the Director, NHLBI to review this protocol, to recommend revisions as needed, and to advise the Institute as to its acceptability. This study will not be implemented until the Director, NHLBI, acting with the advice of this committee, approves this protocol.

This committee will meet prior to initiation of each new study and additionally as necessary to carry out its oversight responsibility.

The Chairman of the Steering Committee, Project Officer, and designated NHLBI staff will participate in these meetings in an ex-officio capacity, to facilitate communication between the Protocol Review Committee and Steering Committee.

The principal investigator of the Coordinating Center and designated Coordinating Center staff will attend these meetings (but will not have a vote) and will be responsible for preparing and presenting up-to-date statistical reports on the progress of the study. These reports will include data on recruitment and randomization, as well as statistical tests and special analyses requested by the committee.

2. Human Subjects Protections:

A. **Informed consent**

Informed consent will be obtained from each participant before they are enrolled in the study. A draft Informed Consent form is found in the Appendix to this protocol. The Consent form will describe the risks and benefits of participating in the study, as well as the responsibilities of the participants and the investigators.

B. **Privacy**

Privacy in the context of this study includes confidentiality of data and personal information at the field center (see individual site descriptions below) and in handling and

reporting of data by the Coordinating Center (see Chapter 11). It also includes discretion on the part of field center staff and arrangements for physical privacy during interviews and examinations.

C. "Right-to-know"

Information obtained at screening, before enrollment, would be available at that time. Abnormal values found at screening would be reported to the participant and also, upon request, to his or her personal physician. During the course of the study, participants occasionally are curious about their progress and about changes in a variable such as serum cholesterol level. Such data will not be available until the very end of the study, because the protocol calls for batch analysis of all samples collected throughout. Information will be provided at the end of the study to participants who are interested.

D. Safety

The DELTA study will evaluate the effects of various diets on blood lipids, and lipoproteins, clotting parameters and related variables. The diets will have varying composition but will not differ greatly from those consumed by many members of the public. All diets will be nutritionally adequate with regard to calories (they will be designed specifically to prevent weight change). All diets will contain at least 90% of the RDA for women 25-50. Unlike those clinical trials evaluating the efficacy of drugs on disease incidence or symptoms, the experimental treatments for the first DELTA study are not expected to pose any particular risk per se. Individuals for whom the diets are contraindicated because of preexisting conditions (for example, liver disease, kidney disease, clotting disorder) would not be eligible for participation according to defined exclusion criteria (see Chapter 6).

Food allergies will be enquired about during screening.

The collection of biological specimens and other information carries a small risk. For example, phlebotomy carries a risk of bruising and discomfort. This will be explained to the participant in the consent form.

Food safety is another concern. The DELTA investigators have an obligation to protect participants against illness due to microbiological contamination of food. The DELTA field centers will guard against this through rigorous application of food handling standards appropriate for their particular institution (hospital-based kitchen; other setting). Individuals will be carefully instructed in the handling and storage of any take-home food or meals for example coolers and "blue ice" will be provided; time X temp strips will be enclosed with instructions not to consume certain foods if the strip indicates that holding temperatures have not been appropriate. (See also Chapter 8). The consent form will include an explanation of the participant's obligation to handle take-out food according to instructions they will be given to maintain adequate sanitary conditions.

Safety oversight

Safety concerns will be addressed during protocol review by the NHLBI-appointed committee and by the individual field center IRBs. After enrollment, the principal investigators will monitor safety issues continuously and report any problems to the Coordinating Center, which will inform the NHLBI Medical Officer. Each field center will have an assigned Medical Officer to ensure the satisfactory disposition of Study-related adverse events (referral to physician; treatment; or decision to drop from Study for medical reasons, which must be made with P.I.).

Medical officers

The Medical Officers for the DELTA Study are:

1. Columbia: Henry N. Ginsberg, M.D.
   Neil S. Shachter, M.D.

2. Minnesota: Aaron Folsom, M.D.

3. LSU/Pennington Donna H. Ryan, M.D.

4. Penn State Thomas A. Pearson, M.D., Ph.D
   W. Channing Nicholas, M.D.

CHAPTER 3

Chapter 3: PROTOCOL 1 HYPOTHESES AND ENDPOINTS

A. Objectives:

The purpose of this study is to determine the effect of reducing dietary total and saturated fat intake on plasma lipids and lipoproteins, and on hemostatic factors. The study will be carried out in healthy male and female, white and black adults. In particular, we are interested in determining if further reductions in total and saturated fat, beyond the levels recommended in the Step 1 diet, are efficacious in these gender and race specific groups.

B. Specific Aims:

1. To compare the effects of three diets, differing in total and saturated fat content, on plasma lipids and lipoproteins in healthy adults.

2. To compare the effects of these diets on plasma hemostatic factors in healthy adults.

3. To determine if males and females, and individuals with different racial origins respond to these diets.

4. To evaluate the role of the menstrual cycle in modulating diet responses in females.

C. Study Outcomes:

1. The primary lipid and lipoprotein endpoints will be plasma concentrations of total cholesterol and triglyceride, low density lipoprotein cholesterol, high density lipoprotein cholesterol, apoprotein B, apoprotein A-I, and lipoprotein (a). In addition, we will determine the apoE genotype of each participant.

2. The primary hemostatic endpoints will be plasma levels of fibrinogen, factor VII and plasminogen activator inhibitor I.

3. Several ancillary studies will be carried out to study a variety of secondary endpoints.

D. Rationale for endpoints:

The rationale for the study design, including the issues of gender and ethnicity are addressed in the Introduction. The choice of endpoints derives from the need to minimize the comparisons in order to maintain statistical power, and the feasibility of carrying out numerous measurements in a large population of subjects. The plasma lipid and

lipoproteins chosen were deemed to be those most likely to affect risk for cardiovascular disease. In addition, issues related to HDL cholesterol are of particular interest, both from the standpoint of gender-specific responsiveness and the response to increasing reductions in total and saturated fat. The inclusion of lipoprotein (a) is based on its emergence as a significant risk factor and the paucity of information related to its response to dietary perturbations. ApoE genotyping will be performed because of the possibility that it affects diet responsiveness. The choice of hemostatic factors was based on data from epidemiologic studies indicating the significance of fibrinogen, factor VII and PAI-1 as risk factors for coronary heart disease.

CHAPTER 4

Chapter 4: STATISTICAL METHODS

The Cross-Over Treatment Design

The details of the study design are given in Chapter 7. In this section we will focus on the statistical issues and analytic methods to be used in comparing two experimental diets, A and B, and a reference diet, R, with respect to hemostasis and plasma lipoproteins. The treatment design is a randomized 3-treatment, 3-period, 6-sequence cross-over. After a brief run-in period, each participant will be randomly assigned to one of six diet sequences: ABR, ARB, BAR, BRA, RAB, and RBA. Each diet period will be eight weeks in duration. For example, the group assigned to the ABR sequence will be fed diet A for eight weeks, then diet B for eight weeks, and finally diet C for eight weeks.

The relative advantages of this treatment design have been evaluated in terms of monetary expense and in terms of statistical power for detecting treatment effects. Monetary costs are proportional to the number of meals served. Sample size comparisons were made under the assumptions that the underlying sources of variation combine additively (rather than multiplicatively, for example.) Compared to an equally capable parallel-arm design, the selected cross-over costs less and requires many fewer subjects to obtain efficient estimates of the direct effects of treatment. This feature allows each Field Center to establish rapport with a small cohorts of well motivated participants. Furthermore, the selected design has satisfactory statistical properties under reasonable assumptions in the setting of this study of serum analytes.

To avoid carryover of effects from one period to the next, an active washout interval will be used. That is, the first four weeks of each diet period will provide time for stabilization of the responses of interest. Blood draws during the last four weeks of each diet period will yield quadruplicates of endpoint measurements. Consequently, baseline measurements at the beginning of each period will not be obtained. We anticipate that lipoprotein responses will have stabilized in four weeks. There is less certainty in regard to the hemostasis variables. In both cases the proposed analysis plans will provide for evaluation of degree of stabilization. As specified in Chapter 7, additional measurements during the last four weeks will be made on premenopausal females. The additional measurements will facilitate the evaluation of cyclical trends in serum analytes attributable to the menstrual cycle.

Statistical Computation and Analysis Methods

The proposed cross-over is a special kind of longitudinal design involving correlations among the repeated measurements made on each individual in the study. This feature must be incorporated in the analyses of the data. "Data analysis" includes selecting and managing analysis strategies, interpreting the subsequent results via written reports, and writing of papers for publication. "Statistical computation" refers to the use of computer systems to produce meaningful calculations, summary tabulations, and graphic figures, as dictated by analysis

strategy. A tremendous variety of statistical analysis methods exist for cross-over studies. Appropriate choices of methods depend upon the scale of the outcome variables (dichotomous, ordinal, interval, time-to-event) and upon the objectives of the study. For example, the parametric methods of linear statistical models ("general" or "generalized") can provide cross-sectional and longitudinal analyses for outcome variables in a wide variety of situations.

The General Linear Mixed Model

For the purposes of this study, analysis methods based on a general linear mixed model with linear covariance structure are preferred. This approach will allow great flexibility in estimation and testing of the expected-value effects (treatment, period, sequence) effects as well as exploration of the covariance structure. Such models are effective in cross-over studies such as this one -- with or without baseline measurements. The linear covariance structure permits use of different variance and covariance parameters for different groups, allows one to assume that specified covariances are zero, and provides for tests of hypotheses about specified variance and covariance parameters. We will use maximum likelihood (ML) and restricted maximum likelihood (REML) estimation methods. The SAS software system will provide the necessary iterative algorithms (via the "MIXED" procedure). Furthermore, the mixed model will cope with any ignorably missing data. (See Laird and Ware (1982), Jennrich and Schlucter (1986), Ware (1985), Neter and Wasserman (1974), Harville (1977).)

This modeling strategy also allows flexibility in detecting and dealing with within-period temporal trends. In the presence of such trends, we will have the option of (a) using only the final (most stabilized) blood sample in the diet period, or (b) using all the data to estimate the final level. We will also have the opportunity to model cyclical trends among the premenopausal females.

Sample Design

Table 1 gives an example of possible recruitment outcome. The centers will attempt to recruit roughly equal numbers of pre- and post-menopausal women (f+ and f-, respectively.) In this chapter we will use "Black" to refer to African American subjects, and "White" to refer to all other subjects in the study. We anticipate that this later category may include a few Asian and Hispanic subjects. Chapter 5 gives details about population frames and recruitment goals for each of the Field Centers.

Table 1

Assumed Sample Design

Center	Gender	Black	White	Total
Columbia U.	F+	3	4	7
	F-	4	4	8
	M	4	5	9
Pennington	F+	4	4	8
	F-	3	3	6
	M	5	5	10
U. Minn.	F+	1	6	7
	F-	1	7	8
	M	1	8	9
Penn. State	F+	1	6	7
	F-	1	6	7
	M	1	9	10
Total	F+	9	20	29
	F-	9	20	29
	M	11	27	38
		29	67	96

M: Males
F+: Premenopausal Females
F-: Postmenopausal Females
Black: African American subjects
White: Non - African American subjects (mostly Caucasian)

Randomization of Subjects to Treatment Groups

At the end of the run-in period, remaining candidate participants will be randomized to treatment groups (diet sequences) in such a way as to provide balance across the three diet periods. Highest priority will be given to balance within field centers to allow for clear interpretation of ancillary studies that will be performed. Balance within the demographic strata (across the field centers) will have next highest priority. Table 2 gives some indication of considerations of balance.

Table 2
Proposed Allocation of Diet-Sequences to Subjects

	Col	LSU	Minn	Penn	Total
ABR	4	4	4	4	16
ARB	4	4	4	4	16
BAR	4	4	4	4	16
BRA	4	4	4	4	16
RAB	4	4	4	4	16
RBA	4	4	4	4	16
Total	24	24	24	24	96

Working Assumptions for Power Analysis

The a priori model for power analysis, planning, and for ultimately testing the primary hypotheses specifies a fairly simple linear covariance structure; namely, the constant-correlation structure. This model is a special case of the general linear mixed model. The usual interpretation of this structure is that the underlying process involves additive errors. In other words, total variance is the sum of two or more sub-components of variance. We anticipate that this model is approximately correct in spite of some sources of variation that are most likely multiplicative in nature.

It should be noted that the additivity assumption is pivotal for many of our important conclusions. Sensitivity of results to this and other modeling assumptions will be evaluated using diagnostics methods for the mixed model.

All power analysis results reported here were based on the assumption that N=96 subjects will be enrolled, 24 from each of the four Centers, as shown in Table 1. We assume that diet sequences will be randomly assigned to subjects as indicated in Table 2. We assume that the actual values of diet differences (and all other model parameters) are fixed and unknown for the study population. Estimates of within-subject and between-subject variability are based on previous single-center studies as shown in Table 3. We assume that the values in Table 3 as a starting point for power computations. For example, we assume that the within-subject variance for the mean of three serum samples for total cholesterol is approximately $108(mg/dl)^2$ (=324/3).

Table 3
Assumed Within-Subject and Between-Subject Variance As Estimated from Single-Center Studies

	Within Subject SD	Within Subject Variance	Between Subject SD	Between Subject Variance	Total SD	Total Variance	Intraclass Correlation
Total Chol.	18.00	324.00	31.18	972.00	36.00	1296.00	75.0%
HDL Chol.	3.60	12.96	7.14	51.04	8.00	64.00	79.8%
LDL Chol.	12.50	156.25	27.27	743.75	30.00	900.00	82.6%
Triglyceride	16.10	259.21	47.34	2240.79	50.00	2500.00	89.6%
Fibrinogen	40.20	1616.04	73.76	5439.96	84.00	7056.00	77.1%
Factor VII	8.40	70.56	21.41	458.44	23.00	529.00	86.7%
PAI-1	7.70	59.29	12.17	148.07	14.40	207.36	71.4%
beta-TG	2.50	6.25	2.99	8.96	3.90	15.21	58.9%
PF4	0.57	0.32	0.52	0.27	0.77	0.59	45.2%
Ox-LDL	2.90	8.41	16.85	284.00	17.10	292.41	97.1%
Vitamin E	1.20	1.44	4.85	23.56	5.00	25.00	94.2%
Mono. cell Chol.	1.70	2.89	3.06	9.36	3.50	12.25	76.4%

All power analysis results are based on the following assumptions: (1) the hypothesis test is performed at the 0.01 level of significance, (2) the sample size is N=96, (3) the sample is distributed as shown in Tables 1 and 2, (4) the endpoint is an average from three weekly blood draws ("REPS=3" in the figures), (5) the resulting within-subject variance is as shown in Table 3, (6) the variance-covariance structure for the repeated measurements has the constant-correlation and constant-variance characteristics, (7) fixed effects for gender, race, center, age, baseline value, period, diet, race*diet interaction, and gender*diet interaction, (7) no carryover effects.

Power Analysis Results

Our analyses indicate that we will have adequate power for detecting the most important clinical differences. Figures 1-5 present a summary of the results in terms of "minimally detectable differences" when power is held fixed at 90%. This concept is illustrated in Fig. 1.

Fig.1 presents power as a nonlinear function of the overall difference between diets A and B for total cholesterol. The power curve ranges from 0.01 (the significance level of the test) to 1.00. If the true difference between diets A and B is about 6.7mg/dl then the power of the intended test procedure is approximately 90%. Our point estimate of the "minimally detectable difference" is then 6.7mg/dl. We use 90% power as the criterion for "minimally detectable" because procedures with smaller power are sensitive to slight inaccuracies in the assumed magnitudes of effects.

Because there is uncertainty about the true value of within-subject variance in the proposed multi-center study, the curve is plotted for three different values of within-subject variance: the assumed value (108), an optimistic value (108*0.5) and a pessimistic value (108*1.5). The upper and lower curves provide an interval estimate of "minimally detectable difference" as illustrated in Fig. 1.

Figures 2-5 present a collection of such interval estimates for the primary comparisons of diets A and B. Estimates of "minimally detectible differences" are given for each of two racial groupings, for premenopausal females (+f), for postmenopausal females (-f), for females overall (f), for males (m), and for White females (wf), White males (wm), Black females (bf), and Black males (mb).

Estimates for total serum cholesterol are given in Fig. 2 (multi-center) and Fig. 3 (single-center). A comparison of Figures 2 and 3 indicates the strategic value of undertaking the

multicenter study. The results for a single-center study assume the sampling distribution in Table 1 for the Columbia University Field Center.

Similarly, Figures 4 and 5 address HDL cholesterol.

Identical results are obtained for A-R and B-R diet comparisons when carryover effects are not included in the model. Inclusion of first order differential carryover effects in the fitted model would reduce the estimated values of statistical power. Equivalently, this means that the detectable differences would be larger when carryover effects were included. For example, when comparing diets A and B for all subjects combined, the minimally detectable difference in total cholesterol would increase from 6.7mg/dl to 7.3mg/dl (an increase of less than 9%.)

Figure 1

Total Serum Cholesterol (mg/dl)

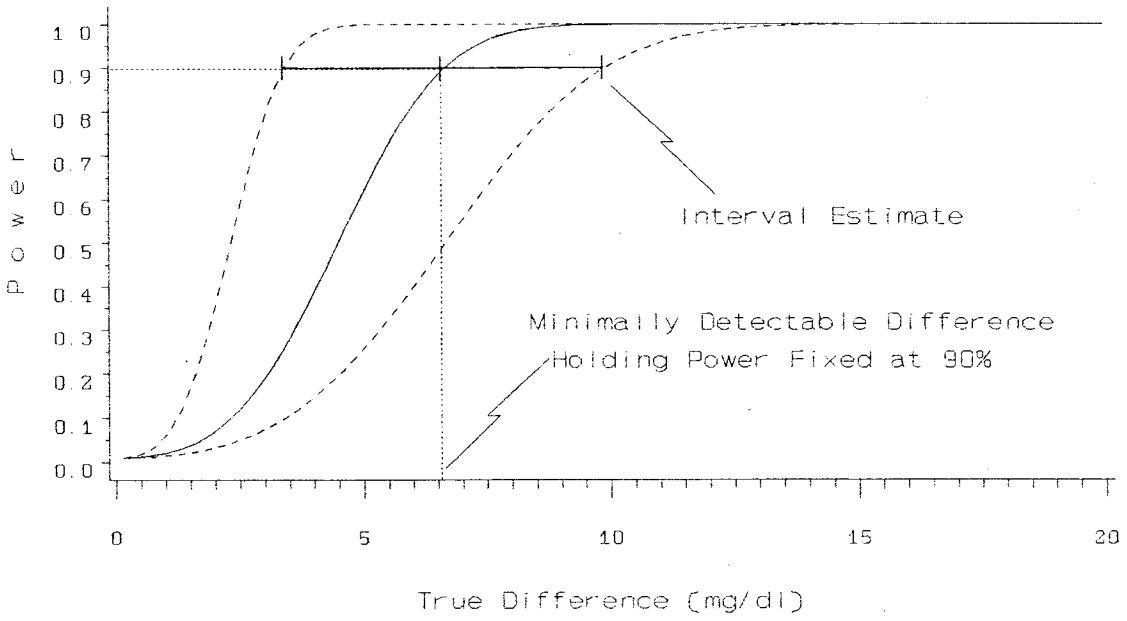
In Response to Diets A, B, and R Among

Black(b), White(w), Male(m) and Female(+f,-f) Subjects

Overall Comparison of Diets A and B

Averaging Over All Centers and Subjects

CENTERS=4 SUBJECTS=96 REPS=3



Significance level of test is 0.01. Upper & lower curves indicate effect of increasing or decreasing within-subject variance by 50%. Within-subject variance for one blood draw is assumed to be 324.00. Results do not depend on the between-subject variance, 972.00, and are identical for (A-R) comparisons and for (B-R) comparisons.

The mixed-model assumptions are as follows:

Fixed Effects --- Gender, Race, Center, Period, Diet, Race*Diet, Gender*Diet

Random Effects --- Intercept only

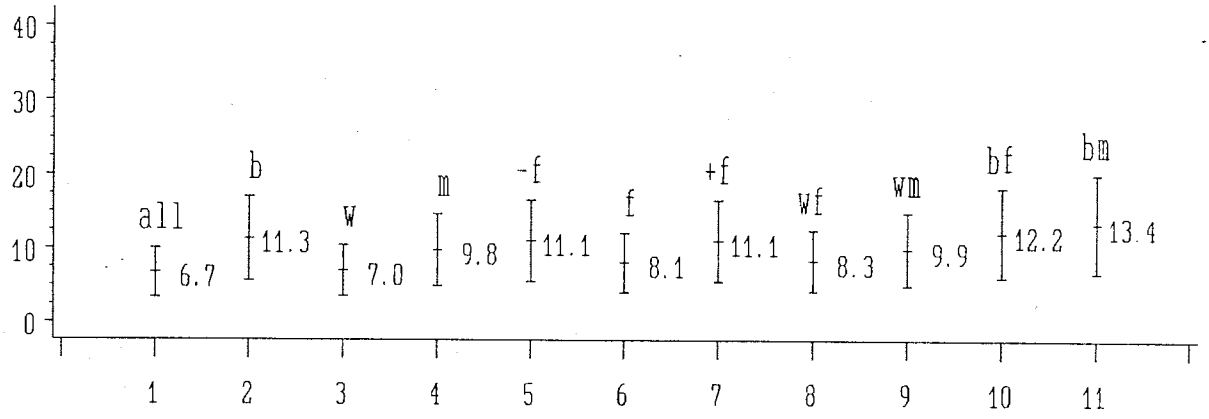
pov503-a

Figure 2

Total Serum Cholesterol (mg/dl)
In Response to Diets A, B, R Among
Black(b), White(w), Male(m), Female(+f,-f) Subjects:

Minimally Detectable Differences
For Comparison of Diets A and B

CENTERS=4 SUBJECTS=96 REPS=3



Minimally detectable differences with power held fixed at 90%.
Significance level of each test is 0.01.
Interval indicates effect of increasing/decreasing within-subject variance by 50%
Within-subject variance for one blood draw is assumed to be 324.00
Results do not depend on the between-subject variance, 972.00
Results are identical for (A-R) comparisons and for (B-R) comparisons.
The mixed-model assumptions are as follows.
Fixed Effects: Gender, Race, Center, Period, Diet, Race*Diet, Gender*Diet
Random Effects: Intercept only

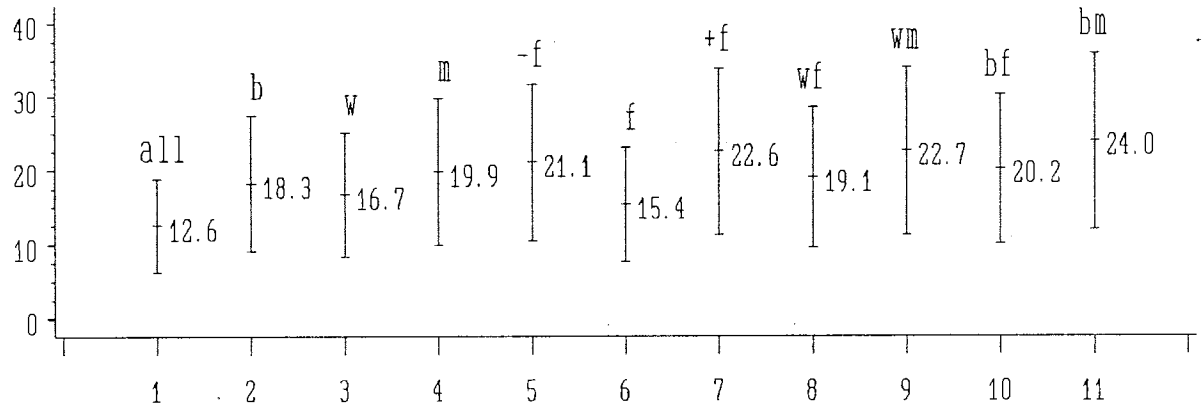
pov502-a

Figure 3

Total Serum Cholesterol (mg/dl)
In Response to Diets A, B, R Among
Black(b), White(w), Male(m), Female(+f,-f) Subjects:

Minimally Detectable Differences
For Comparison of Diets A and B

CENTERS=1 SUBJECTS=24 REPS=3



Group-Specific Comparisons

Minimally detectable differences with power held fixed at 90%.
Significance level of each test is 0.01.
Interval indicates effect of increasing/decreasing within-subject variance by 50%
Within-subject variance for one blood draw is assumed to be 324.00
Results do not depend on the between-subject variance, 972.00
Results are identical for (A-R) comparisons and for (B-R) comparisons.
The mixed-model assumptions are as follows.
Fixed Effects: Gender, Race, Center, Period, Diet, Race*Diet, Gender*Diet
Random Effects: Intercept only

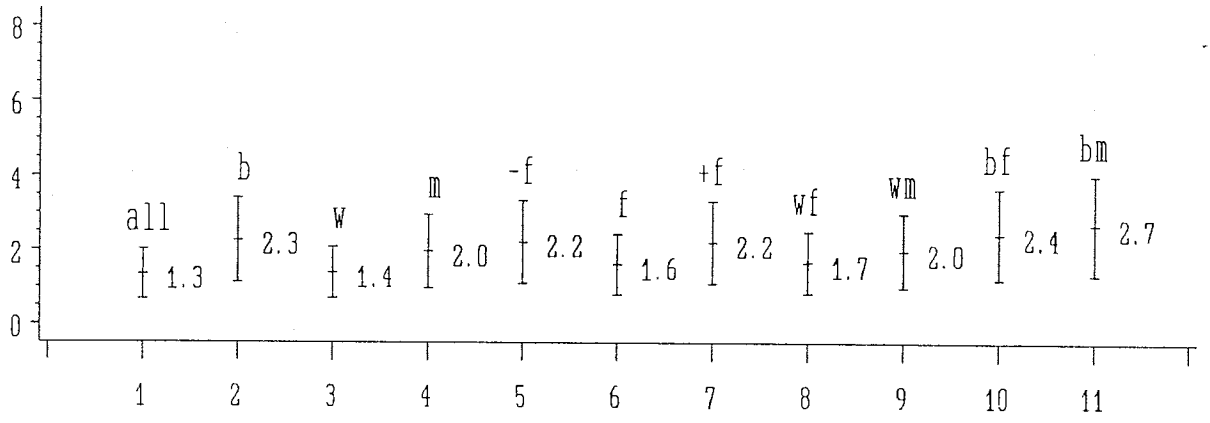
pow502-C

Figure 4

HDL Serum Cholesterol (mg/dl)
In Response to Diets A, B, R Among
Black(b), White(w), Male(m), Female(+f,-f) Subjects:

Minimally Detectable Differences
For Comparison of Diets A and B

CENTERS=4 SUBJECTS=96 REPS=3



Group-Specific Comparisons

Minimally detectable differences with power held fixed at 90%.
Significance level of each test is 0.01.
Interval indicates effect of increasing/decreasing within-subject variance by 50%
Within-subject variance for one blood draw is assumed to be 13.00
Results do not depend on the between-subject variance, 51.00
Results are identical for (A-R) comparisons and for (B-R) comparisons.
The mixed-model assumptions are as follows.
Fixed Effects: Gender, Race, Center, Period, Diet, Race*Diet, Gender*Diet
Random Effects: Intercept only

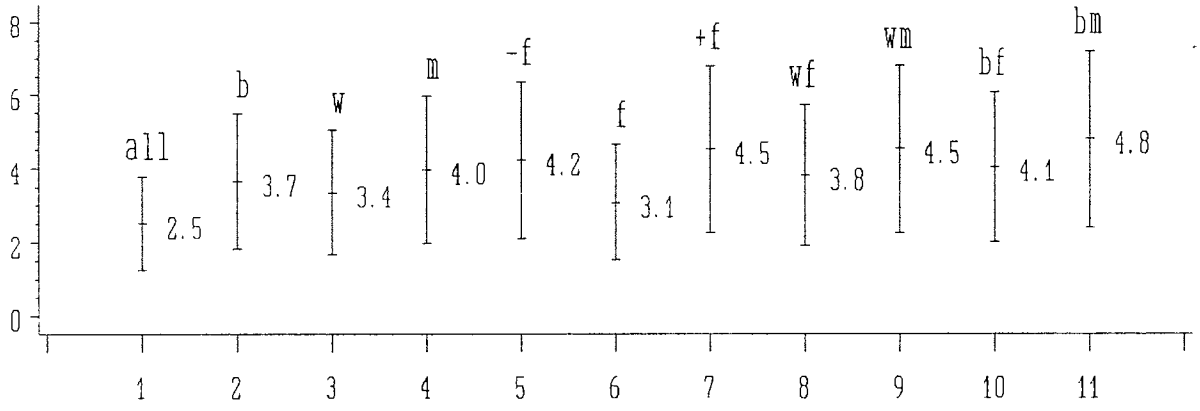
pow502-5

Figure 5

HDL Serum Cholesterol (mg/dl)
In Response to Diets A, B, R Among
Black(b), White(w), Male(m), Female(+f,-f) Subjects:

Minimally Detectable Differences
For Comparison of Diets A and B

CENTERS=1 SUBJECTS=24 REPS=3



Group-Specific Comparisons

Minimally detectable differences with power held fixed at 90%.
Significance level of each test is 0.01.
Interval indicates effect of increasing/decreasing within-subject variance by 50%
Within-subject variance for one blood draw is assumed to be 13.00
Results do not depend on the between-subject variance, 51.00
Results are identical for (A-R) comparisons and for (B-R) comparisons.
The mixed-model assumptions are as follows.
Fixed Effects: Gender, Race, Center, Period, Diet, Race*Diet, Gender*Diet
Random Effects: Intercept only

p-205ad

Effects of Errors in Food Preparation

Measurement errors, variation of nutrients in raw food resources, and other cumulative effects result in inaccuracies in the preparation of experimental meals. As a result, some individuals receive more or receive less of certain nutrients than intended. This source of error can increase both the within-subject variance and between-subject variance. Inaccuracies that are consistent across time and diet can, in theory, cancel out when within-subject diet comparisons are made. However we shouldn't trust that such cancellation will occur. In regard to minimizing food preparation errors or ameliorating their effects, we have four objectives:

1. Perform ancillary research to identify and evaluate sources of variation in food preparation. This is a forward-looking investment of effort and capital that pays off in the long run.

2. Establish and refine food preparation procedures prior to data collection. Our pilot studies address this objective. In the metaphor of industrial quality assurance, this is analogous to building, tuning, and debugging production machinery so that resulting fabrications is "within tolerances" when production begins.

3. Minimize errors via quality assurance strategies. Appropriate strategies include food-purchasing strategies, standard within-kitchen procedures for quality assurance, and assaying sample meals. Feedback from food assays should be as instantaneous as possible.

4. Estimate the mean and variance of the amounts of nutrients served to the study subjects. That is, at the end of the study we will provide information about the actual nutrient levels served. It is important to characterize the actual diets in any publications of the study.

Effects of Serum Assay Measurement Error

Under "additive errors" assumptions, laboratory measurement error is a sub-component of within-subject variance. Any unnecessary increase in within-subject variance reduces the power of the primary hypothesis tests. Therefore implementation of laboratory quality control procedures are crucial for the success of the study. Ideally, such procedures provide the scientist with instantaneous feedback so that gross errors are corrected.

A bias in a laboratory procedure that is consistent over time can, in theory, cancel out when the within-subject comparisons of the diets are computed. However the existence of such a bias is hardly reassuring as it could go hand-in-hand with other errors that do not cancel. Also one worries that the lab procedures might be partially "out of control".

Multi-laboratory studies add an additional source of concern. If the level of laboratory precision varies from one Field Center to another then the variance of the endpoint values will vary from Center to Center. This lack of constancy in variance will rob the collaborative analysis of a small amount of statistical power. It is possible to assume and model non-constant variance via the linear mixed model. However, this complicates the model and reduces degrees of freedom for testing hypotheses.

Use of Statistical Intervals for Quality Assurance

Based on pilot study assays of prepared menus, we will formulate statistical "tolerance intervals" for use in the detection of unusual assays obtained for menu samples during the actual study. For each nutrient assayed, this requires determining upper and a lower limits such that, for example, we have 90% confidence that 95% of future assay values will fall within the limits. In this example the confidence coefficient is $1-\alpha = 90\%$ and the proportion of future assays falling within the interval is $1-P = 95\%$. With this formulation, we anticipate that 5% of future assays will lie outside the interval. The food lab will report to the Field Centers that the corresponding food samples were "unusual". Such occurrences could be attributable to a variety of single or cumulative effects; such as, variation in nutrients in raw food resources. The food lab will also monitor assays of its "reference" materials using tolerance intervals.

The calculation of the tolerance intervals may be updated using additional data obtained during the course of the study. By so quantifying past experience we will be better able to classify assays in future studies. (Please see F.A. Graybill; 1976, page 270-275 for more details about tolerance intervals and prediction intervals.)

Contingency for Improved Statistical Methods

While we expect that established statistical methods will be adequate, it should be noted that it is, generally speaking, not difficult to generate data

for which exact statistical methods have not yet been developed. If conventional statistical methods prove inappropriate or inefficient, statisticians in the Coordinating Center will collaborate with statisticians and investigators in the Field Centers to develop and apply better procedures. They will publish appropriate expository papers explaining the methods to clinical readerships and
to other statisticians.

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CHAPTER 5

Chapter 5: RECRUITMENT INTRODUCTION

The Dietary Effects on Lipoprotein and Thrombogenic Activity study will recruit healthy men and women between the ages of 22 and 65 years of age. The study population will include 60 percent women to obtain adequate numbers of pre- and post-menopausal subjects. Pre- and post-menopausal women will be recruited in approximately equal numbers. Males will be recruited in approximately equal numbers above and below 40 years of age. Minorities will be recruited by all centers in accordance to their availability to participate. The number of African American study participants expected in the Penn State and University of Minnesota centers is quite small; therefore, the Columbia University center and the Pennington Biochemical Research Center will recruit larger numbers of African American subjects to supplement this population group (refer to Chapter 4, Table 1).

Each DELTA center has experience in recruitment and will approach the recruitment phase using sources, methods and strategies that have been most successful for them in the past. Recruitment will take place over approximately two months time and will include the use of flyers, brochures, advertisements and announcements to attract study participants. In addition, lists prepared from recruitment for previous studies, university employee directories and student and community organizations listings will provide targeted sources for mailing purposes. Recruitment will include a variety of sources to produce a wide range in participant age and cholesterol levels.

A recruitment source log will be used by the DELTA centers to record all methods utilized to recruit participants. Prospective participants will be asked to indicate where their knowledge of the study originated. This information will be recorded on their eligibility forms. Use of a recruitment ineligibility log will allow tracking of screenees by the same code should they become ineligible. This process will 1) allow for computer retrieval of information to provide ongoing information reports on the yield of various recruitment methods and sources and 2) provide the ability to generate cost comparisons based on method and yield. Information gained will be extremely valuable in the ongoing recruitment process throughout the various diet protocols and will be used among centers to target recruitment efforts to improve yield and cost effectiveness.

University of Minnesota DELTA Recruitment

I. Population to be recruited

The University of Minnesota DELTA center will recruit 24 participants who are healthy men and women ranging from 22 to 72 years of age who meet the study eligibility criteria. A special effort will be made to include 3-6 African American subjects within the study population of 24 total participants. The recruitment population will include 60 percent women with approximately equal numbers of women being of pre- and post-menopausal status.

II. Sources

Recruitment sources will include lists from previous studies, university employees, age eligible students, and the general population within the surrounding community. Although the minority population in the Twin Cities Area is not large, the University of Minnesota has been successful in recruiting from these groups. Recruitment of African American participants has been enhanced through successful history of recruitment for previous studies and the resulting development of linkages within church and community groups. Care will be taken to target recruitment toward individuals to whom such a study is feasible in an effort to reduce the overall recruitment cost and produce a yield of participants who are likely to be retained throughout the diet periods.

III. Methods and Strategies

The University of Minnesota has successfully recruited subjects for feeding studies through informational advertising on the campus. Flyers, posters, employee and student letters and advertisements in the campus newspapers and newsletters will be the methods utilized to attract interest in this feeding trial among the campus population. Recruitment of the surrounding community will involve mailings to specific zip code areas, posters in community locations and advertisements and feature articles in targeted community newspapers.

Strategies will be used to promote early identification of those individuals who are most likely to be eligible. These strategies are designed to reduce staff time and laboratory costs during the recruitment phase and to minimize study drop out rates. Individuals responding to recruitment efforts will be scheduled for a telephone

screening interview which will 1) identify the recruitment source, 2) provide a brief overview of the basic feeding study expectations and commitment and 3) screen participants who have disease conditions and/or use medications or special diets that exclude their participation.

Columbia University DELTA Recruitment

I. Population and Sources

First year recruitment will be from two pools - healthy male and female students, and healthy male and female employees. The students are aged 20-30, numbering about 200 in the first two years at the medical school, about 100 in the first two years at the dental school and about 40 nursing students. There are equal numbers of males and females in all groups except nursing students, who are 3:1 females to males. The racial makeup is 70 percent Caucasian, 10 percent African American, 10 percent Asian and 10 percent other. Male employees are approximately 500 in number in the security and engineering divisions. Almost all are African American/Hispanic and 30-50 years of age. Female employees are from any department at the medical center and number over 500 between the ages of 30-65.

II. Methods and Strategies

We will recruit students, when they return to school in August, through direct mailings (we can add a notice to the information packet to new students from the Dean's office) and posters. During the two weeks before school, when all students are moving in and being oriented, non-fasting, fingerstick cholesterol screening will be offered. This approach will provide an initial pool of about 300 students, half female. As for the employees, the directors of security as well as the director of engineering have agreed to help in recruitment. The men and women work full time at the campus and can eat one meal during work and one meal either before or after work. Many of them live near the campus. The benefits available to the participants, including free physicals and blood analyses, and the provision of free food for several months, will aid in recruitment.

Pennington Biomedical Research Center DELTA Recruitment

I. Population to be recruited

It is the intention of the PBRC Field Center to recruit a minimum of 24 participants between the ages of 22 and 72 who will meet the eligibility requirements as put forth in Chapter 6. The PBRC Field Center will set as recruitment targets a subject population consisting of 50% Caucasian and 50% African American men and women. Recruitment targets by gender will be 40% men and 60% women. Pre- and post-menopausal women will be recruited in approximately equal numbers.

II. Sources

The PBRC Field Center will recruit subjects from the following sources: PBRC Volunteer Data Base. Over the course of the last 18 months, the PBRC Clinic has received over 2,000 responses from individuals wishing to participate in ongoing Clinical studies. Approximately 750 individuals participated in on-site screenings, of which 360 were enrolled in one of 9 different clinical studies. In excess of 150 individuals have expressed a specific interest in participating in dietary studies.

Louisiana State University. The LSU campus in Baton Rouge is located within 4 miles (15 minute drive) of PBRC. The campus population is comprised of 22,000 undergraduate students, 5,000 graduate students and 5,000 employees. Many of the students and employees of LSU live in the community immediately surrounding the PBRC Field Center.

Southern University. The Southern University Campus is located approximately 20 minutes away from the PBRC Field Center. Southern University is an historically African American university and currently has an enrollment of over 9,000 students. The PBRC has an established record of interaction with Southern University.

Surrounding Baton Rouge Community. To assure adequate subject volunteers, the PBRC Field Center has cultivated a constituency among the Baton Rouge community including ties to its civic, social and religious organizations.

III. Methods and Strategies

The following methods and strategies will be employed to recruit subjects: All recruiting will be coordinated through a full-time Clinical Subject Recruiter. A Minorities Subject Recruiter will join the clinic staff this summer to facilitate recruitment in the African American Community. Advertisements describing the study will be placed in the university and community newspapers.

Publications Director, Ben Phillips, a veteran jouralist, will contact local newspapers and the radio and television stations to publicize the study and volunteer recruitment. Public Education Director, Ruth Patrick, Ph.D., performs a weekly five minute spot on a local television station on nutrition-related issues. Her presentation of research activities at the PBRC will be used to aid in subject recruitment.

Penn State University DELTA Recruitment

I. Population to be recruited

The Pennsylvania State University DELTA center will recruit 24 healthy men and women, aged 22-72 who meet the study eligilibility criteria. Three black participatns will be recruited (see Table 1, Chapter 4). The study population will include 60 percent women of which an equal number of pre- and post-menopausal women will be recruited.

II. Sources

Recruitment sources will include students (there are over 4,000 age-eligible students on the University Park campus - mostly graduate students), Penn State employees, and the general population within the surrounding community. To target postmenopausal women, we will work with the University's Office of Health Promotions (OHP). Over 1,000 women (mostly clerical staff) over the age of 50 have participated in recent health programs and have expressed interest in continued participation. We will work with the OHP's director, who indicates that this is a health-conscious and motivated group. Thus, we will recruit specifically from this pool.

III. Methods and Strategies

The Penn State DELTA center has developed effective recruitment strategies for use in previous studies; these include word-of-mouth and announcements to graduate student organizations, newspaper advertisements (both campus and local papers), newsletter articles, radio announcements and personal letters. Lists and mailing addresses of graduate students will be generated from the PSU registrar. In addition, letters will be mailed to university employees and to those who have participated in past programs held through the OHP. We plan to recruit African American subjects through various minority program offices on campus and by visiting local churches.

We will stimulate interest in the DELTA Study through newpaper, radio, and television(local network) stories. When potential subjects/participants contact us for additional information, we will send them a packet of information that provides a brief overview of the study and describes the expectations and commitment that are required of them.

CHAPTER 6

Chapter 6: ELIGIBILITY AND EXCLUSION CRITERIA

The selection of inclusion and exclusion criteria for DELTA is based on 1) a population target that includes both a wider age range and a larger sample size of women and minorities than included in previous studies; 2) medical exclusions to assure a healthy study population for whom it is safe to participate; 3) a range of cholesterol levels with a higher mean than studied in previous trials; 4) exclusion of conditions or medications that could confound or interact with lipid and/or hemostatic response, and 5) exclusion of factors which may predict poor compliance.

Inclusion Criteria

- Men and women, 22-72 years of age; median age of men, 40 years of age
- Serum cholesterol levels (TC) between the 25th and 90th percentile, adjusted for gender, age and race
- HDL-C between the 10th and 90th percentile adjusted for gender, age, and race
- Triglyceride level < 90th percentile adjusted for gender, age, and race
- Ability and willingness to comply with all aspects and commitments of the feeding study
- Women who are 6 months post-partum or post-lactation

Exclusion Criteria

- Clinical evidence of cardiovascular disease including past myocardial infarction, angina, coronary occlusion, history of coronary artery bypass and/or congestive heart failure
- Diabetes mellitus (use of insulin, oral hypoglycemic agents or a FBS >120 mg/dl)
- Hypertension (use of anti-hypertensive medication or systolic pressure > 140 or diastolic pressure > 90 average on eligibility visits one and two)
- Renal disease including history of nephritis, pyelonephritis, glomerulonephritis, and/or creatinine > 1.8
- Gastrointestinal disease including Crohn's Disease, irritable bowel syndrome, ulcerative colitis, acute ulcer, gastric resection
- Acquired Immunodeficiency Syndrome
- History of cancer within past 5 years
- History of blood clotting disorders
- History of gout requiring medication
- History of allergies or asthma requiring use of steroid medication
- Recent history of depression or mental illness (requiring use of anti-depressants, tranquilizers within the last 6 months)
- Pregnant or planning to become pregnant within next 6 months
- Given birth within last 6 months
- Lactated within last 6 months
- Use of lipid-lowering medication currently or within the last 6 months
- Use of estrogen replacement medication, currently or within the last 6 months
- Use of oral contraceptives from the leveonorgestral category, currently or within the last 6 months
- Use of thyroid medication
- Chronic use of laxatives or antacids
- Ongoing use of aspirin or aspirin containing medications
- Food allergies involving foods in the study design
- Use of special diets prescribed for medical conditions
- Weight loss or gain of > 10 pounds within the past 2 months
- Current involvement in weight loss efforts
- Refusal to discontinue use of any/all vitamin, mineral or fiber supplementation throughout the study
- Body weight > BMI of 32
- Alcohol consumption > 12 drinks/week
- Physical activity extremes including marathon running, competition sports and/or exercise training > 7 hours each week

RATIONALE FOR INCLUSION AND EXCLUSION CRITERIA:

Population Target to be Included

The DELTA study will include participants who are healthy men and women, ages 22-72 DELTA intends to include a wide age range to obtain information on lipid and hemostatic response applicable to a broader population. The study population will include 60 percent women to provide an adequate sample size of both pre- and post-menopausal women in an effort to increase the knowledge base on lipid levels and hemostatic response factors related to menstrual and menopausal status. Minorities will be recruited in approximate proportion to their representation in the local populations of each center and in accordance with their availability to participate.

Medical Exclusions

DELTA will study healthy adult men and women who are free of disease conditions and who do not pose a safety concern related to dietary intake. Criteria aimed at discovery of previously diagnosed medical conditions or indications of impending disease states have been defined. Use of medications which indicate a disease condition and laboratory parameters which are indicative of impending disease states are used as exclusions to assure a healthy population of subjects. Prospective participants who are pregnant or are planning to become pregnant during the study duration will be excluded.

Cholesterol Range for Inclusion

Total serum cholesterol levels between the 25th and 90th percentile, HDL-C between the 10th and 90th percentile and triglyceride < the 90th percentile, all adjusted for gender, age and ethnicity, have been selected for inclusion. Lipids within a higher mean range were selected to 1) promote results that will be more generalizable to groups within the U.S. population and 2) to provide for a potentially larger mean lipid response.

Conditions That Could Affect Lipid and/or Hemostatic Response

Factors that have been known to affect or that could potentially confound or interact with lipid and hemostatic response have been excluded. Current use of prescribed medication, history of use of such medication for a recurrent

condition, and recent use of estrogen replacement medication or lipid-lowering medication are cause for exclusion based on actual or probable interaction with lipid and/or hemostatic response. Obesity above a BMI of 32, gain or loss of weight in excess of ten pounds within the last two months, current involvement in weight loss efforts and physical activity extremes are included as exclusions due to the known effects on lipid levels and possible effect on hemostatic factors. Menopause without use of estrogen replacement medication will not be cause for exclusion; however, menopausal status will be assessed with a series of questions to identify women who are in pre- and post-menopausal states.

Factors That May Predict Poor Compliance

Protection of the integrity of the study design and assurance of optimal response to the various diets necessitates a high level of adherence to the daily food intake and compliance and commitment to the overall goals of a feeding trial. The apparent advantages of utilization of the crossover design are dependent upon maximum participant retention. A variety of factors were identified which may predict poor adherence to the diet or inadequate compliance to the commitment of a feeding trial. Factors identified are: high alcohol intake, excessive use of nutrient or fiber supplementation, extreme or bizarre dietary patterns and extreme levels of physical exercise.

IMPLEMENTATION:

Eligibility for DELTA will be determined in a sequential manner designed to exclude ineligible participants using minimal staff time and laboratory measures (refer to DELTA Visit Flow Chart). A prospective participant's indication of interest by telephone call or postcard return will result in the scheduling of a telephone interview.

Telephone Screening-- Telephone screening will feature a series of questions regarding medical history, medication use, interest, and other general exclusion criteria. The potential participant will be given a general overview of the study including: attendance requirements, time commitment, lab measurements and intake limitations. Based on the information obtained and the prospective participant's interest in involvement, exclusion will be completed or an Eligibility Visit 1 will be scheduled.

Eligibility Visit 1-- The first eligibility visit will involve continued screening. A self-administered questionnaire will include medical history,

medication use, alcohol use, physical activity habits and exclusion criteria. A complete explanation of the study and the participants' expected level of commitment will be presented by clinic staff. Questions will be solicited and addressed. Measurement of blood pressure, height/weight, and a rapid cholesterol screen will be completed. A Participant Agreement will be used to clarify study expectations and to allow the participant to review them with family members or significant others before making a commitment to participate. Based on the information compiled at the first eligibility visit, exclusion will be completed or a second eligibility visit will be scheduled. Potential participants will be given directions to report to the second eligibility visit in a fasted state.

Eligibility Visit 2--The second eligibility visit will begin with a review of the study explanation. Additional questions will be addressed. Blood pressure will be measured and fasted blood chemistries will be drawn. The diet run-in will be explained and the participant will be scheduled. Should the blood chemistry results produce an exclusion value, the person will be called to cancel further study involvement.

Diet Run-In-- A diet run-in phase will allow the prospective participant to experience the routine of a feeding study. The atmosphere, food palatability and intake limitations will be assessed by each participant. Weigh-ins will help with the assessment of caloric levels required for maintenance of body weight. Successful completion of the diet run-in will indicate a viable candidate. Participants who remain interested and provide informed consent will be randomized.

CHAPTER 7

Chapter 7: STUDY DESIGN

A. Design

We will examine the effects of the three selected diets on plasma lipids, apolipoproteins, and hemostasis variables by employing a randomized, double-blinded, three-period, complete crossover design. Each dietary period will be eight weeks in length. The study design is presented in Figure 1. The timetable for the study is presented in Figure 2.

Prospective subjects who meet the eligibility requirements put forth in Chapter 6 will be required to participate in a 6-day diet "run-in" period to take place during the two weeks immediately prior to randomization. The subjects will be provided with each of the three diets during this period (two days each of: Average American Diet (AAD, 16% SFA); Step 1 Diet (9% SFA); Very Low SFA Diet (VLSFA, 5% SFA)). The "run-in" period will serve to familiarize the subjects with the requirements of the study and to allow those subjects who feel that they can not tolerate the demands of the study to drop out prior to randomization. Since it is estimated that approximately 10% of the subjects will drop out during the "run-in" period, the number of prospective subjects entering this phase will be appropriately inflated.

Information regarding subjects who have successfully completed the "run-in" period, have expressed continued interest in participating in the study, and have provided informed consent, will be forwarded to the Coordinating Center. The Coordinating Center will in turn provide a randomization schedule for each Field Center. Subject randomization will provide balanced assignments with respect to gender and race to each of the six dietary sequences. Balanced assignments will occur at the level of the Field Centers (as opposed to across the entire study population) to provided each Field Center with the greatest flexibility in implementing local Ancillary Studies.

Subjects will consume each of the three experimental diets for a period of eight weeks. A eight week dietary period was chosen so that information could be gathered regarding the stabilization of both the lipoprotein and hemostasis variables and to allow menstrual cycle effects to be examined. Subjects will be provided with breaks between diet periods 1 and 2 (from November 20 - January 10) and between diet periods 2 and 3 (from March 5 - April 4). These breaks are designed to provide subjects relief from the demands of the protocol. This is deemed particularly important during the period immediately surrounding Christmas and New Year's Day when problems with subject compliance might be anticipated.
The total length of the study, including the "run-in" period, dietary sequences, and breaks will be approximately 24 weeks.

FIGURE 1

FIGURE 2

B. Diet Protocol

Subjects will be provided with all foods to be consumed during each dietary period. Each center will attempt to provide a minium of 10 meals eaten on site each week including 5 dinners at the designated Field Center dining facility. Weekday packaged meals will be distributed at the previous meal eaten on site. Evening snacks will be distributed at dinner. Weekend meals will be packaged and distributed at the Friday dinner. Cookies or muffins meeting the nutrient specifications of each diet (unit foods) will be provided for discretionary intake. Subjects will record on forms any signs of illness, medication use, the self-selected food items, and any deviations from the diet.

Subjects will not be told of their dietary group assignments. All experimental diets will be similar in overall appearance, smell and taste. Dietary energy adjustments will be made as needed to maintain weight. Each study diet will be prepared at five energy levels (1500, 2000, 2500, 3000, and 3500 kcal/day). Fine tuning of the calories can be accomplished with unit foods. Subjects will be started at the energy level that will most closely match their estimated energy requirement. Body weight (without shoes, jackets or heavy sweaters) will be measured twice weekly prior to the Monday and Thursday breakfast. If a subject's weight varies beyond 1kg of its initial value, the subject will be switched to another energy level until the weight returns to within 1kg of the initial value.

C. Schedule of Measurements

The schedule of measurements is shown in Figure 3. Changes in body weight, compliance to the dietary protocol, and the presence of illness or side effects will be assessed each week for the duration of each dietary period. In men and post-menopausal women, fasting blood samples will be obtained from each subject for endpoint determinations (total cholesterol, HDL cholesterol, triglycerides, apoA-I, apoB, Lp(a), factor VII, fibrinogen, and plasminogen activator inhibitor-I) once during weeks 5, 6, 7, and 8. In pre-menopausal women, fasting blood samples will be obtained for endpoint determinations once during weeks 5, 6, 7, and 8 and an additional sample, timed to coincide with the midpoint of the menstrual cycle, will be obtained once during weeks 6, 7 or 8. A schedule of determinations made at 1 week intervals will allow us to estimate at what time point the measured parameters will have reached a new steady state during each dietary sequence. This information will be used in the development of subsequent dietary protocols.

Due to the funding of an Administrative Supplement for Research on Women's Health Issues, an extended sampling schedule will be implemented in pre-menopausal women (Figure 4). This sampling schedule consists of two fasting blood samples for endpoint determinations obtained during weeks 5, 6, 7 and 8. These additional samples will be

used to further define the effects of the menstrual cycle on the endpoint measurements and to assist in developing sampling strategies to be used in dietary studies involving pre-menopausal women.

Additional blood samples or other assessments may be asked of the subjects as part of their participation in Field Center Ancillary Studies. The timing of these additional assessments will be chosen so as not to interfere with the primary endpoint measurements.

D. Study Close-out

Upon completion of the diet-phase, collected samples will forwarded to the designated Central Laboratories for analysis. Laboratory analysis will be carried out without knowledge of the diet sequence an individual has received. All laboratory and clinic data will be forwarded to the Coordinating Center for statistical analysis.

FIGURE 3

FIGURE 4

CHAPTER 8

Chapter 8: Diet Specifications

We will assess the plasma lipid/lipoprotein and hemostatic responses to the following diets:

	Average American Diet "A"	Step-One Diet "B"	Very Low SFA Diet "C"
FAT, % Calories	37	30	26
SFA, % Calories	16	9	5
MUFA, % Calories	14	14	14
PUFA, % Calories	7	7	7
Cholesterol (mg)	300	300	300

The Average American Diet (A) represents a typical U.S. diet. For men, the level of cholesterol is slightly lower than the national average (which is ~465 mg). For women, however, the amount provided is similar to the national average. The Step-One Diet is lower in saturated fatty acids and total fat. The distribution of MUFA and PUFA and the amount of cholesterol in the diet are similar to Diet A. Thus, we are able to examine the plasma lipid/lipoprotein and hemostatic effects of saturated fatty acids. Diet C is designed to examine the plasma lipid/lipoprotein and hemostatic effects of a marked reduction in dietary SFA. Since MUFA and PUFA (as % of Calories) are held constant, the total fat content of the diet is reduced to 26% of Calories (by virtue of a reduction in SFA). Diet C does not meet the definition of a Step-Two Diet because it provides 300 mg (versus < 200 mg) of cholesterol. In keeping the amount of cholesterol constant, and the distribution of MUFA and PUFA constant in the test diets, this experimental design will enable us principally to examine the plasma lipid/lipoprotein and hemostatic responses to manipulations in SFA (and total fat) in the diet. We will maintain similar ratio of long chain saturated fatty acids in the experimental diets.

Working Definitions

Diet: This term is used to describe the experimental diets that will be fed to subjects. It is defined by the macronutrient composition and levels of fatty acid classes and cholesterol. Each experimental diet will meet the nutrient criteria specified but will vary in energy (to meet the different calorie needs of the subjects).

Food List: A listing of individual foods that comprises a menu. Four 1-day food lists will be developed. Slight modifications will be made in some of the food lists such that 2 highly palatable menus can be developed from one food list. Thus, most foods on a food list will appear on each of the 2-day menus that is developed from one food list. Only a few foods from the food list will appear on just one of the 2-day menus.

Menu: A description of foods served at a meal. Menus refer to descriptions of a single meal, or meals for one or more days. From each 1-day food list, 2 "different" menus will be developed (see below).

Meal: Food served/eaten at breakfast, lunch, dinner, and snack

Menu Cycle: The period of time within which a complete set of menus is served. A 6-day menu cycle will be used in the DELTA study. Each cycle will be developed from a 3-day food list. Thus, a one-day food list will be used to develop a 2-day menu.

Unit Food: A food such as a muffin that is formulated to have the same ratio of control nutrients to calories. It can be eaten as desired by the subject, to meet his or her energy needs, without altering the composition of the diet.

Code: A single meal could have an 8 digit code designating the Diet (A,B,C), period (1,2,3) cycle (0.5 - 6), list (1 - 4), menu (a, b) calorie level (15, 20, 25, 30) and meal (B,L,D,S) (see figure 1).

Menus

Standard menus will be developed and pilot-tested for acceptability during the summer of 1993. The menus will meet the specifications of the experimental diets (defined above) and also be nutritionally adequate (>90% of the RDA for all nutrients). The menus will be developed to provide tasty and appetizing foods that are both culturally and regionally acceptable by subjects at all Field Centers.

Dietitians at each field center developed 4 menus that met the specifications of experimental Diet C which is the most restricted in fat. Members of the Diet Subcommittee reviewed the menus and selected 4 menus (based on perceived acceptability, and procurement, preparation and cost considerations). Food lists from each menu were used to develop a second menu (that includes virtually all of the foods listed). Thus, the menu cycle will consist of four food lists from which 2 menus were developed from each food list to provide 8 variants in each cycle. This approach for menu development was selected to minimize the nutrient variability of the experimental diets while providing additional variety to help maximize compliance with the experimental diets. An example of how the 2-day "companion" menus will be developed is as follows: on day 1, dinner might be a baked chicken breast, rice and mixed vegetables, whereas on day 5, a chicken stir-fry could be served. Diet Subcommittee members will select 3 Food Lists and 6 menus for the study from the 8 menus (and 4

Food Lists) that were validated on the basis of nutrient profile, production effort, cost, and perceived subject acceptability.

FIGURE 1

Since a blinded experimental design is proposed, after the menus are developed for the diet C, they will be modified to meet the specifications of Diets A and B. Therefore, the menus for all experimental diets will be similar in appearance.

All weekend meals (Saturday and Sunday breakfast, lunch, dinner, and snacks) will be packed by the food production staff at each field center for the subjects and sent home with them after dinner on Friday. To simplify food production efforts, the weekend menus have been planned using frozen entreés from Campbell Soup Company. Four one-day weekend menus have been developed. Thus, each one day weekend menu will be served four times during each 8 week feeding period.

Food Preparation and Procurement Procedures

To minimize nutrient variability of the primary nutrients of concern (i.e., fatty acids), all major sources of fat in the experimental diets will be procured centrally. These foods include meat, fish, and poultry, fats (e.g., margarine) and oils, dairy products except fluid skim milk, bread and other grain products. Each Field Center will identify a local dairy that provides skim milk with $<0.5\%$ fat and whole milk with 3.3% fat. Fruits and vegetables will be procured locally and centrally (e.g., frozen, canned, and possibly fresh fruits and vegetables). Food will be purchased, prepared, and stored according to standard procedures.

Food Safety Procedures

Standard procedures for safe procurement, preparation, storage, and shipment/transport of food will be followed by all Field Centers. All staff who prepare and serve food as well as the subjects who will be taking food home or eating it away from the Field Centers' dining facilities will be instructed on relevant safety procedures.

Specific guidelines for preventing food-borne illness will be provided. Bacteria of concern include Salmonella, Campylobacter jejuni, Staphylococcus aureus, Clostridium perfringens, and Shigella. Written materials and food safety lectures/classes will focus on temperature control and cleanliness.

Temperature control issues

Standard procedures for food procurement and storage will be followed by all Field Centers to prevent biological, chemical and physical changes that cause spoilage and contamination of food. Perishable foods will be stored in clean refrigerators or freezers at 34-40° F or 0° F, respectively. Foods will be wrapped appropriately and

stored to ensure adequate air circulation. Cooked and raw foods will be stored separately and fresh meat, fish and poultry will be stored in a manner that prevents contamination of other foods. Frozen meat, poultry and fish will be thawed in the refrigerator. Perishable foods will be kept cold until they are used. After cooking/preparation, food will be served immediately or stored in the refrigerator or freezer.

Meals that are eaten off-site will be packed in insulated bags and coolers. Perishable foods will be cold or frozen before they are packed.

Cooking temperatures for meat, fish, and poultry will be specified. Procedures for cooking eggs to destroy salmonella will be used. Oven temperatures for cooking all foods will be defined as will microwave cooking times.

Sanitation practices

Counter tops, utensils, cutting boards, sinks, and any other surface that comes in contact with food will be washed with hot soapy water before and after food preparation and especially when meat, fish, and poultry are prepared. Employees will wash hands before and during meal preparation and avoid preparing and serving food when ill, especially with a diarrheal illness or open wound.

Handouts that describe food safety procedures will be given to all employees who handle food (these are included in Appendix G). In addition, subjects will be given relevant food safety guidelines for transporting, storing, and preparing meals eaten away from the Field Center dining facility.

Calibrating Oven, Stove, Microwave, Freezer, and Refrigerator Temperatures

Ovens at each Field Center will be calibrated. For all ovens used, the following information will be collected:

a) time required for oven to reach specified operating temperature for all temperatures that will be used to prepare foods for the **DELTA** Study

b) oven temperatures in each quadrant for all temperatures that will be used

c) oven temperature stability

Stove top temperatures will be standardized among Field Centers by specifying the internal temperature of the cooked product and the time required to achieve it.

To standardize microwave cooking temperatures, the size of the microwave (cooking space), its cooking power and the cooking time as well as the cooking utensil and amount of food cooked each time will be specified.

Refrigerator and freezer temperatures will be checked regularly. The temperature for each shelf and drawer will be monitored. Field Centers will follow specific procedures for cooling/freezing hot foods in the refrigerator and/or freezer. Each Field Center will rotate foods as scheduled.

Microwave performance will be checked regularly. FALCC will document power output of microwaves. Each Field Center will provide FALCC with brand names and model numbers.

FOOD ANALYSIS

The primary focus of the food analysis component of **DELTA** will be to standardize the diet across field centers so that the experimental diets are sufficiently comparable to be considered identical treatments. This constitutes a research project in its own right and provides unusual scientific opportunities in the area of food composition and analysis. We divide this research component into two areas. (1) research in methods to improve the quality and efficiency of food assays. (2) validation and characterization of diets including partitioning the variance in components in food.

RESEARCH IN METHODS

In many cases the existing methodology is flawed by reasons of lack of accuracy, lack of precision, high assay costs, poor or lacking analytical quality control procedures and lack of validation for use with composited whole diets. Protocols will be developed and tested for the computation of results for each analyte, and for the reporting of analytical diet data to the PIs and the scientific community. The long term gain of this research will be to provide higher quality nutrient composition data which will benefit the nutrition research community at large.

The following components will be assayed in diets for Diet Protocol One: total fat, cholesterol, moisture, starch, total protein, fatty acids (saturated, monounsaturated, polyunsaturated, omega-3), total dietary fiber, and sugars. Calories will be calculated from the total fat, starch, sugars, and total protein contents of the individual diets.

1. **SAMPLING, STORAGE, and SHIPPING PROTOCOLS**: Development and testing of protocols for sampling of the diets prepared at each field center, protocols for shipments of diet samples from Field Centers to the FALCC,

compositing of the diet samples at the FALCC, storage of diet samples, reference materials, and standards at the FALCC, development of internal FALCC protocols for analytical databases, computation of results, and transmission of results to Coordinating Center.

2. **ASSAY METHODOLOGY**: Selection, testing, modification, and validation of analytical methods for the assay of total fat, cholesterol, moisture, starch, total protein, fatty acids (saturated, monounsaturated, polyunsaturated, omega-3), total dietary fiber, and sugars. Significant effort will be given to the acquisition of validated low cost methods that have adequate accuracy, precision, and analytical quality control components.

The assay methods shown in appendix E of this protocol will be the first candidates for the assay of the critical nutrients. The criteria for acceptable methodology for each analyte are shown in Table 2.

Table 2: Acceptable Analytical Methodology Study 1

Component	Limit of Detection gm/100 g wet	Working Range gm/100 g wet	Precision (Current)	Cost/sample
Total Fat	0.14	2.3 - 5.1	2.5%	
SFA	0.058	0.8 - 1.3	5%	
MUFA	0.164	1.3 - 2.0	5%	
PUFA	0.088	0.6 - 1.0	5%	
Omega-3 FA				
Cholesterol	0.0015	0.0012 - 0.0018	5%	
Sugars	0.04	4.2 - 6.3	5%	
Starch	0.06	6.3 - 9.5	5%	
Total Protein (Nitrogen)	0.06	0.5 - 0.8	5%	
Total Dietary Fiber	0.01	1.0 - 1.6	5%	

The Coordinating Center will carry out a multi-stage program aimed at: (1) compiling a research database for designing future studies; (2) certifying that the experimental diets meet target values; and, (3) providing an ongoing quality assurance program to maintain the integrity of the dietary data.

RESEARCH IN DIETARY VALIDATION AND CONTROL

This phase will be aimed at answering the following questions:

1. What is the actual composition of the diet that is being fed with respect to the target values established for the experimental diets?

2. What is the variance in the nutrients of interest in this study, including nutrients that are controlled and food components that are monitored?

3. What are the sources of variance in the diet composition? Sources of variance that will be examined include daily variation (menu to menu),

market turnover seasonal variation, inter-center variation, preparation variation, assay variation, and calorie level.

4. How valid are current food composition databases in estimating food components of interest in this and future studies in this program?

These research objectives will be met in both short term pilot studies and long term sampling of the diets during the course of the first study.

PILOT STUDY

We performed the first pilot study to answer three questions:

1. How close to target values can the field centers prepare diets using standard procedures, and centrally purchased fats and fat containing foodstuffs?

2. Does central preparation of fat containing foodstuffs offer any advantages in controlling the composition of the diet? Are there disadvantages in acceptability and/or cost.

3. What is the intra and inter-center variance in nutrients of interest in this study?

This pilot study gives preliminary information on how well the centers perform on a series of 3 test menus repeated 3 times with 2 options of delivery. Preliminary results indicate that the centers performed well with respect to the target value for fat. Descriptive statistics for the first cycle of 3 menus is contained in the Appendix F.

VALIDATION

The next phase of the research will be carried out in the context of validating the actual experimental diets that will be used in this first protocol. Eight menus will be generated from four food lists. These menus will be the same for each diet except for added fat to maintain blinding. The same set of 8 menus will be rotated through the entire study.

A Flow chart showing the sequence of activities and a time line are shown in figure 2.

Each center will prepare the 10 menu series for 2 experimental diets or 1 diet and unit foods for all three diets. Thus there will be two samples (prepared at different centers) for each set of menus and unit foods. In addition, each center will prepare an

extra serving of fat containing foods. These will be designated on each menu. A sample of each centrally procured fat will also be archived at the FALCC. Each food sample will be frozen and shipped frozen to FALCC where the samples will be composited, aliquoted, archived and assayed. The initial assay will be for fat and moisture. If the samples are within acceptable range of target values then the foods will be assayed for fatty acids, cholesterol, starch, sugars and nitrogen.

If discrepancies in the nutrient levels occur then actual analysis of the individual foods will be required to find the problem foods (starting with foods thought to be primary sources of the food components which show discrepancies). The data on the individual foods will then be used to reformulate the diets based on the analytical data and finally the new, reformulated diets will be assayed to ensure that the appropriate levels of the key nutrients were present. Some iterations of the process may be necessary.

This validation process, in addition to standardizing the experimental diets will provide important information on the validity of several nutrient databases. It will provide information on inter and intra-center variance over a limited period of time. It will not provide information on season as a source of variability, hence it will not provide information to characterize the average composition of the diet over the entire study period.

CHARACTERIZATION OF THE STUDY DIETS

In order to obtain information on seasonal variability in the diet (which we expect to be different in different regions), we propose an ongoing sampling of the experimental diets during the course of the study. The daily menus would be composited into 8 day menu cycles. This 8 day cycle constitutes the unit of the diet, which would be assayed. Aliquots would be archived for future studies. These aliquots will be a valuable resource, not only for this study, but for future studies as well. The archiving of diet cycle composites of each menu of each diet cycle for each experimental diet at each Field Center provides a key resource to the **DELTA** participants to: 1. Recheck diet composites for cases where the outcome measurements are unexpected, 2. Validate new and/or alternate analytical methods for the assay of **DELTA** nutrients, 3. Do retrospective studies on **DELTA** outcomes for nutrients not on the original assays protocols, 4. Evaluate nutrient data bases for nutrients not studied in the first **DELTA** feeding study, and 5. Obtain data for designing other dietary intervention studies.

This latter research project would be carried out in the context of a quality assurance program to ensure the integrity of the study diets. A tentative sampling plan is described below, subject to modification after statistical design review. Acceptable ranges of variation for each nutrient will also be established following the validation study.

Objective: To partition the variance in the composition of the diets, for the following sources:

Sources:
Inter-center
Market turnover
Seasonal
Calorie level
Diet

Sampling: Each center prepares 1 menu per day according to the following schedule.

Cycle	COL	LSU	MN	PSU
0.4	-	-	-	-
1	A	B	C	A-Hi
2	B-Hi	C-Hi	A-lo	B-lo
3	C-lo	A	B	C
4	A-Hi	B-Hi	C-Hi	A-lo
5	B-lo	C-lo	A	B
6	C	A-Hi	B-Hi	C-Hi
7	A-lo	B-lo	C-lo	A
8	B	C	A-Hi	B-Hi
9	C-Hi	A-lo	B-lo	C-lo
10	A	B	C	A-Hi
11	B-Hi	C-Hi	A-lo	B-lo
12	C-lo	A	B	C
13	A-Hi	B-Hi	C-Hi	A-lo
14	B-lo	C-lo	A	B
15	C	A-Hi	B-Hi	C-Hi
16	A-lo	B-lo	C-lo	A
17	B	C	A-Hi	B-Hi
18	C-Hi	A-lo	B-lo	C-lo

Key A = 2,000 Kcal, diet A
A-Hi = 3,000 Kcal
A-lo = 1,500 Kcal

In this example, Columbia would prepare Diet A 2000 Kcal 8 menus in cycle 1. In cycle 2 Columbia would prepare Diet B, 3000 Kcal 8 menus and cycle 3, Diet C, 1500 Kcal.

Compositing: At the conclusion of each diet cycle (8 days), the collected daily menus (frozen) for each experimental diet (as described above) will be shipped to the FALCC via overnight delivery. The FALCC will provide all materials and protocols required for food collection and shipping. The FALCC will composite each daily menu into a **diet cycle composite** for each experimental diet from each Field Center. Archive samples of each diet cycle composite will be stored at -60°C for retrospective studies.

The FALCC proposal for the assays for protocol 1 is as follows:

Assays:

1. Quality assurance program: The FALCC will determine the total weight, dry weight and total fat of the **diet cycle composite** using rapid assays and a fast turn around time (5 working days from receipt of diet samples).

2. Documentation Program: For documentation purposes The FALCC will assay **diet cycle composites** for total weight, moisture, total fat, cholesterol, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), omega-3 fatty acids, protein, starch, and simple sugars. Calories will be calculated using the data from total fat, starch, sugars, and total protein.

Advantages:

1. Each field center does 1 menu per day
2. Provides rapid feedback for QA
3. Uses calorie level as a source of variation
4. Only 4 composites per week
5. Trend data available weekly for QA
6. Trend data available at 1 - 2 diet cycle intervals for routine assays.

The archiving of diet cycle composites of each menu of each diet cycle for each experimental diet at each Field Center provides a key resource to the DELTA participants to: 1. Recheck diet composites for cases where the outcome measurements are unexpected, 2. Validate new and/or alternate analytical methods for the assay of DELTA nutrients, 3. Do retrospective studies on DELTA outcomes for nutrients not on the original assay protocols, 4. Evaluate nutrient data bases for nutrients not studied in the first DELTA feeding study, and 5. Obtain data for designing other dietary intervention studies.

ANALYTICAL METHODOLOGY RESEARCH

The goal of this research is to develop and/or validate methods for acceptable assays for the nutrients needed for DELTA Protocol 1. The DELTA Protocol 1 nutrients include

assay values for total fat, cholesterol, moisture, starch, total protein, fatty acids (saturated, monounsaturated, polyunsaturated, omega-3), total dietary fiber, and sugars. The DELTA Protocol 1 calculated nutrients include total calories and percent calories from SFA, MUFA, and PUFA. While the current stage of development of the assay and/or calculation of these nutrients does not permit the estimation of the assay cost on a per sample basis at this time, one of the goals for the research of the FALCC is to have sufficient information to develop the protocols such that accurate cost estimates on a per sample basis can be developed. The methodology research for the first year will consist of development and/or validation of the methodology for food collection and shipping, the methodology for menu compositing, the methodology for the assay of the key nutrients for Protocol 1, the methodology for data handling and computation, the methodology for assay quality control and quality assurance. A brief summary of the current status of each of these areas is provided in Appendix E.

STUDIES ON THE COMPOSITION OF INDIVIDUAL FOODS

As was noted in the FALCC response to the RFA there are nutrients for which there are almost no data (e.g., individual sugars) and there are foods which appear to be major sources of components of interest for which there are inadequate amounts of composition data. Thus, in those cases where this lack of data prevents adequate computation of a diet composition some studies will be needed to determine the levels (and variances) of such components in foods. Such studies have the potential of "getting out of hand" and they should be limited to suspected major sources of given nutrient in foods that are being considered for inclusion in the experimental diets.

CHAPTER 9

CHAPTER 9: ADHEREHCE AND PARTICIPANT RETENTION

Assessment of Compliance

Our ability to insure that participants eat all of the food that we provide and do not eat other foods is clearly the key to successful completion of any proposed studies. We will monitor dietary compliance using several different procedures. We will feed a minimum of 10 meals (5 days/week) on site. Complaince will be assessed by a simple tray inspection. A brief compliance form will be completed for each subject after each meal. In addition to brief compliance forms completed at each meal, subjects will be required to keep a record of any other foods they may eat during the week and all food they eat during the weekend (which includes all food we provide). Nutritionists and staff will interact with subjects almost daily and ask casually about dietary compliance (to reinforce the importance of strict dietary compliance and to be of assistance if any subject is having problems adhering to the experimental diet), and provide informal feedback on the participants' weekend food records. At the end of the study, we will administer an anonymous questionnaire to assess overall dietary compliance and validate the objective (food record) and subjective (nutritionists impressions) data.

Body weight measurements will be used to assess overall dietary compliance. Taken at least twice weekly (Monday and Thursday) at the same time every time (before dinner), they will provide important information about gross deviations from the experimental diet. Since weight will be taken on Monday morning routinely, we will have some assessment of dietary compliance during the weekend. Since there are no sensitive biochemical measures of dietary compliance, we will assess adherence to the diet only by the methods described: tray check, food records, brief discussions with subjects, body weight measurements taken twice weekly and an anonymous questionnaire administered at the end of each experimental diet period. Since we will screen potential subjects very carefully, have a run-in experimental diet period and implement strategies to promote compliance (see below), it has been our collective experience that dietary compliance will be excellent.

Strategies to Facilitate Compliance and Retention

To recruit and retain compliant subjects from all segments of the population, we propose a strategy based on careful subject selection, subject and staff education, minimization of inconvenience, comfortable dining facilities and good food, and incentives as outlined below:

- We will assess potential subjects willingness to comply with the experimental diet. Subjects will review the 6-day cycle menu and tell the interviewer if he/she can follow the experimental diets for the duration of the study. We will select subjects who are stable and able to complete the entire study.

- Subjects will be educated about the objectives and design of the diet study and its importance to the American public. Investigators will be available to the subjects for discussion concerning the study, the protocols or other issues at least twice a week during mealtime.

- Efforts will be made to specifically recruit married couples or couples in a stable relationship. Other subjects will be paired in a buddy system to help develop group support. Since the experimental diets will be randomly assigned and since it is possible (and quite likely) that "partners" will have different test diet assignments, all foods that are eaten away from each centers' dining facility will be packaged separately for individual subjects and coded. This procedure will help prevent unintentional deviations from the experimental diet.

- Subjects always will have access to the project staff. Staff members' telephone numbers (home and office) will be given to the subjects. They will be encouraged to call at any time. Staff will provide feedback to the Clinical Coordinator and Principal Investigator and alert them to problems.

- A run-in period will be employed to allow the potential subjects to experience the conditions of the dietary study. At the end of the run-in period, subjects will be carefully re-evaluated to ascertain their commitment to the study and willingness to comply.

- Staff at all centers will work closely with the subjects. They will be concerned and committed individuals who will be able to answer questions about the project, reinforce the importance of adherence to all aspects of the study protocol and be of assistance to subjects when necessary.

- The dining facilities at all centers are pleasant, comfortable, accessible and furnished to encourage group discussions and friendly interactions.

- A staff member will be available to subjects at all times (at the Field Center during business hours and when meals are served at the dining facility and by telephone at all other times). The staff member will deal with any issues that arise during the course of the study that pertain to subjects' ongoing participation. Staff will deal with any problems and provide emergency replacement of any food items when necessary.

- Subjects will be allowed one sel-selected meal per weekend according to guidelines established to maintain a low-fat diet. The advisability of icnreasing the number of self-selected meals to two per weekend will be evaluated if it is deemed to be criticial to continued retention in the study.

Incentives

All Field Centers have planned various activities and will provide incentives to keep both subject and staff morale high throughout the study. In additions to subjects receiving financial compensation for their efforts, strategies such as providing study T-shirts or mugs, developing a newsletter, giving awards and/or prizes to subjects and staff to recognize a special event, games (e.g., Guess the Superbowl winner and score) will be employed.

Other activities include showing movies each weekend, holding "Casino" night or other fun theme activity, and planning games that offer small prizes (e.g., movie tickets, coffee mugs, T-shirts). Birthdays and other special events and holidays will be "celebrated."
Special theme dinners are occasionally held with accompanying dinner music and decorations. Subjects are invited to bring guests on these special nights, and occasionally at other times.

To promote retention, subjects may receive financial incentives for their participation. Plans will vary between centers.

Additional Activities That Will Be Conducted at Each Field Center

Columbia

Field Center staff will print a newsletter for participants every 2 to 3 weeks during the study. This newsletter will answer questions about the study and address problems that arise. Columbia investigators report that a newsletter for subjects is not only a source of information but it establishes good communications. More importantly, it instills pride and ownership in the study.

Minnesota

Field Center staff will seat subjects in small groups of 3 to 4 in a centrally located dining facility. This facility is pleasant and comfortable and will contribute to the development of close friendships among the subjects. Their suggestions and comments about the study will be solicited making them partners in research.

Pennington

Field Center staff at the Pennington Biomedical Research Center also will develop a study newsletter. In addition, they plan to organize social activities for participants to help maintain group morale. If necessary, they will establish a satellite dining facility to

minimize any inconvenience to their subjects. In addition, investigators will provide free-of-charge meals on-site for guests and children living at home with the subjects.

Pennsylvania State

Field Center staff will establish a research partnership with study participants by continually soliciting input on a number of issues (e.g., the meals, snacks, weekend entertainment, etc.). Penn State nutrition students will visit subjects during mealtimes and discuss the study. This activity establishes a sense of ownership in the study among the subjects. Field Center staff also will plan social events and present special awards to subjects on an ongoing basis.

CHAPTER 10

Chapter 10: CLINICAL LABORATORY PROTOCOL

Table of Contents

CLINICAL LABORATORY MEASURES

I. OVERVIEW

A. Introduction

The Delta Study will examine the relationship between the levels of various substances in the blood as well as genes found in blood cells, and diets containing various combinations of fats, carbohydrates, etc. The blood samples will be extensively analyzed initially; storage samples will have further studies performed as new hypotheses and assays come forth. Because the blood samples collected are the foundation for all these tests, both present and future, the proper collection, processing, and storage of these samples is a crucial phase of the entire study. If the blood sample is not correctly drawn and processed, the laboratory results may not be precise or valid. Thus, the research assistant/research nurses who perform the blood drawing and sample processing must be well-trained, competent at drawing and processing the blood, and highly conscientious about the quality of their work.

The DELTA Study requires this considerable effort to standardize blood collection, processing, and storage, since it involves four U.S. field centers and at least five laboratories. If Columbia University and the Stanford/Taiwan group are successful in obtaining support for their collaboration, there will be a fifth field center in Taiwan and an additional laboratory at Stanford. This protocol is necessitated by the importance of all sample collection procedures being feasible and of high quality in order to meet all the investigators' needs. This protocol is derived, in part, from that developed by Russell Tracy, Ph.D., for the Cardiovascular Health Study, for which the University of Vermont serves as central laboratory.

B. Laboratory Organization

1. Description

The laboratories involved in the protocol will consist of those affiliated with the field centers, including Columbia University (CU), Louisianna State University (LSU), Pennsylvania State University/The Mary Imogene Bassett Research Institute (MIBH), Stanford University/Taiwan (SU), and the University of Minnesota (UM), as well as the central coagulation laboratory at the University of Vermont (UV) (Russell Tracy, Ph.D.). For the initial protocol, the following laboratories will perform the following analyses.

	LABORATORY					
ANALYTE	CU	LSU	MIBH	SU	UM	UV
Baseline Chemistry	x	x	x	x	x	
Baseline TSH	x	x	x	x	x	
Baseline Hematology	x	x	x	x	x	
	LABORATORY					

ANALYTE	CU	LSU	MIBH	SU	UM	UV
Fasting Lipid Profile	x	x	x	x	x	
Apoprotein A1			x			
Apoprotein B			x			
Lipoprotein (a)			x			
Baseline Apo E Genotype			x			
Fibrinogen						x
Factor VII						x
PAI-1						x

2. Rationale for Organization

a. Use of field laboratories for lipoprotein profiles.

The rationale for the use of field center laboratories for the analyses of fasting lipid profiles deals with scientific, logistic, and financial issues. The alternative would be to use a central laboratory for this function.

First, the scientific rationale rests with the availability of a program to standardize measurements of all of these laboratories. The Centers for Disease Control Lipid Standardization Program has provided this service for NHLBI-supported laboratories for decades. Two of the laboratories (MIBH and UM) currently participate in this program. This provides the opportunity to decentralize the laboratories while developing and monitoring the program to minimize variance within precision goals.

Second, the effort of shipping these additional bloods to a centralized laboratory is alleviated. Local analyses must be performed for safety purposes (hematology, chemistry, TSH) so that local distribution of samples must be performed in any case. These will not require any standardization procedures.

Finally, most field center budgets have, as part of the personnel budget, support persons to do the sample collection, aliquoting, and storage. These personnel are needed regardless of where the analyses take place. The preservation of some laboratory charges at the field center allows the support for these essential personnel.

b. Pilot testing of field centers.

Prior to the acceptance of a field center's ability to analyze its own lipid profiles, it must meet certification criteria of the CDC program. This will entail sending out blinded reference samples to all laboratories to be performed on four consecutive days (Appendix E). The results of these analyses will be compared between centers to assure that precision and accuracy are at acceptable levels; i.e., those which allow the study sample size to test major study hypotheses.

c. Selection of central laboratories for other analytes.

The apolipoproteins (apo A-1, B, Lp (a)), apo-E genotypes, and coagulation factors (fibrinogen, factor VII, and PAI-1) currently have no standardization programs available to provide laboratory standards. Thus, the optimal way to control precision and accuracy is to use a central laboratory with recognized experience and methods. These laboratories were selected by the principal investigators from the field centers and coordinating centers, and program officers, after submission of competitive bids, based on precision, experience, and cost.

C. Rationale for Analytes Selected.

1. Fasting lipoprotein profile.

The levels of total cholesterol, HDL cholesterol, and triglycerides are analyzed in a standard fashion and constitute the analytes which are recognized as the serum lipids with greatest prediction of cardiovascular risk. LDL cholesterol is calculated using the Friedewald formula (LDL-C = TC-HDL-TG/5) because of a close correlation between LDL cholesterol measured directly using ulracentrifugation methods (in persons with triglyceride levels less than 400 mg per deciliter), and is much less costly than the laborious methods requiring ultracentrifugation. Because of the inclusion of triglycerides in the formula, all samples must be collected after an 8-hour fast.

2. Apolipoprotein A-1.

Apolipoprotein A-1 is the major protein constituent of HDL and is suggested to be a better predictor of cardiovascular risk than HDL cholesterol, in some studies. It also serves as the co-factor in the lecithin cholesterol acyl transferase (LCAT) reaction. The observation that dietary components affect apo A-1 levels may provide insight into mechanisms of actions specific to dietary components.

3. Apolipoprotein B.

Apolipoprotein B is the protein component of LDL and serves to recognize the LDL-receptor, allowing LDL particle uptake by cells. It has also been observed to be a better predictor of coronary disease than LDL-C in some studies. Low LDL cholesterol to apoprotein B ratio suggests the presence of small, dense LDL particles, which could be measured in subsequent studies.

4. Lipoprotein (a) (Lp(a)).

Lipoprotein (a) is structurally similar to LDL, but with a covalently bound protein, apo(a), which has considerable homology with plasminogen. It is proposed to be a link between atherosclerosis and thrombosis. In fact, a number of cross-sectional and case-control studies have shown elevations in Lp(a) levels to be associated with clinical coronary, cerebrovascular, and peripheral vascular diseases. Relationships to dietary factors have been poorly studied, with a suggestion that trans fatty acids may elevate Lp(a) levels.

5. Apo-E genotypes.

The hepatic E receptor binds chylomicron and VLDL remnants. People homozygous for the E-2 phenotype have reduced clearance of chylomicron and VLDL remnants. People homozygous for the E-2 phenotype have reduced clearance of chylomicron and VLDL remnants, resulting in some persons with dysbetalipoproteinemia (Type III). Three isoforms are recognized, E_2, E_3, and E_4. While patients with homozygous phenotypes (E_2/E_2) are recognized to have the above-described lipid abnormality, the E_4/E_4 homozygotes may also be associated with increased cholesterol levels, and it has been suggested to be associated with dietary responsiveness to fats and cholesterol. Thus, the phenotyping of each person may be important in describing the response to specific diets. Genotyping is proposed as a cheaper and more accurate method, which, while recently available, provides a clear improvement over the old isoelectric focusing methods. This needs to be done only once in each participating subject's white blood cell (buffy coat) DNA.

6. Fibrinogen.

Concerning prospective cardiovascular risk, the major risk factor in the hemostasis area is fibrinogen. Northwick Park, Munster, ARIC, and the CHS studies have shown in large cohorts that fibrinogen is related to a variety of other risk factors, including smoking, race, white cell count, diabetes, and negatively, with HDL cholesterol. Northwick Park, Framingham, Munster, Goteborg, Leigh, Caerphilly, and Speedwell have <u>all</u> shown fibrinogen to be an independent, prospective risk factor for CVD. There is little evidence to link fibrinogen plasma levels to diet directly, but several arguments may be made for a potential indirect linkage. One of the key questions is: Is elevated fibrinogen the result of atherosclerosis and ongoing CVD, or a cause? While there is some genetic control of fibrinogen, it is primarily driven by environmental influences, including inflammation associated with acute or chronic conditions. Once elevated, it may cause increased atherosclerosis or thrombosis via one or several pathways: Increased fibrin formation per unit thrombin generation, increased platelet crosslinking, increased platelet viscosity, or decreased rate of fibrinolysis. All mechanisms have been observed in vitro, but the true in vivo story remains unclear. Since the general process of atherosclerosis is diet-related, then an intriguing hypothesis is that by altering diet, one may alter "inflammation" associated with atherogenesis which may, in turn, alter fibrinogen levels.

7. Factor VII.

Factor VII has been related to cardiovascular disease cross-sectionally in a large number of studies, although not all (ARIC and CHS). There is only one prospective study which lists Factor VII as a risk factor, Northwick Park. The second large study is Procam, and they have recently shown that there was a difference in baseline mean Factor VII values between those who developed CHD and those who did not, although this was not statistically significant. Factor VII has a certain genetic control, but this has not been addressed carefully. There is a well-recognized relationship between Factor VII activity and diet, and if major changes are made in the diet concerning fat level, there are clear changes in the Factor VII level in plasma, even in short-term experiments (two weeks or less). However, it remains unclear whether or not small dietary changes are a significant influence on Factor VII.

8. PAI-1.

Plasminogen activator inhibitor-1 (PAI-1), a major plasma regulator of fibrinolysis, is associated with plasma lipids, especially triglycerides, and has been shown to be elevated in those with prevalent CVD. It is also elevated in those who go on to have a second myocardial infarction compared to those who do not. Other than that, there are no prospective data concerning PAI-1, but several studies are ongoing at the present time. PAI-1 is elevated in diabetics and some have proposed that this is due directly to plasma insulin. However, clinical studies have failed to reveal an acute response to insulin, so the insulin connection is currently in question. A second candidate is the plasma triglyceride level, since this is responsive to insulin to a certain extent, and might provide the link to insulin seen cross-sectionally. Since there is little known about the influence of diet on PAI-1, the studies proposed here may shed some light on this issue. PAI-1 levels appear to have a diurnal cycle, requiring the collection of samples between 7 and 10 a.m.

9. Baseline chemistry and TSH.

Liver function tests (AST, ALT), renal profile (BUN, creatinine), glucose, and thyroid stimulating hormone (TSH) are performed at baseline to identify individuals with conditions which could modify dietary responsiveness to fats and cholesterol. Individuals who have these conditions should not participate in the study for both scientific and safety reasons.

10. Hematology.

Hematocrit, hemoglobin, and white blood cell count should be monitored at baseline to assure that the subject will not suffer untoward effects from serial phlebotomy.

11. Other analytes considered.

Additional analytes considered include glucose and insulin, platelet factor 4, and beta thromboglobulin, and a number of other coagulation parameters. For Study I, it was decided that the protocol for laboratory analyses would be rather simple. However, a large number of aliquots of serum plasma and buffy-coat cells will be carefully stored, to allow later analyses. Similarly, 24-hour urine collections will be of interest, but are put off to later studies, especially those which have fats which may affect prostaglandin metabolism.

II. BLOOD COLLECTION -- AN OVERVIEW.

A. Overview.

The DELTA Study requires collection of approximately 25.5 ml of blood from participants at baseline and approximately 36 ml of blood four times at the end of each diet phase. Additional samples may be required from premenopausal women. Since the study depends on the voluntary participation of subjects, both initially and in follow-up, every effort must be made to make the entire procedure as easy and painless as possible, both for the participants and for the field center personnel.

The following schematic describes the blood sampling for a single study subject (Figure 1).

The LDX Capillary Blood Analyzer by Cholestech Corporation (Walnut Creek, CA) will be used for subject screening. This instrument provides accurate and rapid (three minute) results for total and HDL cholesterol and triglyercides (if fasting) at reasonable cost. The Cholestech Corporation has agreed to donate one machine per field center and 500 cassettes at cost for this project. Publications which describe subject screenings should acknowledge use of this instrument in the Methods section and the contribution of Cholestech Corporation in the Acknowledgements. Technicians using this instrument should receive training by OSHA regulations, sample collection technique, and the proper use of this equipment.

Recruitment	Capillary Blood Total and HDL
Baseline	Baseline Package
Diet A, Week 5,6,7,8	Four endpoint packages
Diet B, Week 13,14,15,16	Four endpoint packages
Diet C, Week 21,22,23,24	Four endpoint packages

The baseline package contains serum for a chemistry profile and TSH (6 ml), EDTA plasma for hematology (3.5 ml), EDTA - plasma for buffy-coat (10 ml), and serum for a fasting lipoprotein profile (6 ml) for a total of 25.5 ml of whole blood.

The endpoint package contains a serum for lipid profile, apolipoproteins, and aliquots (9.5 ml), a tube for serum aliquots (9.5 ml), a citrate-plasma tube at room temperature (4.5 ml) for Factor VII, a second citrate-plasma tube at 4°C (4.5 ml) for fibrinogen and PAI-1, and an EDTA-plasma tube at 4°C (7.0 ml) for a total of 35 ml. For the endpoint package, any participants who are concerned about the volume of blood should be reassured that the total amount of blood is 1/15th of the volume given in a typical Red Cross blood collection (450 ml). Each endpoint package should be collected after an 8-hour fast with sample collection occurring between 7-10 a.m.

Serum was selected for analysis of lipoproteins inspite of the fact that many large studies have used plasma in the past. The reasons for this are severalfold. First, the EDTA used as anticoagulant extracts water from red cells, diluting the plasma somewhat. This problem translates into increased variability, since this process seems variable from person to person.

Second, fibrin fragments not pelletted with centrifugation can clog up analysis equipment, lead to errors and equipment failures.

An overall schema of sample processing is shown in figure 2.

B. Blood Collection Trays and Tubes.

1. Blood Collection Tray.

Blood collection trays are prepared in advance for the following day. Each tray is stocked with a full supply of blood-drawing equipment for three to four participants and holds individual blood collection racks for each participant in an ice bath. Several racks are prepared to hold various plastic tubes and vials for the final serum and plasma aliquots. The blood collection tube rack and aliquot tube rack are prelabeled with the appropriate code numbers for the participants drawn that day.

2. Blood Collection Rack.

The collection tray itself is made of hard plastic which is unbreakable and can be easily cleaned. The tray has 10 individual compartments which will be filled with the following supplies:

21 g butterfly needles with luer adapter
alcohol swabs
Band-Aids
Gauze
Tourniquets (2)
Vacutainer holders
Needle/Sharps container
Smelling salts
Timer/Stop watch
Scissors
Adhesive tape
Styrofoam ice bath filled approximately
10 minutes before draw
Pencils/Pens
Latex gloves
Test tube racks (2)

A separate rack contains the necessary draw tubes, etc. for each participant. The tubes are arranged according to the priority of the draw. This rack will fit into the blood collection tray. The blood collection tubes are pre-labeled with identification numbers.

3. Description of blood collection tubes for baseline package.

Tube 1 is a 6 ml siliconized orange-green stopper tube with serum separater. This contains no anticoagulants so that the blood clots to form serum. After drawing, the blood

is allowed to clot at room temperature for 30-40 minutes. This tube is submitted to clinical chemistry for hepatic profile, renal profile, glucose, and TSH.

Tube 2 is a 3.5 ml lavender-stopper tube containing liquid 4.5 mM EDTA as the anticoagulant. After drawing, this tube is mixed by inverting and placed on a tube mixer. The tube will be submitted for hematology assay (hematocrit, hemoglobin, white blood cell count).

Tube 3 is a 10 ml lavender-stopper tube containing liquid 4.5 mM EDTA as the anticoagulant. After drawing, this tube is mixed by inverting on a tube mixer. It is centrifuged at R.T. for five minutes at 3,000 g. The buffy coat is removed and dispensed into three amber-coated vials for DNA analysis.

Tube 4 is a 6 ml siliconized orange-green stopper tube with serum separater. This contains no anticoagulants and is submitted to the field center lipid laboratory for baseline lipoprotein profile. This is centrifuged at 4°C at 3000 g x 10 min and 4-0.5 ml aliquots are frozen at -80°C before analysis.

4. Description of blood collection tubes for endpoint package.

Tube 1 is a 10 ml siliconized yellow/black stopper tube. This tube contains thrombin so that the blood clots to form serum in 5 minutes. Aliquots are coded red. This serum is used for the lipid profile and apoproteins.

Tube 2 is also a 10 ml yellow/black stopper tube. It is processed the same as Tube 1 and is used for aliquots of serum for later studies.

Tube 3 is a 4.5 ml blue-topper tube containing 0.5 ml of 3.8% sodium citrate. It is crucial that this tube remain at room temperature. After centrifugation, plasma is aliquoted into green-coded aliquots. The plasma will be used for Factor VII coagulation assay.

Tube 4 is also a 4.5 ml blue-stopper tube identical to Tube 3. It is different in the way it is processed. Instead of room temperature, this tube must be placed on ice after filling. Plasma from this tube is aliquoted into blue-coated aliquots and will be used for fibrinogen assay and PAI-1 assay.

Tube 5 is a 7.0 ml purple stopper tube. This tube requires mixing after filling and placement on ice. After centrifugation, plasma is aliquotted into yellow-coded cryovials (0.5 ml). The plasma will be used for ancillary and supplemental studies.

Each draw tube is assigned a color-coated aliquot system. There is one type of aliquot used: 0.5 ml cryovials. The cryovials have colored caps.

5. Priority of tubes.

A total of 25.5 ml of blood will be drawn from each participant for each baseline package. The tubes are drawn in the following order: Serum 6 ml - orange/red top,

EDTA plasma 3.5 ml - purple top, EDTA plasma 10.0 ml - purple top, serum 6 ml - orange/red top. All four tubes are required, as they assess inclusion/ exclusion criteria.

The tubes are numbered 1-4 and arranged on the rack in order of draw. Samples from the four tubes are used in approximately eight different biochemical and hematological assays. The buffy-coated aliquots (three) obtained from baseline are amber/brown cryovials, approximately 0.5 ml in each cryovial.

A total of approximately 35 ml will be drawn from each participant in five tubes at each endpoint package. The tubes are drawn in the following order: Serum 10 ml - yellow/black top, serum 10 ml - yellow/black top, citrate plasma 4.5 ml - room temperature blue top, citrate plasma 4.5 ml - 4°C - blue top, and EDTA plasma 7.0 ml - 4°C. Tubes #1, 3, and 4 are of absolute priority and should be collected at each endpoint package, at the minimum.

The tubes are numbered 1-5 and arranged on the rack in order of draw (see diagram in this section). Samples from the five tubes are used in approximately 13 different biochemical and hematological assays.

6. Aliquot tube rack: Labeling and set up.

Each participant will have two aliquot racks set up to correspond with the blood collection tube rack. Rack set up is completed the previous day. All tubes and vials are labeled and arranged appropriately (see diagram in this section). Aliquot assignments:

	Polypropylene	
Tube	Cryovials	Color Code
serum 10 ml	7 (0.5 ml)	red
serum 10 ml	7 (0.5 ml)	red
citrate 4.5 ml	4 (0.5 ml)	green
citrate 4.5 ml	4 (0.5 ml)	blue
EDTA plasma 7.0 ml	6 (0.5 ml)	lavender
TOTALS	28	

Each field center will be provided with a PC-based computer program to allo the inventory of aliquots stored at each field center to be tracked. Each field center will be responsible for the accurate maintenance of the inventory.

7. **Forms.**

a. **Purpose:**

The purpose of the blood collection form is to facilitate the collection of plasma and serum samples from participants. This collection must be done in a rapid and efficient manner, with maximum protection for the participant. In addition, the process must facilitate the monitoring of phlebotomy and other quality assurance parameters as well.

Note: All forms are to be completed in ink.

b. **Description:**

There are two parts of the Phlebotomy Processing Form associated with blood drawing. The top half is the participant/phlebotomy question about fasting status. If the person has eaten less than eight hours earlier, he/she should be rescheduled on an alternate day, within three days of the initially scheduled date.

The bottom half deals with phlebotomy and processing of samples.

These forms have the following purposes: 1) Assure the most efficient and safest possible venipuncture for participants. 2) Allow the monitoring of the quality of the above procedures. 3) Allow more efficient processing of the samples at central laboratories. 4) Provide information critical to the interpretation of assay results.

c. **Preparation for Specimen Collection:**

Preparation for specimen collection is done in the following manner:

Early morning, prior to arrival of any participants:

1) Check to make sure that blood collection tray is properly equipped. Every item on the checklist must be ready before proceeding.

2) Check that each vacutainer tube is properly labeled with the appropriate participant number and number 1-4 (baseline) or 1-5 (endpoint) in order of draw. A sheet of numbered labels will be provided for each participant.

3) Check that the sample processing station is properly equipped. Eve item on the checklist must be ready and in its proper position.

4) Check that each sample aliquot tube is labeled with its appropriate participant identification number and in its proper rack.

5) Check that the participant Phlebotomy Processing Forms are labeled and included with the blood collection tray.

6) Perform quality control check on refrigerator temperature (refrigerator temperature log).

7) Perform quality control check on freezer temperature (freezer temperature log).

8) Make sure the phlebotomy area is tidy and stocked with extra smelling salts, basin, disposable wash clothes, and that the blood mixer is functional.

Approximately 10 minutes before scheduled participant arrival:

1) Fill styrofoam bath 3/4 full with crushed ice.

At participant arrival:

1) Check that the ID number on the tubes matches the participant ID.

d. Venipuncture

1. Precautions for handling blood specimens:

In accordance with OSHA regulations on blood borne pathogens (see appendix for the complete OSHA regulations), the following laboratory safety protocol for field-center laboratories is recommended: Use of non-permeable lab coats, Latex gloves, and face shields when handling any blood in any situation where splashes, spray, splatter, or droplets of blood may be generated and eye, nose, mouth contamination can be reasonably anticipated. Follow universal precautions when handling any blood products. Contaminated needles and sharps shall be immediately placed in a puncture-resistant, leak-proof container. Never re-cap or break needles. Hepatitis B vaccine should be offered to all technicians handling blood.

2. Phlebotomy area:

The blood drawing area should take place in an isolated room or participants should be separated by room dividers. The room should be equipped with all the necessary blood drawing supplies. A separate counter or work cart should be equipped with all the materials and vials that are used for blood handling and processing. The centrifuge, refrigerator, and freezer should be near by.

3. Participant preparation:

Informed consent must be obtained before drawing blood. This procedure is followed to ensure that the subjects understand the purpose of blood drawing and the possible complications of venipuncture. A standard informed consent has been prepared for this study. With regard to laboratory procedures, the consent statement informs study subjects that there is a small risk of bruising at the spot on the arm where the blood is taken and that about 2 tablespoons of blood are drawn. The consent statement also informs study subjects that they will be contacted if clinically important test results are abnormal.

4. Participant phlebotomy question:

Minimal fasting time required for testing is 8 hours (optimum is 12 hours), and all samples for endpoint package should be collected between 6-10 a.m. Record the time of last food and the time of blood drawing. If the participant is not fasting, the blood sample will not be drawn and the subject should be rescheduled for phlebotomy within the next three days. Similarly, if the collection time is outside the 6-10 a.m. period, the subject should be rescheduled for phlebotomy.

5. General:

Blood drawing is standardized for the sitting position. The venipuncture performed with the 21-gauge butterfly needle with 12 inches of plastic tubing between the venipuncture site and the blood collection tubes. The butterfly is a small, thin-walled needle which minimizes trauma to the skin and vein. The use of 12 inches of tube allows tubes to be changed without any movement of the needle in the vein. If the participant is concerned about the venipuncture, he/she may be reassured to know that such care is taken. The participant should be given enough time to feel comfortable both before and after the blood collection. In many cases, the most memorable part of the experience for the participant will be the contact with the person who draws the blood and their general attitude and competence. If the participant is nervous or excited, the technician briefly describes the procedure; e.g., "I am going to be drawing about 2 tablespoons of blood. This blood will be used for tests for lipids and cholesterol and blood clotting factors."

HANDLING PARTICIPANTS WHO ARE EXTREMELY APPREHENSIVE ABOUT HAVING BLOOD DRAWN.

Do not under any circumstances force a participant to have blood drawn. It may help to explain to the participant that the blood drawing is designed to be as nearly painless as possible. It is sometimes best to let the participant go on with another part of the examination. It may also be helpful to have the participant relax in the blood drawing chair just so the phlebotomist can check the veins in the participant's arms, without actually drawing blood.

6. Venipuncture procedure: Wear Latex gloves and lab coat

a) Arrange draw tubes in order of draw on the table top within easy reach. Assemble butterfly apparatus and vacutainer holders, gauze, and alcohol prep prior to tourniquet application.

b) Apply tourniquet to either arm.

c) Examine participant's arms for the best site for venipuncture. Release tourniquet.

d) Cleanse venipuncture site. Prepare area by wiping with alcohol swab in a circular motion from center to periphery. Allow area to dry.

e) Reapply tourniquet and start timer.

f) Grasp the participant's arms firmly using your thumb to draw the skin taut. This anchors the vein. The thumb should be one or two inches below the venipuncture site.

g) With the needle bevel upward, enter the vein with a smooth continuous motion.

h) Make sure the participant's arms are in a flat or dominant position while maintaining the tube below the site when the needle is in the vein. It might be helpful to have the participant make a fist with the opposite hand and place it under the elbow for support.

i) Grasp the sheath of the needle holder and push the tube forward until the butt end of the needle punctures the stopper, exposing the full length of the needle.

j) Note the blood flow into the first collection tube. If blood is flowing freely, the butterfly needle can be taped to the participant's arm for the duration of the study. If the blood flow is very slow, the needle may not be positioned correctly.

k) Remove the tourniquet at two minutes. Note the time on the PP form. Once the draw has started, do not change the position of the tube until it is withdrawn from the needle. If blood flow ceases after the tourniquet is removed, it may be reapplied for another two minutes, but this is noted on the Phlebotomy Processing Form.

l) Keep a constant, slight amount of pressure (in the direction of the needle) on the end of the tube (especially tubes #1 and #2). This prevents release of the shut-off valve and stopping of blood flow, do not bring pressure or reintroduce pressure after completion of the draw.

m) Fill each vacutainer tube as completely as possible; i.e., until the vacuum is exhausted and the blood flow ceases. If a vacutainer tube fills only partially, remove the vacutainer and attach another without removing the needle from the vein. As each tube is filled, mix by gently inverting before placing tube on the mixer. (See Section Blood Mixing During Venipuncture.)

n) When the blood flow ceases, remove the tube from the holder. Shut off valve recovers the point, stopping blood flow until the next tube is inserted (if necessary).

o) Average venipuncture time is 3-6 minutes, but any difficulties may increase this time to 10 or 15 minutes.

7. Removing the Needle:

To remove the needle, lightly place clean gauze over the venipuncture site. Remove the needle quickly and immediately apply pressure to the site with a gauze pad. Discard needle into puncture-proof sharps container. Have the participant hold the gauze pad firmly for 2 minutes to prevent a hematoma. Remove tube from the blood mixer and place on ice (#4,#5) and at room temperature (#1,2,3).

8. Bandaging the Arm:

a) Under Normal Conditions:

1) Set the gauze pad down over the site, continuing mild pressure.

2) Apply an adhesive or gauze bandage over the veni puncture site after making sure that blood flow is stopped.

3) Tell the patient to leave the bandage on for at least 15 minutes.

b) If the patient continues to bleed:

1) Apply pressure on the site with a gauze pad. Keep the arm elevated until the bleeding stops.

2) Wrap a gauze bandage tightly around the arm over the pad.

3) Tell the patient to leave the bandage on for at least 15 minutes.

9. Procedures for difficult draw and complications of blood drawing.

If a blood sample is not forthcoming, the following manipulations may be helpful.

a) If there is a sucking sound, turn the needle slightly or lift the holder in an effort to move the bevel away from the wall of the vein.

b) If no blood appears, move needle slightly in hope of entering vein. Do not probe. If not successful, release tourniquet and remove needle. A second attempt can be made on the other arm.

c) Loosen the tourniquet. It may have been applied too tightly, thereby stopping the blood flow. Apply the tourniquet loosely. If the tourniquet is the Velcro type, quickly release and press back together. Be sure, however, that the tourniquet remains on for no longer than 2 minutes at a time.

d) The phlebotomist should not attempt to venipuncture more than twice.

e) Reassure the participant that the inability to obtain a clean venipuncture is not any sign of a medical problem on his/her part.

f) If venipuncture is unsuccessful, the participant must be rescheduled at a later date with a different field center phlebotomist.

10. Syringe Technique for Venipuncture

Collection of blood sample using syringes may also be used if the phlebotomist, upon examination of the participant's veins, feels that sample will be difficult to obtain by vacutainer method. Blood collection using syringes should be used only if the phlebotomist anticipates a difficult draw (vein collapse, small veins, etc.).

11. Blood Mixing During Venipuncture:

Each tube should be treated as follows:

a) Serum (#1,2) - Invert once, place on rack at room temperature.

b) Citrate (#3) on mixer for 30 seconds then place on rack at room temperature.

c) Citrate (#4) on mixer for 30 seconds then place in ice bath.

d) EDTA (#5) on mixer for 30 seconds then place in ice.

12) Precautions:

a) When a participant feels faint or looks faint following the blood drawing:

1) Have the participant remain in a chair, if necessary, h him/her place head between knees.

2) Provide the person with a basin if he/she feels nausea.

3) Have the person stay reclined until the color returns and he/she feels better.

4) Place a cold wet cloth on the back of the person's neck.

5) If the person faints, use smelling salts to revive by crushing the ampule and waving it under the person's nose for a few seconds.

6) If the person continues to feel sick, contact the medical staff member who will advise you on further action.

13. Completing the Blood Draw Procedure:

a) Dispose of the needle and tubing:
Dispose of needle and tubing in the appropriate biohazard needle/sharps containers. (It may be necessary to use Hemostats to remove tubing from vacutainer holders.) Complete the first page of the Phlebotomy Processing Form. Clean up th venipuncture area (if necessary). Bring blood collection tray to the processing area with the filled vacutainer tubes.

Blood collection tray checklist per tray:

10 - 21 gauge butterfly with lure adaptors
10 - alcohol swabs
15 - Band-Aids
15 - gauze pads
5 - Vacutainer holders
2 - tourniquets
1 - smelling salts
1 - timer or stop watch
2 - pencil/pen
- Latex gloves
1 - hemostat
1 - adhesive tape
1 - scissors

Approximately 10 minutes before draw:

1 - styrofoam ice bath filled with ice.

Optional:

10 cc plastic syringes
20 cc plastic syringes
needles for syringes

Per participant:

1 - blood tube rack with four draw tubes (labeled and numbered)

1 - Phlebotomy Processing Form
Have available on phlebotomy cart:
basin
cold cloth
tube mixer
biohazard containers
needle/sharps container
paper towels

III. PROCESSING THE BLOOD SAMPLES

A. General:

Processing should be done immediately following venipuncture. Personal protective equipment (non-permeable lab coats, double gloves with at least one Latex pair, spatter shield) must be worn. Flowchart is included at the end of this section to diagram this process (Figure 2).

B. Processing of Baseline Samples:

The red stopper tube #1 (6 ml) with serum separator is placed on a rack at room temperature; the purple stopper tube #2 is mixed for 30 seconds and placed on a rack at room temperature. These tubes are submitted to the field center's chemistry and hematology laboratories. The buffy-coat aliquot is obtained from 10 ml EDTA tube #3 at baseline, after centrifugation at R.T. for 10 minutes at 3000 x g. After the plasma is removed, using an MLA pipette, carefully suction off the white cell layer. Try not to disturb the red cells. The buffy coat is aliquoted into three amber vials. The second red stopper serum tube (6 ml) with serum separator is also allowed to clot at room temperature for 30 minutes and then centrifuged x 3000 g for 10 minutes. The tube is then submitted to the field center's lipid laboratory for fasting lipid profile; it should be refrigerated if not delivered to the laboratory within three hours of phlebotomy. Alternatively, 4-0.5 ml aliquots can be frozen at -80°C prior to analyses at the lipid laboratory.

C. Immediate Processing of Endpoint Samples:

Upon reaching the blood processing station, remove the blood tube drawing rack and ice bath containing tube from the blood collection tray. The rack should contain three

tubes: #1, #2, #3. The ice bath should contain two tubes: #4, #5. All tubes should be processed within 20 minutes of phlebotomy. The corresponding aliquot racks (two per patient) should be ready. Rack #1 (with color codes red, blue, and lavender) should be placed in an ice bath. Rack #2 (with color code green) remains at room temperature.

D. Centrifugation:

Tubes #1, #2, #4, and #5 are centrifuged at 4°C for 10-20 minutes at 3,000 g. The centrifuge may need to be precooled to 4°C. 4.5 ml and 7 ml balance tubes are required for tubes #4 and #5. Simultaneously, tube #3 is centrifuged at room temperature for 10-20 minutes at 3,000 g. A 4.5-ml balance tube is required. Once centrifuged, the maximum time allowed before aliquoting is 10 minutes. While these tubes are spinning, the blood collection tray can be restocked with tube rack and blood collection tubes, ice, and forms for the next participant. Recheck labels on the two aliquot racks. Perform any necessary clean-up.

E. Aliquots:

Allow the centrifuge(s) to come to a complete stop. Remove tubes #1, #2, #4, and #5 from the 4°C centrifuge being careful not to shake the tubes. Tubes are put on ice. Remove tube #3 from the room temperature centrifuge. Place in tube rack at room temperature. Assess the plasma. Mark on the Phlebotomy Processing Form if lipemic (L), icteric (I), hemolyzed (H), clotted (C). Follow the outline on the Phlebotomy Processing Form for aliquoting the samples. Checkmark on the form if the aliquot is completed, and mark any different volumes. Be careful not to disturb the other white cell layers. If plasma is a lower than expected volume, use one less aliquot rather than disturb the red cells or buffy coat. Do not have any red cells in the aliquots. Use a new pipette tip for each tube type. Recap aliquots after each draw tube has been pipetted.

		ALIQUOT
Tube #5	Use 500 uL MLA Cryovials (6) - 0.5 mL plasma/cryovial	Rack 1 (on ice) Lavender code
Tube #4	Use 500 uL MLA Cryovials (4) - 0.5 mL plasma/cryovial	Rack 1 (on ice) Blue code
Tube #3	Use 500 uL MLA Cryovials (4) - 0.5 mL/cryovial	Rack 2 (room temp.) Green code
Tube #1	Use 500 uL MLA pipette Cryovials (7) - 0.5 mL serum/cryovial	Rack 1 (on ice) Red code
Tube #2	Use 500 uL MLA pipette Cryovials (7) - 0.5 mL serum/cryovial	Rack 1 (on ice) Red code

* Indicates minimum volumes specified are required.

Checkmark on the Phlebotomy Processing Form if the aliquot is complete, then mark any different volumes. Original blood collection tubes can be properly disposed of in biohazard waste bags.

F. **Freezing:**

Upon completion of the processing steps, aliquots must be frozen within 10 minutes. Green aliquots (4 cryovials) at room temperature are added to the rack at 4°C. That rack is removed from the ice bath and placed upright in the freezer at -70°C for at least ½ hour (preferably until the end of the day). Make sure the aliquots are not wet when they are placed in the freezer. If the -80°C freezer is not immediately available, the aliquots can be placed on dry ice or snap frozen in liquid nitrogen/methanol mixture.

G. **Completed Forms:**

The completed Phlebotomy Processing Form can be set aside in the daily work folder. These forms are copied. Originals are filed at each field center; copies are enclosed with each shipment of samples to the various laboratories.

H. **End of the Day Procedures:**

Frozen aliquots and racks are packaged with prelabeled freezer box. One box contains the aliquots from a single endpoint series on each patient (28 aliquots per total). The boxes containing aliquots are stored at -80°C freezer by date. The copy of the Phlebotomy Processing Form is kept in a file to be included with the shipment of samples. Restock blood collection tray with samples. Label with next day's participants. Blood draw tubes, aliquots, forms. Arrange draw tubes and aliquots in their proper racks. Wipe down all work areas with 10% Clorox solution.

I. **Summary of Processing Time Limitations:**

On blood drawn prior to processing:

1) Serum 9.5 ml - 40 minutes;
2) Serum 9.5 ml - 40 minutes;
3) Citrate 4.5 ml - room temperature - 30 minutes;
4) Citrate 4.5 ml - 4°C - 30 minutes;
5) EDTA 7.0 ml - 4°C - 30 minutes;

Once centrifuged, maximum time before aliquoting is 10 minutes; after aliquoting all samples, freeze within 10 minutes.

J. **Sample Processing Checklist:**

Pipette: MLA 2,000
Pipette: MLA 1,000
Pipette: MLA 500
Pipette tips
Pasteur pipette with bulbs
Ice bath/ice
Latex gloves
Lab mat

Kimwipes
Biohazard waste bags
Clorox (10% in wash bottle)
Pen/pencils
Study participant aliquot racks (2) with prelabeled aliquots
Phlebotomy Processing Form (page 1 completed)
Freezer
Refrigerator
Centrifuges -- room temperature and 4°C
Balance tube
Thermometer
Zip-lock bag
Lab coats, gloves, face shields

IV. SHIPPING THE BLOOD SAMPLES

A. General:

Blood samples are stored at the field centers at -80°C and shipped to the various laboratories according to the schedule in Figure 3.

B. Methods:

The samples to be shipped are removed from boxes stored at -80°C and packaged in bags for each subject. Copies of the corresponding Phlebotomy Processing Forms should be sent for that shipment. A transmittal form is completed to include those samples that are actually included in the shipment. The proper mailing/shipping labels (provided by carrier) are filled out. The UV mailing address is: Laboratory for Clinical Biochemistry Research, Colchester Medical Research Facility, Room T-205, University of Vermont, 5580 South Park Drive, Colchester, VT 05446, phone number: (802) 656-8963 (Elaine Cornell). The MIBH mailing address is: Lipid Research Laboratory - 4th Floor, M.I. Bassett Research Institute, One Atwell Road, Cooperstown, NY 13326, phone number: (607) 547-3048 (Leslie Davidson). The styrofoam mailer is lined with absorbent material; i.e., paper towels. Approximately half the dry ice is placed on the bottom of the mailer. Check the ID number against the transmittal form. Carefully place the zip-lock bags, each containing the samples from a single subject, in the mailer. Remaining dry ice (6 pounds total) is placed on the sample. The top of the styrofoam is sealed up with tape. It is closed with the outer cardboard sheath. The Phlebotomy Processing Form is placed on top of the styrofoam before the outer sheath is closed by tape or strap. Fix shipping labels. Place the entire box in the refrigerator if pick-up is not immediate (samples should not be on dry ice for more than 24 hours). Do not ship samples for more than 30 participants per box. Note: The number of samples per box may vary depending on the size of the container used. We recommend using your judgment and 6 pounds of dry ice.

C. Sample Shipping Checklist:

Styrofoam mailing container with outer cardboard sleeve
Absorbent material

Zip-lock bag
Packaging tape
Dry ice (6 pounds per shipment)
Labels (provided by carrier)
Completed Phlebotomy Processing Form
Blood sample transmittal form

V. STORAGE OF ALIQUOTS

Blood samples sent to Field Lipid Laboratories, Central Lipid Laboratory (MIBH), and Central Coagulation Laboratory (UV) will be stored at -80°C until analyzed. This should be a brief period of time.

Numerous additional aliquots are to be stored at the Field Centers for later analyses, as yet not proposed. The rationales for using the Field Centers for storage:

1. This reduces the risk of loss of endpoint samples, since some samples would be retained by the Field Centers,

2. The labotories analyzing these samples are as yet unknown, and they could be shipped directly at the time needed.

Each Field Center should identify a -80°C freezer which is protected from thawing through a reliable alarm system or back-up power system. The loss of aliquots through thawing may result in exclusion of that center's samples from important analyses.

A software program will be developed for the purpose of inventory control. The maintenance of this inventory is the responsibility of the field centers. All samples collected, stored, and shipped shall be accounted for on this program. Training on the use of the program will be included in the training sessions.

VI. SAMPLE RECEIPT AND ANALYSES

A. Sample Receipt:

Samples received at MIBH and UV will be preceded by a FAX of the transmittal form. Upon receipt of the shipment, the attached blood sample transmittal form is checked against the fax, to assure that no confusion has occurred and to allow for telephone verification if there are any lingering doubts. The samples are removed from the package and immediately frozen at -80°C for later analyses. Any problems in the shipment in terms of thawing, breakage, etc. should be noted.

Samples are thawed prior to analyses per protocol, either at room temperature or in a warming bath.

B. Lipid Profiles:

1. Columbia University.

All determinations of cholesterol and triglycerides in whole serum and in HDL will be carried out in the Core Lipid Laboratory of the Atherosclerosis Score at Columbia. Dr. Ginsberg is the Director of this laboratory, which is a participant in the Standardization Programs of the Centers for Disease Control. Cholesterol and triglyceride levels will be determined by enzymatic methods using the Hitachi 705 automated spectrophotometer. The interassay coefficients of variation for these two measurements are less than 3% at present. Whole HDL cholesterol will be measured at the precipitation of plasma apo-B-containing lipoproteins at 10 gm per liter Dextran sulfate and 0.5 M magnesium Mg Cl2 (0.91 mg per ml and 0.045 M final concentrations, respectively).

2. Louisiana State University.

All routine lipid analyses (total cholesterol, triglycerides) will be performed on the Beckman Synchron CX5 automated chemistry analyzer. HDL cholesterol is performed in the Beckman TH5 after precipitation of the non-HDL fractions by Dextran sulfate (50,000 MW) (DMA, Dallas, TX) following the protocol of Warnick, et al. Assay controls by DMA are used to verify accuracy. LDL cholesterol is calculated using the Friedewald formula.

For automated analyses, daily quality control is performed prior to all analyses which will be run on that day. Acceptable results must be obtained on quality control before any results are allowed to be reported. The results are logged into a computer bases' quality control monitoring package (Lyphline, BioRad Laboratories). Results for the intralaboratory comparisons by BioRad for lipid analyses have been very good. For cholesterol, there is a cumulative CV of 1.5% for level 1 and 2.5% for level 2. Comparison with other Beckman CX5 users shows the SDI is -1.0 and -0.9 for levels 1 and 2, respectively (-2.0 and +2.0 acceptable). For triglyceride, there are cumulative CVs of 1.8% and 3.7% for levels 1 and 2. The SDIs are negative for 0.1 and 0.2 when results are compared to others CX5 users. For HDL, controls are assayed from DMA. They are consistently within the acceptable ranges for these values. CVs are 2.9% and 6.4% for levels 1 and 2.

3. The MI Bassett Research Institute.

Lipid profiles consist of measurement of total cholesterol, triglycerides, HDL cholesterol after precipitation of apo-B-containing lipoproteins using 50,000 MW Dextran sulfate. LDL cholesterol is calculated today using the Friedewald formula. All analyses are done on a Roche MIRA random access automated analyzer. Cholesterol and HDL cholesterol are assayed by enzymatic method based on a cholesterol esterase and a cholesterol oxidase system using a peroxidase / 4 amino antipyrene detection system. This is manufactured as Roche reagent for cholesterol (Roche Diagnostic Systems). For HDL cholesterol detection, non-HDL lipoproteins are precipitated by treatment of 500 microliters of serum with 50 microliters of Dextran sulfate (Sigma Chemical). Triglycerides are assayed by Sigma Chemical triglyceride reagent based on hydrolysis by lipase and detection of glycerol by glycerol kinase, coupled with glycerol-1 -

phosphate oxidation to produce peroxide, which is detected by peroxidase conversion of the aminoantipyrene to quinonemia.

Calculation of cholesterol is based on a serum-based calibrator certified for accuracy by cross-over with Abell-Kendall reference method. Calibration is done in triplicate once a month. Calibration of triglyceride is based on reaction with pure glycerol expressed as equivalent triolein (Sigma Calibrators). For total cholesterol, two levels of quality control material (from Dade) are included with each run. For HDL cholesterol, two levels of quality control material are precipitated and analyzed with each run. For triglyceride, two levels of quality control material are included in each run. A run is considered out of control, using Westgard rules and precision ranges based on MTP guidelines (3% for total choles terol, 5% for triglycerides, 3% for HDL cholesterol). If it runs out of control, the entire run is repeated.

4. University of Minnesota.

Cholesterol is measured on a Roche Cobas FARA analyzer using Boehringer Mannheim enzymatic reagent. A frozen serum pool which has been measured on multiple occasions by the Abell-Kendeall method is used as calibreator. Accuracy of cholesterol measureemnts are also verified by the CDC and are in close agreement with their reference Abell-Kendeall method with fresh patient samples. Total serum triglyceride is measured on a Roche Cobas FARA analyzer using Boehringer Mannheim GB reagent and calibrator. This method gives a "true" triglyceride level which has been corrected for free glycerol concentratio Triglyceride measurements are standardized by the Centers for Disease Control (CDC) Lipid Standardization Program and are in close agreement with the CDC reference triglyceride method. LDL cholesterol is estimated by the Friedewald formula. This equation assumes a ratio of five for plasma triglyceride to the VLDL cholesterol; while this ratio is correct for the typical American diet, it may change for other proposed research diets.

The HDL cholesterol is measured enzymatically after precipitation of VLDL and LDL with Dextran sulfate (molecular weight 50,000) and magnesium chloride. The method is the same as described by Warnick, et al. We have chosen the Dextran sulfate/magnesium precipitation method over the Heparin/Manganese procedure because Manganese interferes with enzymatic cholesterol methods and must, therefore, be removed with sodium bicarbonate. This adds additional steps and imprecision to the method. In addition, the Dextran/Magnesium method is less sensitive to variations in temperature and centrifugation.

C. Apoprotein A-1 and BIOO (MIBH).

The analytical method is rate nephelometry using antisera specific for Apo-A1 or Apo-B-100. The formation of antigen-antibody complexes are monitored with time; the initial reading of light scatter serves as a baseline for change. Instrument monitors the rate of change and rejects any sample where change in turbidity with time is outside an acceptable margin of error, thus assuring that the presence of particulate matter drifting through the measurement field or the occurrence of lipemia does not produce artificially modified results. Analysis is performed on a Beckman Array using Beckman reagents, calibrators, controls (Beckman Instrument Company, Fullerton, CA). Fresh or frozen serum may be used. Reagents are polyclonal antisera specific for either apolipoprotein A-1 or apolipoprotein B-100. Titers of the antisera are determined by the manufacturer and preparation is optimized for reaction in the volumes of buffer and sample used with the Array system. Each lot of antisera is accompanied by programming information which is entered into the instrument to fit the calibration curve and set the margin of error allowable for the rate of reaction. Calibrators are standardized to the internationally developed reference material designated by CDC-IUIS (Centers for Disease Control and International Immunological Society). The instrument is calibrated every two weeks using this material. Control materials are included at the beginning, middle, and the end of all runs to assure reproducibility of results.

Quality control samples are included at the beginning and the end of each run and are analyzed in duplicate. If the average value is not within the +/-2 standard deviation range for the QC samples or if reproducibility in these samples is unacceptable (samples at the end of the run deviated by more than one standard deviation from those at the beginning), the entire run is repeated. The precision for Apoprotein A-1 at 117 mg per deciliter over the long-term (run to run) is 4.9% and within run is 0.7-1.8%. Apo-B 100 precision at 170 mg per deciliter over the long run (run to run) is 3.8% and within run is 0.3-0.9%. The samples that give an error signal, indicating that they fall outside an acceptable time course development of turbidity are diluted and reanalyzed to assure this reaction did not occur at antigen or antibody access.

D. Lipoprotein a (MIBH).

Analytical method is an ELISA (Enzyme Linked Immunosorbent Assay) using the Macra LPa kit manufactured by Terumo (Elkton, MD). Monoclonal antibody to Lp (a), immobilized on microtiter wells, serves as the capture antibody. Bound Lp (a) is detected using a monoclonal anti LP (a) antibody conjugated with horseradish peroxidase. The complex is detected and quantified by chromagen formation upon incubation of peroxide and o-phenylenediamine substrate. A 100 microliter aliquot of serum, stored at -80°C, is used for this assay. The antisera, calibrators, and controls are provided by Terumo (Elkton, MD) in their Macro LP (a) kit. ELISA plates are read on an automated ELISA plate reader, Dynatech model MR600 set to monitor at 492 nanomoles. A calibration curve, consisting of six ampules ranging from 0-80 mg/dL Lp (a) is run in each batch. Each calibrator is run in duplicate and averaged to generate a standard curve. Each sample is analyzed in duplicate.

Two quality control samples are included in each run. If these do not match the target value within +/-2 standard deviation, the run is repeated. The precision of LP(a) at 15

mg per deciliter on the long term (day-to-day) is 3.9% and within run is 1.4%. LP(a) of 36 m per deciliter has a long-term precision (day-to-day) of 3.8% and within run of 1.8%. Samples LP(a) concentrations above the highest calibrator or absorbing at over 2.0 absorbing units are diluted 1-to-1 with saline and repeated on another run.

E. Coagulation Factors.

1. *Fibrinogen*. Fibrinogen will be measured by the clot-rate method of Clauss, using a semi-automated instrument, the ST4 from Stago. The assay will be standardized using the College of American Pathologists Standardized Reference Plasma, which the UV laboratory helped establish.

In addition to outside quality assurance programs, such as the Coagulation Program of the College of American Pathologists, quality control will be established with three control materials (normal, elevated, and low) and so-called Westgard Rule (the multi-rule Shewart QC System) will be applied. Longitudinal drift will be assessed using lyophilized control plasma. In one recent large study in the UV laboratory, the fibrinogen assayed at an average monthly CV of approximately 3.09% and blind duplicate analyses included the technical error of 7.4% with a correlation calibration of 0.8727. The fibrinogen assay will be done each test plasma in duplicates at one dilution (1/10). If the fibrinogen concentration is too high, the sample is assayed again at 1/20, 1/40 dilution. Assay requires a minimal of 0.25 ml of citrated plasma.

2. *Factor VII Activity*.

Factor VII activity will be assessed using one-stage clot-rate assay based upon the prothrombin time, using immunodeficient human plasma. The thromboplastin will be human placental thromboplastin, and the assay will be standardized using World Health Organization reference plasma. The semiautomated Coag-A-Mate X2 instrument from General Diagnostics will be used.

Quality control will be established with two control plasmas (both approximately normal) and so-called Westgard Rules (a multi-rule Shewart QC system) will be applied. Longitudinal drift will be assessed using lyophilized control plasma. In one recent large study in the UV laboratory, the Factor VII assay had a monthly average CV of approximately 5.31% and blind duplicate analyses indicated a technical error of 6.2% with a correlation of coefficient 0.9133. Factor VII assay will be done on each test plasma as singleton measurements on each of two dilutions, 1/20 and 1/40. If the Factor VII concentration is too high, the sample is assayed again at a higher dilution. The assay requires a minimum of 0.50 ml of citrated plasma. The plasma should be centrifuged as soon as possible following phlebotomy, using at least 30,000 g min of centrifugation. Ideally, the sample should not be cooled on ice following phlebotomy, nor should the centrifugation occur at 4°C, but rather at room temperature. Factor VII may be activated in the cold and this should be avoided whenever possible. Following preparation of plasma, the samples should be frozen as soon as possible. When thawed, the samples will be thawed quickly and assayed immediately to minimize the possibility of Factor V[illegible] activation.

3. *PAI-I*.

PAI-I will be assayed in plasma using an ELISA method originally developed by Coller and colleagues. This method is sensitive to free and latent forms of PAI-I, but not complexed forms. The robotic system in our laboratory, Hewlett Packard Microassay System) automates virtually all aspects of this assay, including sample dilution, incubations, enzymatic color generation, absorbance reading, and data reduction. Quality control will be established with two control-plasma (both approximately normal), and so-called Westgard rules (a multi-rule Shewart QC System) will be applied. Longitudinal drift will be assessed using lyophilized control plasma. In routine use in the UV laboratory, this assay has a CV of approximately 9%. PAI-I ELISA will assay each sample at a fixed solution, in duplicate. If the sample results do not fall in an acceptable range based on a standard curve, then additional dilutions will be made and a sample rerun. The standard curve will be made from pooled plasma calibrated to agree with the laboratory of Dr. Desire Coller in Leuven, Belgium. There are no generally agreed upon standards at this time. The minimum sample of volume required is 0.25 ml of concentrate plasma, prepared as listed for fibrinogen.

F. Apoprotein E-Genotype (MIBH).

The analytical method is identification of the genotype based on the amplification of a key portion of the gene for apo-E using PCR (polymerase chain reaction) and identification of the gene (apo-E2, apo-E3, apo-E4) based on the pattern of DNA fragments produced by cleavage with the restriction enzyme (HhaI). This method is a modification of a published method (J.E. Hixson and D.T. Vernier, Restriction isotyping of human apolipoprotein-E by gene amplification with cleavage with HhaI, J Lipid Research 31:545-548, 1990). White blood cells from whole blood collected in EDTA serve as the source of DNA. One hundred microliter aliquot of whole blood is stored in a small centrifuge tube and 500 ul of a Tris-EDTA buffer is added to lyse the red blood cells. The sample is mixed by inversion and centrifuged in a table-top centrifuge to pellet the intact white blood cells. Supernatant is carefully removed, and the pellet is washed with Tris-EDTA buffer again. The super natant is discarded, and the resultant pellet is stored frozen. Reagents include Proteinase K (Signe Chemicals, St. Louis) for the isolation of DNA from leukocytes, TAQ polymerase (Promega) and the deoxynucleoside triphosphates (Boerhinger Mannheim) for amplification, the HhaI enzyme (Promega) for cleavage into fragments and standard pBR 322 DNA-Msp1 digest (New England Biolabs, Beverly, MA) to use as a standard marker on electrophonetic gels used to separate DNA fragments. We have prepared our own sense and antisense primers using the PCR-mate DNA synthesizer (Biorad).

G. Supplemental Analyses

If funds should become available, a number of additional analysis will be performed. These analyses will not require additional analyses, as sample collection and processing took these possible analyses into account.

1. LDL Subfractions (Louisiana State University - Pennington)

These analyses will require one EDTA-plasma cryovial (0.5 ml) from each endpoint package; 100 uL/assay is needed to be done in duplicate, for a total of 200 ml per endpoint on subjects from all four field centers.

LDL and HDL size distribution will be determined in frozen plasma samples obtained from each of the last three weeks of each dietary period from each subject. Size distribution will be determined by nondenaturing gradient gel electrophoresis as described by Musliner and Krauss (1) and Blanche et al. (2) with the exception that in-house 2-30% concave acrylamide gels will be used (3). The format of these gels is such that LDL and HDL size distribution can be determined from a single gel. All samples from a given individual will be analyzed at the same time on the same batch of gels and, to the extent possible, on the same gel. Two quality-control samples, obtained from single-use aliquots of frozen (-80°C) plasma, will be included on each gel. The quality-control plasma will be chosen to provide different LDL phenotypes and high and low HDL_2 ($HDL_{2a}+HDL_{2b}$) levels.

Gels will be stained with Sudan black B as described by McNamara et al. (4). The lipid distribution, as a function of gel migration (R_f) will be determined by densitometry at a resolution of 84 uM employing a BioRad GS-670 Imaging Densitometer. The R_f-based distribution will be converted to a particle size-based distribution employing the pardigm developed by Williams et al. (5) or by custom software available at this Field Center. From the distribution of relative lipid-stain intensity versus particle diameter, the following parameter will be determined:

a. LDL phenotype (A, B, or Intermediate) based upon the peak diameter for LDL.

b. "LDL score" as defined by McNamara et al. (4) and based upon the weighted distribution of LDL among seven size classes as described by Krauss and Burke (1).

c. Relative LDL subpopulation distribution based upon Gaussian deconvolution (PeakFit Software) of the LDL pattern. Data will be expressed as percent of total LDL lipid staining intensity distributed among the seven LDL size classes.

d. Relative HDL subpopulation distribution based upon Gaussian deconvolution of the HDL pattern. Data will be expressed as percent of total HDL lipid staining intensity distributed among five HDL size classes as defined by Blanche et al. (2).

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2. HDL Subfraction (Columbia University)

For assay of HDL-2/HDL-3, two or three EDTA-plasma cryovials (0.5 ml each) from each endpoint package will be needed. Each assay requires 500 uL and are done in duplicate per endpoint on subjects for all four field centers. HDL-2 cholesterol will be determined as the difference between whole HDL cholesterol and HDL-3 cholesterol after differential precipitation of HDL-2 from the dextran sulfate/Mg++ supernatant with 10 g/L dextran sulfate and 1.5 M $MgCl_2$ following the procedure of Gidez et al., J. Lipid Res. 23, 1206-1223 (1982).

3. LDL Resistance to Oxidation (University of Minnesota)

For assy of LDL resistance to oxidation, two serum cryovials (0.5 ml each) for each endpoint package will be required from subjects at all four field centers.

Measurement of LDL resistance to oxidation with hemin and H_2O_2. LDL (1.019-1.063 g/ml) will be isolated from 1 ml of serum by sequential ultracentrifugation. LDL will be oxidized with hemin and H_2O_2 in 96-well Immulon 1 microtiter plates (Dynatech, Chantilly, VA). The oxidation of LDL will be monitored by measuring the decreasing absorbance of hemin at 405 nm. The decrease in hemin absorbance parallels the increase in thiobarbituric acide reactive substances (TBARS) and conjugated dienes. TBARS will also be measured. The final assay concentrations for the micotiter assay will be 40 ug/ml LDL protein, 2.5 uM hemin, and 50 uM H_2O_2 in HEPES-NaCl (10-50mM) pH 7.4 buffer in a final assay volume of 0.15 ml. The assay will be started by the addition of H_2O_2. Each LDL sample will be assayed in quadruplicate. After the addition of hydrogen peroxide, the plate will be read at 43 second inter vals for four hours in a Vmax kinetic microtiter plate reader (Molecular Devices, Menlo Park, CA). The resistance of LDL to oxidation will be measured as the time required for LDL oxidation to reach maximum velocity, i.e., time to Vmax. The time to Vmax will be computed by computer software linked to the plate reader (Molecular Devices, Menlo Park, CA). The correlation (r) between lag time and time to Vmax in 56 samples was 0.992, however, time to Vmax was approximately 10 minutes longer than lag time. The analytic and analytic plus biologic coefficients of variation for the method were 5.9 and 9.6 percent, respectively.

4. Assays of Sex Hormones in Premenopausal Women

Three hormone assays will be performed on serum from premenopausal women: progesterone, estradiol, and luetinizing hormone. These will be used to assess the menstrual cycle and identify the particular phase (follicular, late follicular, and lutea) in order to

assess any effect of the menstrual on the outcome parameters being measured. Two serum cryovials (0.5 ml each) will be required from premenopausal female subjects from two field centers (Penn State and LSU).

a. Progesterone.

Progesterone will be measured by an RIA method using ^{125}I tracer (Count-a-Count from Diagnostic Products Corporation, Los Angeles, CA). This method is a solid phase immunoassay. The assay uses 10 ul of serum or plasma and the analyte is stable for six months at -80°C.

Reference ranges

Follicular	0.1 to 1.5 ng/mL
Luteal	2.5 to 28 ng/mL

b. Estradiol.

Estradiol will be measured by an RIA method using ^{125}I tracer (Coat-a-Count from Diagnostic Products Corporation, Los Angeles, CA). This method is a solid phase assay with virtually no cross reactivity with other steroid hormones. The assay uses 100 ul of serum or plasma and the analyte is stable for six months at -80°C.

Reference ranges

Early follicular	30-100 pg/ml
Late follicular	100-400 pg/ml
Luteal	50-150 pg/ml

c. Luetinizing Hormone (LH).

LH will be measured by an RI method using ^{125}I tracer (Coat-a-Count from Diagnostic Products Corporation, Los Angeles, CA). This method is a solid phase assay with virtually no cross reactivity with other steroid hormones. The assay uses 100 ul of serum or plasma and the analyte is stable for six months at -70°C. LH maintains a low and constant level in the blood during the follicular phase, rise rapidly at ovulation and fall to a constant level during the luteal phase. The rise of LH at ovulation is of about a three-day duration.

Reference ranges

Follicular	0-14 mIU/mL
Midcycle peak	20-70 mIU/mL
Luteal	0-16 mIU/mL

VII. QUALITY CONTROL PROCEDURES

A. Overview

To assure validity of the results in this study requires rigid adherence to collection, labeling, and shipping protocols. To ensure that these are well understood, a training session will be held to provide training and experience before the beginning of the project. Adherence to these protocols, including periodic reviews and retraining, are the responsibility of each field center.

In addition, other quality control measures will be taken to assess the integrity of the stored samples once they are removed from storage for analysis (sections VI.B and C.) in addition to individual laboratory quality control programs that are in place for each analyte (section VI.D.).

B. Sample Integrity

Aliquots of samples will be frozen upright at -80°C before being placed in boxes for long-term storage and shipping. Upon removal from freezers for shipping or analysis, the sample should be inspected to for evidence of thawing and leaking. If the liquid is no longer frozen (with top of frozen layer being horizontal in the vial) in the bottom of the vial or if there is evidence that the sample has leaked, based on less than the original volume remaining in the vial, the sample will be discarded and replaced by another sample that is acceptable.

Upon receipt at local or central laboratories, samples will be inspected upon unpacking to assure that they remained frozen in transit. Any sample showing evidence of being thawed will be noted. If transportation to the local labor atory occurs immediately upon removal from the -80°C freezer (within three hours) the sample needs to be kept frozen. Any sample that showed evidence of thawing but remained cold during transport may be analyzed for lipids and lipoproteins, but not for coagulation factors. Analysis should occur within three days of receipt of these samples. Any sample that leaked during transport will not be analyzed.

Any sample that has been identified as thawed will not be included in the data analysis if the analytical results are greater than 3SD (biologic plus analytical) from the average of the other three in that set of four.

Expected biologic plus analytical SD for triplicate analysis (Clin. Chem. 36,209, 1990)

	1 SD
Cholesterol	5%
Triglyceride	13%
HDL-chol	7%
Apo A-1	10%
Apo B-100	11%

Sample identity will also be verified based on packing lists and FAXed inventory lists. Any shipment that shows a discrepancy between the packing list and the actual contents may be sent back to the originating center to correct if the discrepancy cannot be resolved by a phone call.

C. Analytical Integrity

Samples will be coded such that all samples from a single subject can be identified and grouped into three sets, from each of the three diet phases. These will be analyzed for ea analyte on a single run for the analyte. If any sample of a set from the end of a single diet trial is more than 3SD (biologic plus analytical) from the average of the others, it will be reanalyzed promptly to confirm the value. It is the responsibility of the laboratory performing each test to review results on a run to run basis to identify such outliers and schedule a repeat analysis.

D. Analytical Quality Control Procedures

All analytical runs will include appropriate quality control samples to assure the validity of the run. Quality control samples will be analyzed at specific intervals with each run as specified by the manual of operation for the laboratory performing the analysis. If the quality control materials do not meet the criteria set by the laboratory's manual of operation, the run must be rejected and repeated in total.

E. Longitudinal Control Procedures

To be able to assess comparability of analyses in earlier years and later years for this study, samples other than diet study samples, will be used as not to deplete the archival stores of study subject samples.

Three volunteers would be recruited from each center to provide a series of "endpoint" samples which would be treated and aliquoted as a modified endpoint set to provid six sets of vials for each of the core analytes. These would be drawn and processed in the same way as a regular endpoint series (Section III), except that two extra blue-top tubes will be drawn and processed, to assure adequate blue and green aliquots. A series would be sent for analysis with the samples at the end of the baseline/recruitment phase. The remainder would be held and anlyzed at the end of each successive diet trial for the core analytes - lipid profile, apolipoproteins A-1, B-100, and Lp(a) and for fibrinogen, PAI-1 and factor VII. These samples (12 in all) would reflect the comparability of results over the course of the entire project.

All field center laboratories will, in addition, participate in the Lipid Standardization Program of the Centers for Disease Control, which provides some control for longitudinal samples of lipoprotein profiles.

VIII. FORMS

A number of forms will be required for the tracking of sample collection and management of data (copies in Appendix F):

A. Baseline Lipid Profile Form

This form should be completed on all subjects from whom a baseline lipid profile is collected, and sent with the sample to the field center lipid laboratory.

B. Baseline Apoprotein E Genotyping Form

This form should be completed on all subjects from whom an Apo E Genotyping is collected at baseline, and a copy sent to the field center with the amber cryovial containing Buffy Coat.

IX. DATA RECORDING AND TRANSFER

A hard copy of all results will be retained by all laboratories for the duration of the study. Values from the field center lipid laboratories and the MIBH Lipid Laboratory will be submitted to each field center by FAX, regular mail, or electronically, using predesigned forms. The UV will send hard copy plus data entered on floppy disk.

X. ALERT VALUES

Results beyond these values should be flagged for review by the field center physician, who may elect to contact the patient's physician.

Assay	Call-Back Value
Creatinine	Above upper limit of normal
Hematocrit	Below lower limit of normal for age/sex
TSH	Above upper limit of normal
AST/ALT	Above 2x upper limit of normal
Total cholesterol (mg/dl)	greater than 200 mg/dl
Triglycerides	greater than 250 mg/dl (immediate contact if greater than 1,000 mg/dl)
Glucose (mg/dl)	less than 60, greater than 140

X. TRAINING PROCEDURES

A. Field Center Research Nurse/Technician Training and Certification.

Field center technician training and certification is accomplished by a standardized training course at the Coordinating Center with performance monitoring at the individual field centers. This initial training includes a 1½-day seminar with both lecture and laboratory components. At the completion of the course, the technician will successfully complete a written and practical examination. The technician's performance is also evaluated at each field center by observation by the field center investigators and by periodic examination by supervisory personnel. Recertification of field technicians takes place prior to each blood-drawing year or when new technicians are enrolled.

B. Training Course by MIBH and UV Staff.

The purpose of the training course is to provide standardized methodology for venipuncture and blood processing for the field centers. Training will also include collection of capillary blood specimens by finger-stick and use of the LDX. Standardization of procedures is important for the quality of blood samples from participants. The UV training session is preceded by a training video and consists of a three-hour lecture component followed by a four

hour laboratory session, on day 1 of the course. The second day's activities are concerned with certification of field center technicians through a written and practical examination. Prior to th UV training courses, the field center technicians are required to review the field center manu of operations and view a training video of the procedures used for the sample collection.

C. Lecture Component Objectives.

1. Overview and purpose of blood sample collection for UV.

There will be a half-hour discussion of the importance of blood collection processing phases and the success of the DELTA Study. The role of the MIBH and UV laboratories in the study will be reviewed; some informational laboratory testing with participants is included.

2. Venipuncture techniques.

This lecture presents information relating to the collection of blood samples: infection control, safety precautions including new OSHA regulations, management of the participant, special problems associated with finger-stick and venipuncture, handling of equipment, procedures for fingerstick and venipuncture, and completion of phlebotomy forms. Demonstration of finger-stick using LDX and venipuncture using the butterfly apparatus is seen.

3. Processing of blood samples.

This lecture presents information on the proper procedures for processing venipuncture blood samples: purpose and proper management of each blood collection tube, aliquot rack setup, detailed instruction for the preparation of aliquots from each tube, centrifugation and temperature requirements for each tube, correct completion of blood processing form, and procedures for local storage.

4. Sample shipment to the central laboratories.

This lecture presents information on shipment schedules and packaging of blood samples.

5. Quality assurance.

a. Didactic session will be given which summarizes and explains all QC procedures which are to be used, blood collection, processing, and shipment.

D. Laboratory Component Objectives:

1. Preparation for venipuncture.

The field center research assistant/nurses prepare the blood collection trays and aliquot racks for blood drawing. The technician should be able to state the order of tube drawing, the purpose of each tube, the temperature of tube during and post phlebotomy, appropriate aliquot tubes for each collection tube, temperature, centrifugation, and the final number of aliquots to be forwarded.

2. Finger-stick and venipuncture on volunteer subjects.

The technicians practice on one volunteer at a time following finger-stick and phlebotomy protocol. If the technician does not feel comfortable, he or she may observe for a longer period. At least one practice finger-stick and venipuncture will be done by each technician.

3. Sample processing.

Following the venipuncture on each volunteer, the technician proceeds to process the samples as outlined in the manual. The technicians can practice sample centrifugation and pipeting, gain familiarity with aliquot tube manipulation and color coding, and complete the processing form. This exercise will be supervised to assure proper procedural guidelines are understood by technicians.

4. Local storage and sample shipment.

Once the processing is completed, the technicians place their aliquot racks in the freezer. Once samples have frozen, the aliquots are packaged in mailing containers and prepared for shipment.

E. Certification of Training.

The second day of training is a half-day session. During the session UV and MIBH personnel evaluate the performance of the field center technicians. The technicians are given a written and practical examination. The written examination consists of multiple choice and true/false questions covering information presented in the field center manual. The practical examination requires performance of both phlebotomy on the volunteer and processing of the sample collection tubes. UV and MIBH supervisory personnel monitor the venipuncture and processing performance and evaluate the technician's performance using the standardized certification form. Both written and practical examinations must be successfully completed, as well as an on-site observation, in order for CHF certification to be granted to the technician. An example of the written examination and certification form are included in the appendix. Once the field center technician has been fully certified, including observation on-site, he/she is qualified to certify other technicians in the complete or partial process. The three steps for certification are:

1. Successful completion of written exam (prepared by UV).
2. Successful completion of practical exam (using certification form).
3. Observation by certified personnel of complete phlebotomy/processing procedures on the volunteer/participant.
   Completed written exams will be corrected and kept on file at the UV.

F. Monitoring Field Center Technicians.

Supervisory personnel, such as the study coordinator, monitor the performance of the field center technician through observation of phlebotomy on participants at each field center. Visits to each field center during the study provide a quality assurance check on blood collection and processing of participant samples. Review of blood collection forms by MIBH/UV will also be done, providing additional monitoring of field center activities.

G. Maintaining Certification:

In order to maintain certification, the technician must complete phlebotomy and processing in one-full-day's worth of participants every two week period, not necessarily on a single day.

CHAPTER 11

Chapter 11: DATA MANAGEMENT FOR DELTA

1. Data Collection

The data collected for the DELTA study will be recorded on paper forms at each of the participating field centers. The local study data coordinator will be responsible for the secure and confidential management of original paper forms. Data unique to site-specific ancillary studies will be kept at the local DELTA field center with data management and analysis performed by individual study principal investigators. A microcomputer based data management system for DELTA developed by the Collaborative Studies Coordinating Center (CSCC) will provide all of the capabilities required for research data management for the multi-center trial, including: data entry, data editing, data transfer, and database processing.

2. Data Entry

Data forms will be keyed using a full-screen FoxPro data entry system developed specifically for the DELTA study. The electronic screens will closely resemble the paper forms in format to facilitate online management and review of the data. Each data value will be edited during entry. The data entry system will flag each data value with a "status character" documenting the current editing status of the value (empty, skipped, passed edit, questionable, confirmed, etc.). Users will have the ability to flag, or annotate questionable data items. Updated data values will be entered and edited in the same manner as the original data values. Central Lipid laboratory data will be keyed centrally at the CSCC. Written data queries will be generated on a periodic basis for resolution by the local data or laboratory coordinator. Once the local center, or laboratory has responded in writing on the query, the necessary action to resolve the query will be implemented by the Coordinating Center.

3. Data Transfer

Data files for the DELTA study will be generated by the microcomputer based data management system onto diskette.
Copies of the electronic data files will be transferred by diskette to the following address:

Collaborative Studies Coordinating Center
Attention: DELTA Central Receiving
Suite 203, 137 E. Franklin St.
Chapel Hill, N.C. 27514

Each data transfer diskette should be sent in a protective diskette mailer. The original paper forms will be kept at the field center site by the study data coordinator. Do not send paper forms to the Coordinating Center unless specifically requested. The frequency of data transfer by the field centers to the Coordinating Center should be at least once a month.

Central agencies and laboratories (i.e., Food Analysis and Hemostasis laboratories) may also send data on diskette to the CSCC; however, all information transferred electronically must be in a format that conforms to DELTA data transfer standards to insure complete and accurate processing.

The central Lipid laboratories will send the results of the blood lipid determinations to the CSCC on original paper forms at the address given above, while retaining one legible copy for local archival. Lab data forms will be shipped in batches from the originating lab on a regular basis. Each batch of forms will have an inventory form that details the number of participant samples analyzed and transmitted with the batch. The receipt of each batch will be logged, and the contents reconciled against the accompanying inventory form within one working day of receipt. Discrepancies will be communicated to the originating laboratory coordinator in writing.

4. Database Processing

The receipt of each data transfer diskette will be acknowledged by the CSCC, after being logged into the study inventory system. Any discrepancy discovered while processing the data will be communicated to the originating data coordinator or central agency in writing. The study data will be stored in a single collaborative database. All the data dictionary / schema definition facilities available by the data base management system will be used to document and format the database. Standard transaction validity checks will be applied to all updates to the database (e.g., to prevent the addition of records with duplicate keys, etc.). A journal file of all updates to the database will be maintained. Database backups will be made daily with the archival of the data files occurring monthly.

All data (paper and electronic) will be stored, processed, and analyzed within the CSCC office suite. At the CSCC, all access to office space containing data is controlled through manned reception areas. Visitors are screened by the receptionists and cannot move about without a CSCC escort. All office space is locked after working hours. Access to computer data files is controlled by passwords released only to those CSCC personnel who use the files. In addition, critical data files are encrypted.

A backup of the study database will be made daily to a separate file server on the CSCC local area network. Magnetic tape backups of the database will be made weekly, using a father/grandfather cycle with 5 generations. Once a month, the current backup tape will be removed from the cycle and permanently archived at the CSCC's off-site data storage facility.

Output mailed to clinic staff will identify participants only by ID number. No individually identifiable information will distributed to clinical sites.

Data will be processed, and analyzed using in-house computers by CSCC staff.

CHAPTER 12

Chapter 12: ANCILLARY STUDIES

A. Introduction

Ancillary studies have the potential to enhance the scientific value of the DELTA study in many ways, such as mechanistic studies, adherence evaluations, evaluation and development of methods for measurement of compliance, and evaluation and comparison of nutrient data bases.

B. Criteria

Ancillary studies should not interfere with execution of the main study. If ancillary studies will require modification of the daily routine of the main DELTA study, specific arrangements must be made by the responsible Principal Investigator to extend dietary periods for the purpose of conducting ancillary studies.

C. Evaluation

Ancillary studies will be evaluated by the Principal Investigators, when they make recommendations regarding the feasibility and merit of the ancillary studies, as well as by the external Protocol Review Committee.

D. Authorship

Authorship for ancillary studies will include only members of the participating Center(s), but acknowledgement will be given to the overall DELTA study.

Proposed ancillary studies, as submitted, are compiled in Appendix D according to individual Centers. Final selection of ancillary studies will take place at a later date.

APPENDICES

APPENDIX A
CONSENT TO PARTICIPATE

CONSENT TO PARTICIPATE
IN THE

Dietary Effects on Lipoprotein and Thrombogenic Activity Study
(DELTA)

_____, Principal Investigator
(Clinic name and address)

Guide to the Department of Health, Education and Welfare Policy on Protection of Human Subjects DHEW Publication No. (NIII) 72-120, pg.7.

I agree to participate as a research participant in the Diet Effects on Lipoprotein and Thrombogenic Activity Study (**DELTA**). This research program is the first multi-center controlled feeding study of dietary effects on coronary heart disease risk. Four centers in the U.S. will enroll a total of 120 participants for this study, who will be given a controlled feeding regiment that is identical in all centers..

Purpose of the Study

Heart disease is the leading cause of illness and death in Americans, affecting more than __ million men and women. Coronary heart disease (CHD) affects the heart muscle, mainly as a result of atherosclerosis and its complications. Atherosclerotic plaques and thrombosis combine to interrupt blood flow to the heart, producing the clinical symptoms of heart disease and heart attack. Elevated blood cholesterol (fat in the blood), is a primary risk factor for heart disease. Abnormalities in blood clotting factors contribute to thrombosis. Dietary measures that have a beneficial effect on blood cholesterol and clotting could reduce the burden of CHD in Americans. In order to determine how dietary modification will benefit the general public, large numbers of participants need to be studied under very well controlled conditions.

Procedures

I understand that my participation in DELTA will involve the following procedures:

1. At the beginning of the study, I will be asked questions about my medical history, current medications and questions about my dietary habits, dietary restrictions and physical activity.

2. I will be asked to follow a diet in which all foods and beverages will be provided. I will be asked to eat everything provided and not to eat any food or beverages from

outside sources. The diets will consist of wholesome foods that meet the Recommended Dietary Allowances (RDA) for essential nutrients and adequate calories to maintain my present weight. The experimental diets will be modified in type and amount of fat.

3. I will be asked to fill in a daily food record check list when I come to the feeding site. I will be asked to eat a minimum of 10 meals per week at the feeeding center, 5 of which are dinners and will be provide take out meals for the remaining meals and snacks. I will have my weight measured two times every week.

4. I will be asked to provide adequate refrigeration to store foods that are provided for offsite consumption.

5. I will also have blood samples drawn from a vein in my arm at regular intervals during the study, for the measurement of total cholesterol, HDL, and LDL cholesterol blood clotting factors and for a variety of blood tests which will evaluate my overall state of health. Each blood sample will involve approximately 2 Tablespoons of blood. A total of ___ samples will be collected at regular intervals during the study.

I understand that all food will be provided at **(name and address of center)**. On-site meals will be served between _____ and _____ (breakfast) _____ and _____ (lunch) _____ and _____ (dinner). Filling in the daily food record will take about 5 minutes.

Risk/Discomforts

The diets that will be fed will consist of wholesome foods and contain adequate levels of essential nutrients. Foods will be prepared according to accepted standards of sanitation and provision made to ensure the safety of foods provided for off site consumption. However, it is possible that incorrect food handling during shipping, storage or preparation if not detected could result in food borne illness. Every effort will be made to safeguard against this possibility.

Feeding studies that require on site eating of meals and strict adherence to the diets provided may interfere with social activities centered around eating such as dining in restaurants. While rotating menus will provide some variety in the diets, the number of food items will be more limited than available in an average grocery store. The limited variety may become boring over the course of the study.

Other risks of the study involve those of taking blood. These include: commonly, the occurrence of discomfort and/or bruise at the site of puncture; and less commonly, the formation of a small blood clot or swelling of the vein and surrounding tissue and/or bleeding from the puncture site.

Possible Benefits

Elevations in cholesterol values may be associated with a greater risk of developing atherosclerosis (fatty deposits in the arteries) and coronary heart disease such as angina or heart attack. The benefits of participation in this study include the possibility that I will experience lowering of my blood cholesterol improvement in some clotting factors and that I may enhance scientific knowledge concerning the most effective diet modifications to lower the risk of CHD. No benefit from this treatment can be guaranteed.

Alternative Therapies

This is not applicable, because there are no alternative therapies. This is a diet study, not a drug study.

Costs

There will be no cost to me for my participation in the study. I understand that I will be reimbursed for some travel expenses such as local taxicab fare, parking at the clinical center, the fare involved in traveling to the center by subway and/or mileage to and from the clinic. I will receive all food free of charge during the study.

Participation and Termination

Participation in this study is entirely voluntary. Participants are completely free to withdraw from the study at any time without adversely affecting their future care by Drs. ( )

I will be informed of any new information that may affect my willingness to participate. Any questions that I may have concerning any aspect of this study will be answered by Drs. ( ) and by other members of the clinic staff. The clinic's address is ( ). I also understand that I am free to refuse to participate or to withdraw from participation in this study at any time and that it will in no way affect my relationship with, or treatment by these physicians or the ( ).

Drs. ( ) should be notified immediately of any new condition or injury which develops during the course of the study. The (name of University) which is sponsoring the research, does not furnish any funding for medical treatment or compensation for human subjects in the event the research resulting in physical injury. In the event of such injury the University will make available only immediate and essential medical treatment including hospitalization. For further information about this, I may call the office of the (Institution) Institutional Review Board at (phone number).

I understand that I may be asked to leave the study at any time if I do not comply, and if I am discontinued, there will be no compensation because the study was not completed.

Confidentiality

My research records will be handled as confidentially as is possible within the law. All records are coded with an I.D. number and no names are transmitted to the central data processing center. Records containing names or other identifying information are kept under lock at the clinical center, and only study investigators have access to them. Under certain circumstances, monitors from the National Heart, Lung and Blood Institute or members of the (Institution) Committee on Human Research may need to see some specific records in order to verify the study data. No individual identities will be used in any reports or publications resulting from this study. At the end of the study, I will be given my laboratory results without cost, informed of the results of the study, and advised on their implications for my future care.

Rights of Investigators

-Right not to enroll a subject
-Right to terminate for following reasons: (list)

I have read and understand the consent form and have had an opportunity to discuss this study with a member of the clinic staff. All my questions regarding my rights as a research participant concerning this study have been answered to my satisfaction and I hereby willing consent to participate in this study. A copy of this consent form has been given to me.

_____ _____

Signature of Participant Signature of Witness

_____ _____

Date Date

_____

Signature of Investigator

Date

APPENDIX B

LIST OF DELTA FORMS TO BE DEVELOPED

APPENDIX B

LIST OF DELTA FORMS TO BE DEVELOPED	
1.	Recruitment Log
2.	Telephone Screening Log
3.	Visit 1 Screening Questionnaire
4.	Explanation of Study Checklist
5.	Food Record
6.	Participant Agreement
7.	Visit 2 Clinic Form with Lab Checklist (blood chemistries and urine)
8.	Clinical Form with Diet Adherence Checklist and Participant Assessment
9.	Randomization Form
10.	Consent Form
11.	Food Analysis Data Entry Form
12.	Shipping Forms for Food Samples
13.	Diet Preparation Verification
14.	Diet Compliance and (Daily) Weight
15.	Central Food Preparation
16.	Shipping Forms
17.	Data Entry Form for Lipids and Lipoproteins
18.	Data Entry Form for Hemostasis Variables
19.	Clinic Visit Form for Phlebotomy and BP (post randomization)
20.	Hedonic Scale (for Pilot Study)
21.	Form to Use when Calibrating Weights for Scales (for Pilot Study)
22.	Close-out Form
23.	Validation of system to track lab procedures
24.	Lab Standardization Form

APPENDIX C

CONFLICT OF INTEREST

THE UNIVERSITY OF NORTH CAROLINA
AT
CHAPEL HILL

Collaborative Studies Coordinating Center
Department of Biostatistics
School of Public Health

137 E. Franklin St., Suite 203, CB# 8030
The University of North Carolina at Chapel Hill
Chapel Hill, N.C. 27514-4145

Telephone: (919) 962-6971
FAX: (919) 962-3265

M E M O R A N D U M

TO: DELTA Principal Investigators

FROM: Ed Davis

DATE: December 29, 1992

RE: Conflict of Interest Statement

At the December 15, 1992 meeting it was decided that no formal guidelines for a conflict of interest for DELTA investigators would be stated. However, the Coordinating Center was given the charge of maintaining a list of the major associations of the PI's with the food industry. With this in mind, please respond to the following:

1. List all consulting activities in the calendar year 1992 with a food industry company. Consulting is defined as providing advice for which you received financial compensation.

2. List all food industry companies which provide financial support for your research program(s).

3. List all food industry companies in which you or your immediate family (spouse or children) own stock.

Please send your lists to me at your earliest convenience. I will file them at the Coordinating Center in a confidential file which will only be forwarded to NHLBI, if requested.

APPENDIX D

ANCILLARY STUDIES
(will become part of Chapter 12)

1. Influence of Diet Compostion on HDL Subpopulations
(Dr. Paul S. Roheim, Principal Investigator)

2. Effect of Dietary Saturated Fatty Acids on HDL Function (Michael Lefevre, Ph.D., Principal Investigator)

Reductions in the intake of dietary fat, especially saturated fat have been recommended as a means to lower plasma and low density lipoprotein (LDL) cholesterol levels. However, these dietary recommendations also tend to lower plasma high density lipoprotein (HDL) levels. While recent experimental and epidemiologic data clearly show the benefits of lowering LDL levels, the physiologic consequences of reductions in HDL levels are less well understood. HDL are thought to play a pivotal role in reverse cholesterol transport, i.e., the delivery of peripheral cholesterol to the liver for excretion. We hypothesize that diet-induced changes in HDL concentration and composition are associated with alterations in the ability of HDL to promote net cholesterol efflux from cells. To test this hypothesis, we propose to determine the effects of dietary saturated fatty acid reductions on the ability of HDL to promote net cholesterol efflux from GM3468 cultured fibroblasts.

A subset of 12 subjects will be recruited from the study population enrolled at the PBRC Field Center (PBRC). The subset will be balanced with respect to race and gender. A single plasma sample will be obtained from each subject during the last two weeks of each of the three dietary periods. N-ethylmaleimide and PPACK will be added to plasma to inhibit lecithin cholesterol acyltransferase (LCAT) and thrombin, respectively.

The ability of HDL to promote cholesterol removal from cells will be examined by measuring the bi-directional flux of cholesterol between HDL and GM3468 fibroblasts as described by Johnson et al. (*J. Biol. Chem.* 261:5766, 1986). ApoE, apoB-depleted plasma (prepared by heparin-affinity chromatography or heparin-Mn^{++} precipitation) will be used for these studies to minimize potential artifacts resulting from ultracentrifugation. ApoE, ApoB-depleted plasma will be characterized with respect to the content of free cholesterol (FC), cholesterol ester (CE), plasma lipoproteins (PL), triglycerides (TG), apoA-I and apoA-II. Data will be corrected back to the concentration of whole plasma using albumin as a recovery marker.

Media containing [^{14}C]cholesterol-labeled plasma, diluted to 1% of original concentration, will be incubated with the [^{3}H]cholesterol-labeled cells for 4 hours. Following incubation, the media and cells will be harvested and analyzed for isotopic content. Rate constants for cholesterol influx and efflux will be derived from the fractional change in media constants for ^{14}C-cholesterol or cellular ^{3}H-cholesterol. Rate constants obtained will be converted to cholesterol flux (ng free cholesterol/minute or ng free cholesterol/minute/mg cell protein) into or out of the cell. Additionally, rate constants for cholesterol efflux and influx will be used to determine the estimated

increment (or decrement) in HDL free cholesterol content at equilibrium. Data will be analyzed as a function of plasma concentration and apoA-I concentration. Values based on plasma concentration will likely be dependent on both HDL concentration and composition and will reflect the intrinsic ability of plasma to support net cholesterol transport from cells. Calculations based on apoA-I concentration will reflect changes in HDL composition and subpopulation distribution.

3. Dietary Compliance During DELTA Studies as Measured by PABA Incorporation (Marlene J. Windhauser Ph.D)

4. Body Composition Determination Using TOBEC Instrumentation
(Bernestine Mcgee, Ph.D., Deputy Principal Investigator)

5. Comparison of Nutrient Databases (Catherine M. Champagne, Ph.D., Principal Investigator)

Rationale and background. The DELTA study is an appropriate study for comparing nutrient evaluations of recipes and menus being used for subjects. The rationale for the proposed study is that appropriate comparisons can be made between different databases being used in the study so that we may assess the differences between databases. No specific database has been selected at the present time for use in this study. This factor encourages initiation of the proposed study of database comparison.

Hypothesis and specific aims. Our hypothesis is that computerized nutrient evaluations of proposed menus using the Extended Table of Nutrient Values (ETNV) will be closely aligned with laboratory values. We arrived at this hypothesis as a result of recent comparisons of the Army Computerized Analysis of Nutrients (CAN) with laboratory analyses of small and large batch military recipes (Champagne et al., 1992). Results showed good agreeability with both the laboratory and with CAN.

Specifically, the aim is to carry the above work a step further to show that there is good agreeability with other nutrient databases. Use of a specific database in any given situation is dependent upon nutrient content of the database and economic issues related to its use.

Methods. It is our intention to analyze all recipes and menus for the study using the ETNV. This will enable comparisons between our database and the database selected for use in this project. We will ascertain identification codes being used by others who will analyze recipes to ensure that appropriate specific codes that identify the ingredients being used are obtained for these analyses. Using our recipe programs, we anticipate our results will closely match laboratory data. Spot checks of computerized data will be verified by laboratory results using the Pennington Biomedical Research Center food chemistry laboratory.

Number of analyses required. We anticipate that all recipes and menus that will be analyzed by other participating sites and using software programs will be analyzed using the ETNV.

Data analysis. We will analyze data with the assistance of the PBRC biostatistician, using the most appropriate method of statistical analysis. Because we

have previously published similar data, we anticipate that analyses will be very similar. Reports will be presented at scientific meetings and published in the literature.

Reference. Champagne, et al. Comparison of military recipes using computerized nutrient data bases and laboratory values. *Seventeenth National Nutrient Databank Conference*, 1992.

1. Measurement of LDL Receptors on Peripheral Mononuclear Cells (Penny Kris-Etherton, Ph.D., Principal Investigator)

Rationale/background. Peripheral blood mononuclear cells, although not employed routinely in human diet and lipoprotein studies, have proven to be a valuable model for mechanistic studies that examine LDL-receptor function. The advantages associated with their use are accessibility and resemblance to hepatic lipoprotein metabolism. We will use this cell system to examine changes in LDL-receptor levels induced by the experimental diets.

Hypothesis. Mononuclear cell LDL-receptor levels will decrease in response to experimental diets that elicit a decrease in plasma LDL-cholesterol levels.

Methods. Mononuclear cells will be isolated and LDL-receptors quantified (by measuring LDL-receptor protein mass) by ELISA as described by May et al. (*J. Lipid Res.* 31:1683, 1990). We will confirm any treatment effects seen with the ELISA methodology by doing Western blots that will allow us to determine whether there are any changes in molecular size of receptor protein. We will collaborate with Dr. Allen D. Cooper from the Palo Alto Medical Foundation Research Institute and Stanford University on these studies. Dr. Cooper will provide the LDL-receptor antibody.

Number of studies. This study can be conducted on all subjects who participate in the first feeding study at Penn State. Alternately, we can study a subset of subjects or only those who have a plasma LDL-cholesterol response to the experimental diets. We also can conduct these studies in subjects at other centers.

Data analysis. Statistical analyses will include analysis of variance with follow-up tests of pairwise comparisons to examine diet effects on subjects' mononuclear cell LDL-receptor levels.

2. Oxidative Status of LDL Fatty Acids (C. Channa Reddy, Ph.D., Principal Investigator)

Rationale/background. There is a growing body of evidence to suggest that oxidative modification of low density lipoproteins (OX-LDL) plays an important role in the pathophysiology of atherogenesis. In recent years, a number of molecular mechanisms of different oxidation pathways leading to modification of LDL-C have been proposed. It is now clear that the earliest step in the generation of OX-LDL is peroxidation of its polyunsaturated fatty acids (PUFA). It has been proposed that oxidative breakdown products, such as malonaldehyde and 4-hydroxynoneal, interact with apoB of LDL-C and specifically modify lysine residues. Modified apoB is readily recognized by the acetyl LDL receptor of the macrophages. This affects normal ***in vivo*** cholesterol metabolism and leads to a conversion of macrophages into the lipid-laden foam cells, characteristic constituents of atherosclerotic plaques on vascular endothelium. Foam cell formation and genesis of atherosclerotic plaques are thus dependent on the presence of highly oxidized PUFA in the low density lipoprotein-C (LDL-C) particles. One of the promising strategies for beneficially affecting coronary heart disease (CHD) risk is to alter plasma lipid composition by manipulating dietary fatty acid composition. For example, recently it has been reported that enrichment of diets with monounsaturated fatty acids (MUFA) results in LDL that are resistant to oxidative modification. Thus, the proposed ancillary study will test the hypothesis that dietary manipulation of fatty acids can be used to effectively reduce the oxidative potential of the LDL fraction.

Hypothesis. Dietary manipulation of fatty acids can reduce the oxidative potential of LDL.

Methods. The plasma LDL fraction will be isolated by single spin density ultracentrifugation. The peroxidation potential of LDL will be quantified by both malonaldehyde formation and diene conjugation. Approximately 150-200 μg LDL protein will be incubated with ascorbate-ADP-Fe^{+2}. Thiobarbituric acid reactive substances will be quantified and expressed as malonaldehyde equivalent content (nmoles/mg protein). For the determination of conjugated dienes, the above reaction will be terminated after 15 minutes by the addition of 6 N HCL. Oxidized fatty acids will be extracted and separated. The organic phase will be evaporated, redissolved in methanol, and the conjugated dienes will be quantified by measuring absorbance at 234 nm.

Number of subjects. This study can be conducted on all subjects at one, more, or all Field Centers.

Data analysis. Data will be analyzed by analysis of variance.

3. Dietary Assessment
(Helen Smicklas-Wright, Ph.D., Principal Investigator)

Rationale/background. Energy intake on experimental diets provided at levels to achieve weight maintenance are often higher than subjects' self-reported energy intakes. These and other sources of data suggest that some, but not all subjects, underreport energy intake. There is little information about specific sources of reporting error. Studies of reported versus actual intake have generally been limited to observations of a single meal. Few studies have partitioned error terms for food item versus portion size errors. Portion size errors may lead to general underreporting of energy and nutrients; omission of selected food items may have a differential impact on reported intakes of some food components, such as fats and saturated fats. Thus, there is a need to conduct a dietary assessment study to evaluate the contribution of item omission and portion size error to differences between self-reported and actual intakes.

Hypothesis. Subjects underreport their energy intake by omitting items and/or underestimating portion sizes.

Methods. To examine the accuracy of self-reported energy intake, we will compare actual and self-reported intake. We will conduct interviewer-initiated dietary recalls (3-day recalls during weeks 1 and 2 of the feeding study) using the Nutrient Calculation System (NDS) developed at the University of Minnesota. We will partition differences in actual and reported intake estimates into either differences in item omissions or reported serving sizes.

Number of subjects. This study will be conducted on all subjects in one feeding study. It can be done with subjects at one or more field centers.

Data analysis. Data will be analyzed by categorical data analysis (for omissions) and analysis of variance (for total intake and underestimated portions).

4. Family Feeding Studies (Madeleine Sigman-Grant, Ph.D., Principal Investigator)

Over the 2 1/2 years that feeding studies are planned in the DELTA project, we plan to recruit some men and women from the same household unit. Some of these "married couples" will have children. In an attempt to learn more about the genetics of dietary responsiveness, we will study a small number of healthy, prepubescent children over a 2 1/2-year period. We will look primarily at the response of plasma lipids and lipoproteins to diet in children and their biological parents and relate this information to Apo E genotype. This small pilot study will provide some information about dietary responsiveness inc hildren and the role of age and genetics in the magnitude of this resposne. This ancillary study may be used subsequently to plan larger-scale experiments designed to understand the genetic basis for dietary responsiveness.

5. Effect of Energy from Fat and Saturated Fat in the Diet on Sensitivity and Hedonic Response to Fat in Selected Foods
(Jean-Xavier Guinard Principal Investigator)

1. Platelet Studies
(Aaron Folsom, M.D., Patricia Elmer Ph.D., John Eckfeldt Principal Investigators)

2. Hemostasis Chage in Relation to Genotype
(Aaron Folsom, M.D., Patricia Elmer Ph.D., Principal Investigators)

APPENDIX E

METHODS FOR FOOD ASSAYS

METHODS FOR FOOD ASSAYS

Fatty acids (saturated, monounsaturated, polyunsaturated)

Fatty acid composition will be determined by gas-liquid chromatographic analysis of fatty acid methyl esters prepared from the saponified lipid extracts of diet composites: Diet composite samples are hydrolyzed, then the hydrolyzate is extracted with ethyl ether or petroleum ether, and the fatty acids are derivatized to methyl esters. The fatty acid methyl esters are separated and quantitated by GLC. Data will be reported for SFA (sum of fatty acids n:0), MUFA (sum of fatty acids n:1), PUFA (sum of fatty acids n:≥2), and omega-3 fatty acids (EPA, DHA, ∝-linolenic; reported separately).

Total fat

Quality assurance assays: Total fat in diet cycle composites will be monitored using a semi-automated gravimetric assay, in which lipids are extracted with either chloroform/methanol (modified Folch procedure) or methylene chloride (AOAC #), then weighed after evaporation of the solvent.

Documentation data: Total fat will be calculated as the sum of the triglyceride equivalent of the fatty acids (i.e. grams fatty acid x correction factor to convert to triacylglycerol), measured as described above. Alternatively tota fat will be determined gravimetrically using a modified Folch procedure.

Cholesterol

Cholesterol will be measured in chloroform/methanol extracts of diet composites using gas-liquid chromatography as described by Thompson and Merola [USDA Nutrient Composition Laboratory, Beltsville, MD, 1992].

Moisture

Moisture content of the diet composites will be determined with a microwave moisture/solids analyzer. Alternatively, a vacuum oven and an analytical balance will be used. Moisture values will be used to calculate nutrient data on a dry weight basis.

Starch

Starch will be calculated from glucose measured after treatment of diet composites with amyloglucosidase. Glucose is measured using a glucose oxidase assay (reference). Starch is calculated as grams glucose x 0.9, after correction for glucose content in the absence of enzymatic digestion.

Total Protein

Protein will be determined as total nitrogen x 6.25. Nitrogen will be measured by the Kjeldahl method.

Sugars

Sugar methods are being developed. The assay of the simple sugars will probably be done by high-performance liquid chromatography (HPLC) or a combination of enzyme treatment and HPLC.

Total Calories

Total calories will be calculated using total fat, starch, sugars, and total protein values (determined as described above):

kcals = (grams fat x 9) + (grams starch x 4) + (grams sugars x 4) + (grams protein x 4)

Until the sugar assays are brought on-line, the computed energy values from the menu planning database will be used (modified for deviations in the fat content?).

APPENDIX F

MEMORANDUM

TO: **DELTA** Steering Committee

FROM: **DELTA** Coordinating Center

DATE: April 13, 1993

RE: **DELTA Diet Pilot Study EZ Cycle 1 Analysis**

Attached are descriptive statistics of data from the DELTA Diet Pilot Study for the EZ food preparation strategy in cycle 1. Data available for the analysis were dietary measures from three menus prepared once at the four US field centers using the EZ preparation option which requires central procurement of fat-containing items and local preparation of menus according to standard specifications.

Nutrients reported from FALCC were total fat, saturated fatty acids (SFA), monounsaturated fatty acids (MFA), and polyunsaturated fatty acids (PFA). Total fat was determined by gravimetric assay. The SFA, MFA, and PFA values were determined by Dr. Raymond (Rick) Thompson at the Nutrient Composition Laboratory-Beltsville. The nutrient values were presented in grams per 2000 kcal of the daily menu and percent kcal. The computed value for total kcals used in the menu design (i.e., 2000 kcal) was used for calculation of each nutrient on a percent kcal basis. Total weight of the composite (grams), percent moisture in composite, and total dry weight of composite (grams) were also provided.

Data Collection

Foods were procured centrally and locally for the EZ preparation option. Recipes, ingredients by brand, food descriptions, weights, and instructions for preparation were distributed to the field centers by the diet subcommittee. Each center performed all the cooking and baking, weighing, and storage. Each one day menu was packaged, frozen, and shipped to the FALCC for food analysis. Also, fat containing food items from each menu were packaged separately, frozen, and shipped to the FALCC.

Results of the food analysis for EZ cycle 1 were received on March 26 at the DELTA Coordinating Center (DCC) on data collection forms designed by the FALCC and DCC. The data sent included total fat and fatty acids results for each menu.

DELTA PROTOCOL 1

Deviations from Protocol

Notifications of deviations from standard operating procedure were sent to the FALCC from the four centers following the preparations of the three menus. The comments provided are listed in Table 1.

Note that the calories for green beans in EZ cycle 1 menu 2 were not correct in the calculations of the menu. The menu was actually approximately 1600 kcals instead of 2000 kcals. For the descriptive statistics, the percent calories were recalculated for menu 2 using the number grams reported in the numerator and 1661.33 kcals in the denominator.

Descriptive Statistics

Tables 2 and 3 present the mean, standard deviation, minimum and maximum, and coefficient of variation from the SAS MEANS procedure, by menu and by center respectively. Also, a copy of the data from the data collection form is included as Table 4. Note that percent calories of all nutrients for menu 2 were recalculated due to the problem with the green beans as noted above.

Menu Comparison

Table 2 shows the field center means for individual menus, calculated to be at target levels. Menus varied in the assayed amounts for total fat and fatty acids. Menu 1 was on target but menu 3 was low. Menu 2 was higher than target because of the error in calorie level (1600 rather than 2000). Inter-center variance was similar for menus 1 and 3 but considerably less for menu 2. The variance in dry weight was also similar in menus 1 and 3 and very small in menu 2 suggesting more variability in sample weighing in menus 1 and 3. If this is true, this is a good indicator for quality assurance. Fatty acids were lower than target. This is attributable to differences in assay methods. Total fat extraction includes phospholipid as well as triglyceride whereas fatty acids are derived from triglyceride.

Center Comparison

Table 3 shows the means of three menus for each center. Three centers were on target for total fat. Fatty acids among these three centers were identical. PSU had somewhat lower total fat, however, the ratios of fatty acids to total fat were similar to the other centers.

Deviations from Analytical Values using Different Food Composition Databases

Figure 1 and tables 1, 2, and 3 present preliminary data comparing analytical values with calculated values from 4 different databases. All the databases underestimated fatty acids. The deviation was largest in menu 3 especially for SFA and MFA. Overall for the nutrients reported, ETNV performed somewhat better for the foods in these menus.

DELTA PROTOCOL 1
TABLE 1

Notification of Deviations from Standard Operating Procedures (abbreviated) For EZ 1, Cycle 1

Penn State	
Menu 1	Pineapple juice was drained, but the pineapples were not rinsed.
Menu 2	Amount in grams of egg yolk for turkey sandwich was listed as 12 gms in one place and 11 gms in another. Used 11 gms.
Menu 2	Meat for spaghetti entree was microwaved at high for 1 minute (rare, all juices in bowl). Sauce was added to meat, and whole mixture was microwaved at high for 0.5 minutes. Cooked spaghetti was then added to meat sauce.
Menu 3	Only 2 servings of butter were requested for day 3, but 'we' sent 3 servings.
Menu 3	Sirloin tips baked in oven at 350_0F for 12 minutes (rare), gravy then added, and mixture baked another 10 minutes at same temperature.
All	All meats were defrosted in the refrigerator for 6 hours.
All	Frozen vegetables defrosted at room temperature. Carrots and green beans were not thoroughly defrosted before weighing and cooking.
PBRC	
Menu 1	All Bran used instead of complete bran flakes.
Menu 2	Pepperidge Farm French bread used instead of Italian bread.
Columbia	
Menu 2	Pepperidge Farm "Distinctive White" used in place of Italian bread.
Menu 2	Egg yolk, 12 gms, used for lunch and not 11 gms. Calculations included 12 gms, but specifications stated 11 gms.
Menu 3	Bird's Eye carrots used instead of Green Giant.
Menu 3	Bottled Ocean Spray cranberry juice cocktail used instead of canned.

DELTA PROTOCOL 1

Menu 3 Mott's unsweetened applesauce used instead of Sexton.

Columbia used 4 new ziplock bags, because the bags became slightly wet before food was placed in them. Accuracy in labelling was maintained.

DELTA PROTOCOL 1
TABLE 1

Notification of Deviations from Standard Operating Procedures (abbreviated) For EZ 1, Cycle 2

Columbia

Menu 1	Used Dole pineapple chucks and unsweetned pineapple juice.
Menu 1	BARLETT Pears -- SHOPRITE in pear juice.
Menu 2	Libby's peaches in pear juice were used.
Menu 2	Spaghetti with meat sauce for "Prepared Fat Sources" could not be prepared as we did not have any meat left.

PBRC

Menu 1	All Bran used instead of complete bran flakes (30g).
Menu 2	Pepperidge Farm French bread used in place of Italian bread.

TABLE 2: Descriptive Statistics, L[illegible]4, for the DELTA Diet
Pilot Study, EZ Cycle 1

MENU	N Obs	Variable	Label	N	Minimum	Maximum	Mean	Std Dev	CV
1	4	TOTWT	Total Weight (gms)	4	1721.00	1974.00	1876.75	109.55	5.84
		MOISTURE	Moisture (%)	4	76.69	77.83	77.25	0.53	0.69
		TOTDRYWT	Total Dry Weight (gms)	4	386.00	448.00	427.00	27.83	6.52
		FAT	Fat (gms)	4	60.00	68.00	65.75	3.86	5.87
		FATPCT	Fat (% kcal)	4	27.00	31.00	29.75	1.89	6.36
		SFA	SFA (gms)	4	15.00	19.00	17.75	1.89	10.66
		SFAPCT	SFA (% kcal)	4	7.00	9.00	8.00	0.82	10.21
		MFA	MFA (gms)	4	18.00	21.00	19.75	1.26	6.37
		MFAPCT	MFA (% kcal)	4	8.00	9.00	8.75	0.50	5.71
		PFA	PFA (gms)	4	10.00	13.00	12.25	1.50	12.24
		PFAPCT	PFA (% kcal)	4	5.00	6.00	5.75	0.50	8.70
2	4	TOTWT	Total Weight (gms)	4	1380.00	1420.00	1398.75	17.23	1.23
		MOISTURE	Moisture (%)	4	74.94	75.27	75.05	0.15	0.20
		TOTDRYWT	Total Dry Weight (gms)	4	346.00	351.00	348.75	2.22	0.64
		FAT	Fat (gms)	4	60.00	63.00	61.25	1.50	2.45
		FATPCT	Fat (% kcal)	4	32.50	34.13	33.18	0.81	2.45
		SFA	SFA (gms)	4	15.00	16.00	15.75	0.50	3.17
		SFAPCT	SFA (% kcal)	4	8.13	8.67	8.53	0.27	3.17
		MFA	MFA (gms)	4	20.00	21.00	20.75	0.50	2.41
		MFAPCT	MFA (% kcal)	4	10.83	11.38	11.24	0.27	2.41
		PFA	PFA (gms)	4	11.00	11.00	11.00	0.00	0.00
		PFAPCT	PFA (% kcal)	4	5.96	5.96	5.96	0.00	0.00
3	4	TOTWT	Total Weight (gms)	4	1717.00	1943.00	1867.25	101.94	5.46
		MOISTURE	Moisture (%)	4	77.83	79.48	78.53	0.69	0.88
		TOTDRYWT	Total Dry Weight (gms)	4	352.00	421.00	401.50	33.19	8.27
		FAT	Fat (gms)	4	56.00	63.00	59.25	2.87	4.85
		FATPCT	Fat (% kcal)	4	25.00	28.00	26.75	1.26	4.70
		SFA	SFA (gms)	4	14.00	15.00	14.50	0.58	3.98
		SFAPCT	SFA (% kcal)	4	6.00	7.00	6.50	0.58	8.88
		MFA	MFA (gms)	4	17.00	19.00	18.00	0.82	4.54
		MFAPCT	MFA (% kcal)	4	8.00	8.00	8.00	0.00	0.00
		PFA	PFA (gms)	4	10.00	12.00	11.00	0.82	7.42
		PFAPCT	PFA (% kcal)	4	4.00	5.00	4.75	0.50	10.53

Calculated total weight: 2030 (menu 1), 1441 (menu 2), 1994 (menu 3)
Target: Fat 30 % kcal, SFA 9 % kcal, MFA 14 % kcal, PFA 7 % kcal

TABLE 3: Descriptive Statistics, by C , for the DELTA Diet
Pilot Study, EZ Cycle 1

CENTER	N Obs	Variable	Label	N	Minimum	Maximum	Mean	Std Dev	CV
Columbia	3	TOTWT	Total Weight (gms)	3	1380.00	1923.00	1733.67	306.54	17.68
		MOISTURE	Moisture (%)	3	74.94	77.83	76.49	1.46	1.90
		TOTDRYWT	Total Dry Weight (gms)	3	346.00	448.00	405.00	52.85	13.05
		FAT	Fat (gms)	3	59.00	68.00	63.00	4.58	7.27
		FATPCT	Fat (% kcal)	3	27.00	33.59	30.53	3.32	10.87
		SFA	SFA (gms)	3	15.00	19.00	16.67	2.08	12.49
		SFAPCT	SFA (% kcal)	3	7.00	8.67	7.89	0.84	10.64
		MFA	MFA (gms)	3	19.00	21.00	20.00	1.00	5.00
		MFAPCT	MFA (% kcal)	3	8.00	11.38	9.46	1.73	18.34
		PFA	PFA (gms)	3	11.00	13.00	12.00	1.00	8.33
		PFAPCT	PFA (% kcal)	3	5.00	6.00	5.65	0.57	10.01
PBRC	3	TOTWT	Total Weight (gms)	3	1404.00	1911.00	1734.67	286.58	16.52
		MOISTURE	Moisture (%)	3	75.04	78.41	76.79	1.69	2.20
		TOTDRYWT	Total Dry Weight (gms)	3	350.00	436.00	399.67	44.52	11.14
		FAT	Fat (gms)	3	63.00	67.00	64.33	2.31	3.59
		FATPCT	Fat (% kcal)	3	28.00	34.13	30.71	3.13	10.18
		SFA	SFA (gms)	3	15.00	18.00	16.33	1.53	9.35
		SFAPCT	SFA (% kcal)	3	7.00	8.67	7.89	0.84	10.64
		MFA	MFA (gms)	3	18.00	21.00	19.67	1.53	7.77
		MFAPCT	MFA (% kcal)	3	8.00	11.38	9.46	1.73	18.34
		PFA	PFA (gms)	3	11.00	13.00	11.67	1.15	9.90
		PFAPCT	PFA (% kcal)	3	5.00	6.00	5.65	0.57	10.01
Minnesota	3	TOTWT	Total Weight (gms)	3	1420.00	1974.00	1779.00	311.29	17.50
		MOISTURE	Moisture (%)	3	75.27	78.40	77.17	1.67	2.16
		TOTDRYWT	Total Dry Weight (gms)	3	351.00	438.00	403.00	45.92	11.40
		FAT	Fat (gms)	3	59.00	68.00	62.33	4.93	7.91
		FATPCT	Fat (% kcal)	3	27.00	32.50	30.17	2.84	9.43
		SFA	SFA (gms)	3	14.00	19.00	16.33	2.52	15.41
		SFAPCT	SFA (% kcal)	3	6.00	9.00	7.89	1.64	20.85
		MFA	MFA (gms)	3	18.00	21.00	20.00	1.73	8.66
		MFAPCT	MFA (% kcal)	3	8.00	11.38	9.46	1.73	18.34
		PFA	PFA (gms)	3	11.00	13.00	11.67	1.15	9.90
		PFAPCT	PFA (% kcal)	3	5.00	6.00	5.65	0.57	10.01
Penn State	3	TOTWT	Total Weight (gms)	3	1391.00	1721.00	1609.67	189.38	11.77
		MOISTURE	Moisture (%)	3	74.96	79.48	77.33	2.27	2.93
		TOTDRYWT	Total Dry Weight (gms)	3	348.00	386.00	362.00	20.88	5.77
		FAT	Fat (gms)	3	56.00	60.00	58.67	2.31	3.94
		FATPCT	Fat (% kcal)	3	25.00	32.50	28.17	3.89	13.80
		SFA	SFA (gms)	3	14.00	15.00	14.67	0.58	3.94
		SFAPCT	SFA (% kcal)	3	6.00	8.13	7.04	1.06	15.10
		MFA	MFA (gms)	3	17.00	20.00	18.33	1.53	8.33
		MFAPCT	MFA (% kcal)	3	8.00	10.83	8.94	1.64	18.30
		PFA	PFA (gms)	3	10.00	11.00	10.33	0.58	5.59
		PFAPCT	PFA (% kcal)	3	4.00	5.96	4.99	0.98	19.65

Calculated total weight: 2030 (menu 1), 1441 (menu 2), 1994 (menu 3)
Target: Fat 30 % kcal, SFA 9 % kcal, MFA 14 % kcal, PFA 7 % kcal

TABLE 4: Raw Data, Sorted by Center and Menu, for the DELTA Diet
Pilot Study, EZ Cycle 1

OBS	Center	Diet	Menu Number	Cycle Number	Food Collection Date	Total Weight (gms)	Moisture (%)	Total Dry Weight (gms)	Fat Assay Number	Fat (gms)	Fat (% kcal)	SFA Assay Number	SFA (gms)	SFA (% kcal)	MFA Assay Number	MFA (gms)	MFA (% kcal)	PFA Assay Number	PFA (gms)	PFA (% kcal)
1	Columbia	EZ	1	1	02/24/93	1923	76.69	448	1	68	31.0	RT2	19	8.0	RT2	20	9.0	RT2	13	6.0
2	Columbia	EZ	2	1	02/24/93	1380	74.94	346	2	62	33.6	RT2	16	8.7	RT2	21	11.4	RT2	11	6.0
3	Columbia	EZ	3	1	02/24/93	1898	77.83	421	2	59	27.0	RT2	15	7.0	RT2	19	8.0	RT2	12	5.0
4	PBRC	EZ	1	1	03/02/93	1889	76.92	436	1	67	30.0	RT2	18	8.0	RT2	20	9.0	RT2	13	6.0
5	PBRC	EZ	2	1	03/03/93	1404	75.04	350	2	63	34.1	RT2	16	8.7	RT2	21	11.4	RT2	11	6.0
6	PBRC	EZ	3	1	30/03/93	1911	78.41	413	2	63	28.0	RT2	15	7.0	RT2	18	8.0	RT2	11	5.0
7	Minnesota	EZ	1	1	02/24/93	1974	77.83	438	1	68	31.0	RT2	19	9.0	RT2	21	9.0	RT2	13	6.0
8	Minnesota	EZ	2	1	02/24/93	1420	75.27	351	1	60	32.5	RT2	16	8.7	RT2	21	11.4	RT2	11	6.0
9	Minnesota	EZ	3	1	02/24/93	1943	78.40	420	2	59	27.0	RT2	14	6.0	RT2	18	8.0	RT2	11	5.0
10	Penn State	EZ	1	1	02/24/93	1721	77.56	386	1	60	27.0	RT2	15	7.0	RT2	18	8.0	RT2	10	5.0
11	Penn State	EZ	2	1	02/24/93	1391	74.96	348	1	60	32.5	RT2	15	8.1	RT2	20	10.8	RT2	11	6.0
12	Penn State	EZ	3	1	02/24/93	1717	79.48	352	2	56	25.0	RT2	14	6.0	RT2	17	8.0	RT2	10	4.0

Calculated total weight: 2030 (menu 1), 1441 (menu 2), 1994 (menu 3)
Target: Fat 30 % kcal, SFA 9 % kcal, MFA 14 % kcal, PFA 7 % kcal

DELTA PROTOCOL 1

Diet Pilot Study - EZ Cycle 1 Data

FAT
Mixed Model Analysis
Response = (FAT - 66.67)

NAME	ESTIMATE	STD_ERR	P_VALUE
Between-subject Variance (Vb)	4.58333	4.85869	0.34551
Within-subject Variance (Vw)	3.88889	2.24525	0.08326
total variance: Vb + Vw	8.47222	.	.
Correlation: Vb / (Vb+Vw)	0.54098	.	.
Ratio: Vb / Vw	1.17857	.	.
Amt Menu1 was off target: M1	-0.92000	1.45535	0.55060
Amt Menu2 was off target: M2	-5.42000	1.45535	0.00980
Amt Menu3 was off target: M3	-7.42000	1.45535	0.00222
Difference: M1 - M2	4.50000	1.39443	0.01798
Difference: M1 - M3	6.50000	1.39443	0.00346
Difference: M2 - M3	2.00000	1.39443	0.20149
Ho: { M1 = M2 = M3 }	.	.	0.00904
Ho: { M1 = M2 = M3 = 0 }	.	.	0.00558

DELTA PROTOCOL 1

Diet Pilot Study - EZ Cycle 1 Data

SFA
Mixed Model Analysis
Response = (SFA - 21.00)

NAME	ESTIMATE	STD_ERR	P_VALUE
Between-subject Variance (Vb)	0.52778	0.68562	0.44143
Within-subject Variance (Vw)	0.86111	0.49716	0.08326
total variance: Vb + Vw	1.38889	.	.
Correlation: Vb / (Vb+Vw)	0.38000	.	.
Ratio: Vb / Vw	0.61290	.	.
Amt Menu1 was off target: M1	-3.25000	0.58926	0.00149
Amt Menu2 was off target: M2	-5.25000	0.58926	0.00011
Amt Menu3 was off target: M3	-6.50000	0.58926	0.00003
Difference: M1 - M2	2.00000	0.65617	0.02257
Difference: M1 - M3	3.25000	0.65617	0.00257
Difference: M2 - M3	1.25000	0.65617	0.10544
Ho: { M1 = M2 = M3 }	.	.	0.00727
Ho: { M1 = M2 = M3 = 0 }	.	.	0.00013

DELTA PROTOCOL 1

Diet Pilot Study - EZ Cycle 1 Data

MFA
Mixed Model Analysis
Response = (MFA - 25.50)

NAME	ESTIMATE	STD_ERR	P_VALUE
Between-subject Variance (Vb)	0.52778	0.51744	0.30774
Within-subject Variance (Vw)	0.30556	0.17641	0.08326
total variance: Vb + Vw	0.83333	.	.
Correlation: Vb / (Vb+Vw)	0.63333	.	.
Ratio: Vb / Vw	1.72727	.	.
Amt Menu1 was off target: M1	-5.75000	0.45644	0.00002
Amt Menu2 was off target: M2	-4.75000	0.45644	0.00005
Amt Menu3 was off target: M3	-7.50000	0.45644	0.00000
Difference: M1 - M2	-1.00000	0.39087	0.04300
Difference: M1 - M3	1.75000	0.39087	0.00420
Difference: M2 - M3	2.75000	0.39087	0.00041
Ho: { M1 = M2 = M3 }	.	.	0.00118
Ho: { M1 = M2 = M3 = 0 }	.	.	0.00002

DELTA PROTOCOL 1

Diet Pilot Study - EZ Cycle 1 Data

PFA
Mixed Model Analysis
Response = (PFA - 14.00)

NAME	ESTIMATE	STD_ERR	P_VALUE
Between-subject Variance (Vb)	0.33333	0.46269	0.47126
Within-subject Variance (Vw)	0.63889	0.36886	0.08326
total variance: Vb + Vw	0.97222	.	.
Correlation: Vb / (Vb+Vw)	0.34286	.	.
Ratio: Vb / Vw	0.52174	.	.
Amt Menu1 was off target: M1	-1.75000	0.49301	0.01208
Amt Menu2 was off target: M2	-3.00000	0.49301	0.00090
Amt Menu3 was off target: M3	-3.00000	0.49301	0.00090
Difference: M1 - M2	1.25000	0.56519	0.06899
Difference: M1 - M3	1.25000	0.56519	0.06899
Difference: M2 - M3	0.00000	0.56519	1.00000
Ho: { M1 = M2 = M3 }	.	.	0.11002
Ho: { M1 = M2 = M3 = 0 }	.	.	0.00197

DELTA PROTOCOL 1

Diet Pilot Study - EZ Cycle 1 Data

FATpct
Mixed Model Analysis
Response = (FATpct - 30.00)

NAME	ESTIMATE	STD_ERR	P_VALUE
Between-subject Variance (Vb)	1.09033	1.13405	0.33633
Within-subject Variance (Vw)	0.85200	0.49190	0.08326
total variance: Vb + Vw	1.94233	.	.
Correlation: Vb / (Vb+Vw)	0.56135	.	.
Ratio: Vb / Vw	1.27974	.	.
Amt Menu1 was off target: M1	-0.25000	0.69684	0.73206
Amt Menu2 was off target: M2	3.18125	0.69684	0.00383
Amt Menu3 was off target: M3	-3.25000	0.69684	0.00345
Difference: M1 - M2	-3.43125	0.65268	0.00191
Difference: M1 - M3	3.00000	0.65268	0.00371
Difference: M2 - M3	6.43125	0.65268	0.00006
Ho: { M1 = M2 = M3 }	.	.	0.00020
Ho: { M1 = M2 = M3 = 0 }	.	.	0.00042

DELTA PROTOCOL 1

Diet Pilot Study - EZ Cycle 1 Data

SFApct
Mixed Model Analysis
Response = (SFApct - 9.50)

NAME	ESTIMATE	STD_ERR	P_VALUE
Between-subject Variance (Vb)	0.09029	0.15531	0.56100
Within-subject Variance (Vw)	0.26750	0.15444	0.08326
total variance: Vb + Vw	0.35779	.	.
Correlation: Vb / (Vb+Vw)	0.25235	.	.
Ratio: Vb / Vw	0.33753	.	.
Amt Menu1 was off target: M1	-1.50000	0.29908	0.00242
Amt Menu2 was off target: M2	-0.96768	0.29908	0.01779
Amt Menu3 was off target: M3	-3.00000	0.29908	0.00006
Difference: M1 - M2	-0.53232	0.36572	0.19576
Difference: M1 - M3	1.50000	0.36572	0.00635
Difference: M2 - M3	2.03232	0.36572	0.00144
Ho: { M1 = M2 = M3 }	.	.	0.00358
Ho: { M1 = M2 = M3 = 0 }	.	.	0.00032

DELTA PROTOCOL 1

Diet Pilot Study - EZ Cycle 1 Data

MFApct
Mixed Model Analysis
Response = (MFApct - 11.50)

NAME	ESTIMATE	STD_ERR	P_VALUE
Between-subject Variance (Vb)	0.04514	0.05524	0.41380
Within-subject Variance (Vw)	0.06265	0.03617	0.08326
total variance: Vb + Vw	0.10779	.	.
Correlation: Vb / (Vb+Vw)	0.41882	.	.
Ratio: Vb / Vw	0.72064	.	.
Amt Menu1 was off target: M1	-2.75000	0.16416	0.00000
Amt Menu2 was off target: M2	-0.25901	0.16416	0.16569
Amt Menu3 was off target: M3	-3.50000	0.16416	0.00000
Difference: M1 - M2	-2.49099	0.17698	0.00001
Difference: M1 - M3	0.75000	0.17698	0.00545
Difference: M2 - M3	3.24099	0.17698	0.00000
Ho: { M1 = M2 = M3 }	.	.	0.00000
Ho: { M1 = M2 = M3 = 0 }	.	.	0.00000

DELTA PROTOCOL 1

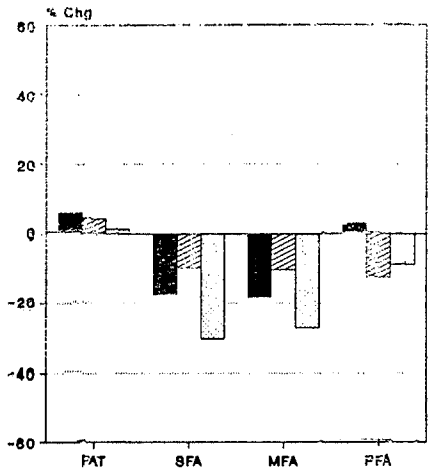
Diet Pilot Study - EZ Cycle 1 Data

PFApct
Mixed Model Analysis
Response = (PFApct - 6.30)

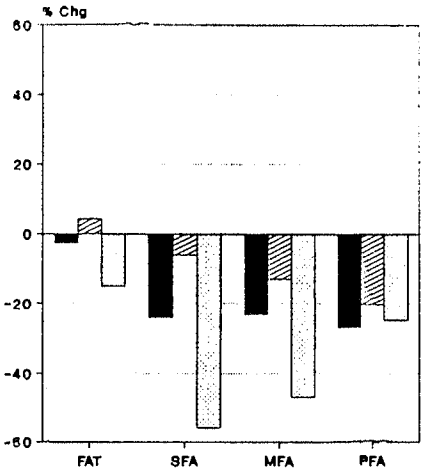
NAME	ESTIMATE	STD_ERR	P_VALUE
Between-subject Variance (Vb)	0.08333	0.09213	0.36571
Within-subject Variance (Vw)	0.08333	0.04811	0.08326
total variance: Vb + Vw	0.16667	.	.
Correlation: Vb / (Vb+Vw)	0.50000	.	.
Ratio: Vb / Vw	1.00000	.	.
Amt Menu1 was off target: M1	-0.55000	0.20412	0.03584
Amt Menu2 was off target: M2	-0.34092	0.20412	0.14593
Amt Menu3 was off target: M3	-1.55000	0.20412	0.00027
Difference: M1 - M2	-0.20908	0.20412	0.34521
Difference: M1 - M3	1.00000	0.20412	0.00271
Difference: M2 - M3	1.20908	0.20412	0.00103
Ho: { M1 = M2 = M3 }	.	.	0.00221
Ho: { M1 = M2 = M3 = 0 }	.	.	0.00134

Deviations from Analytical Value

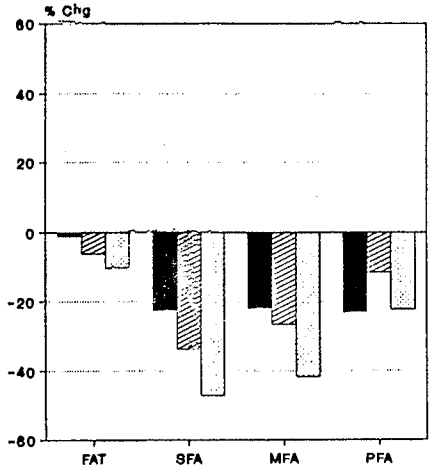
in ETNV Database



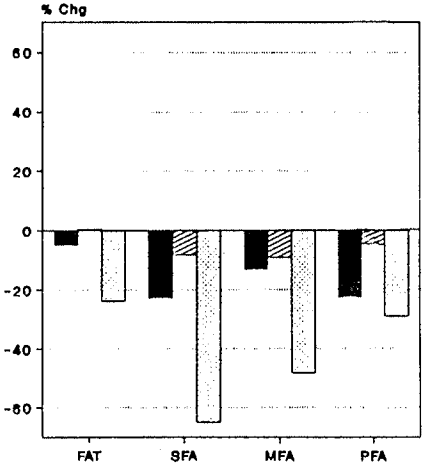
in NDS Database



in Diet Planner Database



in Nutritionist III Database



Menu 1 Menu 2 Menu 3

DELTA Diet Pilot Study
Summary of Comparison of Different Databases
With Analytical Values: EZ Cycle 1, Menu 1

Nutrient	Analytic (A)	ETNV (B)	A-B	% CHG	NDS (C)	A-C	% CHG	Diet Planner (D)	A-D	% CHG	Nutr III (E)	A-E	% CHG
Calories (kcal)	.	1978.0	.	.	1972.0	.	.	1924.7	.	.	1963.0	.	.
Protein (g)	.	70.4	.	.	74.6	.	.	74.9	.	.	73.9	.	.
Fat (g)	65.7	61.6	4.1	6.2	67.5	-1.8	2.7	66.5	-0.8	1.2	69.0	-3.3	5.1
Carbohydrate (g)	.	302.1	.	.	285.2	.	.	275.1	.	.	281.0	.	.
SFA (g)	17.8	20.9	-3.1	17.4	22.1	-4.3	24.2	21.8	-4.0	22.5	21.9	-4.1	22.8
PUFA (g)	12.3	12.0	0.3	2.4	15.6	-3.3	27.0	15.1	-2.8	23.0	15.1	-2.8	22.5
MUFA (g)	19.8	23.4	-3.6	18.3	24.4	-4.6	23.2	24.1	-4.3	21.9	22.4	-2.6	13.2
Cholesterol (mg)	.	282.0	.	.	320.5	.	.	305.9	.	.	289.0	.	

DELTA Diet Pi. tudy
Summary of Comparison of Different Databases
With Analytical Values: EZ Cycle 1, Menu 2

Nutrient	Analytic (A)	ETNV (B)	A-B	% CHG	NDS (C)	A-C	% CHG	Diet Planner (D)	A-D	% CHG	Nutr III (E)	A-E	% CHG
Calories (kcal)	.	1631.0	.	.	1615.0	.	.	1661.3	.	.	1778.0	.	.
Protein (g)	.	59.2	.	.	61.0	.	.	58.4	.	.	61.4	.	.
Fat (g)	61.2	58.5	2.7	4.3	58.6	2.6	4.3	64.9	-3.7	6.1	60.9	0.3	0.5
Carbohydrate (g)	.	224.6	.	.	217.3	.	.	218.3	.	.	255.0	.	.
SFA (g)	15.8	17.3	-1.5	9.6	16.8	-0.9	6.0	21.1	-5.3	33.7	17.1	-1.3	8.3
PUFA (g)	11.0	12.4	-1.4	12.4	13.2	-2.2	20.3	12.3	-1.3	11.5	11.5	-0.5	4.7
MUFA (g)	20.8	22.9	-2.1	10.2	23.5	-2.7	12.9	26.3	-5.5	26.4	22.7	-1.9	9.2
Cholesterol (mg)	.	273.0	.	.	323.6	.	.	303.3	.	.	280.0	.	.

DELTA Diet Pilot Study
Summary of Comparison of Different Databases
With Analytical Values: EZ Cycle 1, Menu 3

Nutrient	Analytic (A)	ETNV (B)	A-B	% CHG	NDS (C)	A-C	% CHG	Diet Planner (D)	A-D	% CHG	Nutr III (E)	A-E	% CHG
Calories (kcal)	.	1912.0	.	.	1937.0	.	.	1954.9	.	.	2020.0	.	.
Protein (g)	.	70.8	.	.	68.6	.	.	74.9	.	.	76.7	.	.
Fat (g)	59.2	58.5	0.7	1.3	68.0	-8.8	14.9	65.6	-6.4	10.8	73.2	-14.0	23.6
Carbohydrate (g)	.	283.7	.	.	273.1	.	.	276.4	.	.	274.0	.	.
SFA (g)	14.5	18.9	-4.4	30.2	22.6	-8.1	55.8	21.3	-6.8	47.1	23.9	-9.4	65.0
PUFA (g)	11.0	12.0	-1.0	8.7	13.7	-2.7	24.7	13.4	-2.4	22.1	14.2	-3.2	28.8
MUFA (g)	18.0	22.9	-4.9	26.9	26.4	-8.4	46.9	25.5	-7.5	41.7	26.7	-8.7	48.2
Cholesterol (mg)	.	272.0	.	.	296.9	.	.	295.8	.	.	298.0	.	.

TABLE 5
DELTA Diet Pilot Study, Option EZ
Descriptive Statistics, by Center and Cycle

Total Fat (g/100 g dry wt)

CENTER	CYCLE	N	MEAN	STD	MIN	MAX
Columbia	1	3	15.74	1.97	14.10	17.93
	2	3	16.25	2.16	13.91	18.16
	3	3	16.35	2.26	14.06	18.57
PBRC	1	3	16.15	1.51	15.15	17.88
	2	3	15.97	1.99	13.95	17.92
	3	3	16.75	1.32	15.98	18.27
Minnesota	1	3	15.62	1.49	14.16	17.14
	2	3	16.25	1.74	14.48	17.95
	3	3	15.91	1.32	14.87	17.39
Penn State	1	3	16.20	0.96	15.44	17.27
	2	3	15.43	1.62	13.64	16.81
	3	3	16.22	1.83	14.83	18.29

Total Dry Weight (grams)

CENTER	CYCLE	N	MEAN	STD	MIN	MAX
Columbia	1	3	405.00	52.85	346.00	448.00
	2	3	395.83	50.15	339.40	435.30
	3	3	390.27	46.34	340.40	432.00
PBRC	1	3	399.67	44.52	350.00	436.00
	2	3	399.37	46.87	348.50	440.80
	3	3	395.23	54.94	332.90	436.60
Minnesota	1	3	403.00	45.92	351.00	438.00
	2	3	402.80	52.06	343.80	442.30
	3	3	402.60	44.11	352.70	436.40
Penn State	1	3	374.33	22.85	348.00	389.00
	2	3	405.67	48.57	350.30	441.10
	3	3	394.33	48.96	341.40	438.00

TABLE 6
DELTA Diet Pilot Study, Option EZ
Descriptive Statistics, by Center and Menu

Total Fat (g/100 g dry wt)

CENTER	MENU	N	MEAN	STD	MIN	MAX
Columbia	1	3	16.10	0.79	15.19	16.67
	2	3	18.22	0.32	17.93	18.57
	3	3	14.02	0.10	13.91	14.10
PBRC	1	3	15.81	0.35	15.41	16.04
	2	3	18.02	0.21	17.88	18.27
	3	3	15.03	1.02	13.95	15.98
Minnesota	1	3	15.78	0.46	15.46	16.31
	2	3	17.49	0.41	17.14	17.95
	3	3	14.50	0.36	14.16	14.87
Penn State	1	3	15.61	0.20	15.44	15.83
	2	3	17.46	0.76	16.81	18.29
	3	3	14.78	1.12	13.64	15.88

Total Dry Weight (grams)

CENTER	MENU	N	MEAN	STD	MIN	MAX
Columbia	1	3	438.43	8.45	432.00	448.00
	2	3	341.93	3.56	339.40	346.00
	3	3	410.73	11.44	398.40	421.00
PBRC	1	3	437.80	2.62	436.00	440.80
	2	3	343.80	9.47	332.90	350.00
	3	3	412.67	3.71	408.80	416.20
Minnesota	1	3	438.90	3.05	436.40	442.30
	2	3	349.17	4.72	343.80	352.70
	3	3	420.33	1.82	418.70	422.30
Penn State	1	3	421.70	30.96	386.00	441.10
	2	3	346.57	4.62	341.40	350.30
	3	3	406.07	18.42	389.00	425.60

TABLE 7

DELTA Diet Pilot Study, Option EZ
Descriptive Statistics, by Cycle and Menu

Total Fat (g/100 g dry wt)

CYCLE	MENU	N	MEAN	STD	MIN	MAX
1	1	4	15.40	0.15	15.19	15.56
	2	4	17.55	0.41	17.14	17.93
	3	4	14.82	0.85	14.10	15.88
2	1	4	16.21	0.36	15.83	16.67
	2	4	17.71	0.61	16.81	18.16
	3	4	14.00	0.35	13.64	14.48
3	1	4	15.86	0.45	15.46	16.43
	2	4	18.13	0.51	17.39	18.57
	3	4	14.94	0.79	14.06	15.98

Total Dry Weight (grams)

CYCLE	MENU	N	MEAN	STD	MIN	MAX
1	1	4	427.00	27.83	386.00	448.00
	2	4	348.75	2.22	346.00	351.00
	3	4	410.75	14.93	389.00	421.00
2	1	4	439.87	3.12	435.30	442.30
	2	4	345.50	4.90	339.40	350.30
	3	4	417.37	7.88	408.80	425.60
3	1	4	435.75	2.60	432.00	438.00
	2	4	341.85	8.17	332.90	352.70
	3	4	409.23	9.79	398.40	418.70

TABLE 6

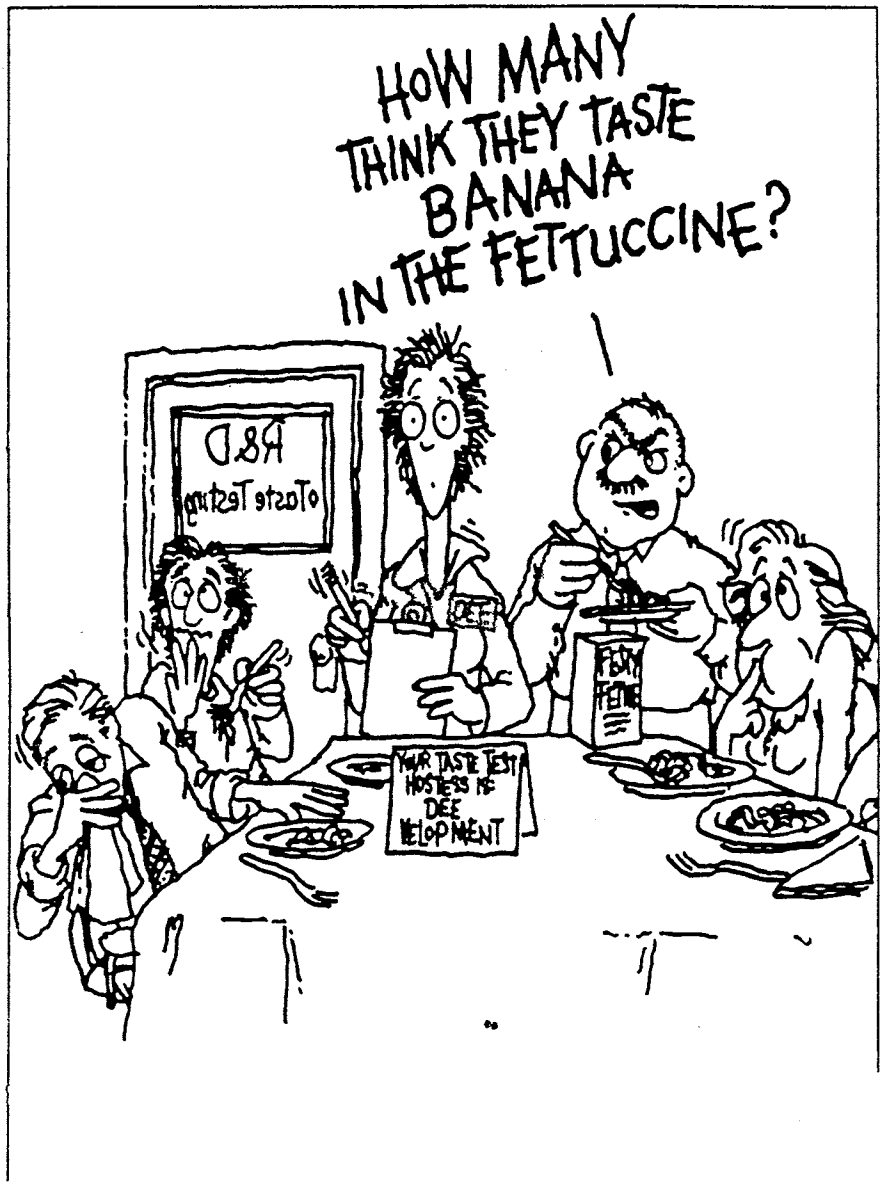
DELTA Diet Pilot Study, Option EZ
Descriptive Statistics, by Cycle

Total Fat (g/100 g dry wt)

CYCLE	N	MEAN	STD	MIN	MAX
1	12	15.93	1.33	14.10	17.93
2	12	15.97	1.65	13.64	18.16
3	12	16.31	1.50	14.06	18.57

Total Dry Weight (grams)

CYCLE	N	MEAN	STD	MIN	MAX
1	12	395.50	38.90	346.00	448.00
2	12	400.92	42.35	339.40	442.30
3	12	395.61	41.84	332.90	438.00


HOW MANY THINK THEY TASTE BANANA IN THE FETTUCCINE?
R&D
Taste Testing
YOUR TASTE TEST HOSTESS IS DEE VELOPMENT

APPENDIX G

FOOD SAFETY

Safe Food to Go

Millions of meals and snacks are carried to work and school every day. Whether you call it brown-bagging, briefcasing, or dashboard dining, it's the way lots of us eat when we're away from home. It's a handy and often cost-effective way to eat, but it is extremely important to keep those meals safe from the bacteria that cause foodborne illnesses.

Food poisoning is caused by bacteria that are widely present in land and aquatic environments, on humans, animals, and birds. If they are in or on food, bacteria can multiply quickly at warm temperatures (between 60 and 125°F). The bacteria that cause most cases of foodborne illness include *Salmonella, Campylobacter jejuni, Staphylococcus aureus, Clostridium perfringens*, and *Shigella*. Some of them cause illness when sufficient numbers are present in food and multiply in the body after we eat the food; others produce toxins in the food that cause illness after we eat them. There are differences among these bacteria in the way illness develops and the time required for symptoms to occur. The symptoms, however, are similar and include severe abdominal cramps and diarrhea, nausea, and sometimes vomiting. Healthy adults will usually recover in a few days, but these illnesses can be life-threatening for elderly persons, young children, or immunocompromised individuals.

The most common foodborne illnesses are caused by the mishandling of food in our homes or in commercial settings such as food services and other retail food distribution sources. When you are carrying food it is possible to prevent the growth of bacteria by paying attention to temperature control and cleanliness.

Ways to Control Food Temperature

Use insulated bags or coolers. Unless the food is going to be eaten within 2 hours of cooking or refrigeration, you will need a way to keep it very hot or cold. Insulated bags or coolers are available in many sizes. Use ice or a reusable freeze-pack to keep foods cold for several hours.

Newton Muffins

- Be sure foods are cold or frozen before you place them in the cooler or a cold thermos. If you don't have a cooler, simply freezing a sandwich will help keep it safer. Preparing carried lunches the night before and refrigerating them will help you be sure that the foods are sufficiently chilled before packing.
- Keep the lunch bag or cooler in the coolest possible place. For example, a car trunk might be the coolest place in the winter, but in the summer, it will be very hot. In hot weather, keep a cooler (or any perishable food) inside the car, preferably on the floor. Keep foods out of direct sun where higher temperatures will develop.
- Put frozen yogurt cups or drink boxes in lunch bags to help keep foods cool; by lunchtime, they'll be defrosted but still cool.

Use wide-mouth thermos containers for hot foods. Rinse the thermos with very hot water before you fill it with hot foods. Soups, sloppy joe mix, taco filling, stews, hot dogs in chili or hot water, and even casserole mixtures can be safely carried this way, provided they are extremely hot (above 165°F) when you fill the thermos. A good thermos will keep food at safe temperatures for several hours.

Safe Food to Go

To protect your foods when on the go:

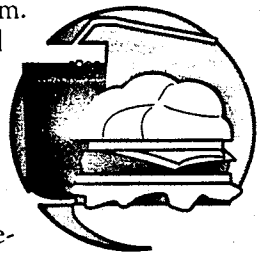
- ❒ Pack foods in a refrigerated, frozen, or hot condition.
- ❒ Use insulated bags, coolers, or wide-mouth thermoses to keep foods cold or hot.
- ❒ Check "use by" dates on processed meats and poultry, and on deli selections.
- ❒ Wash fruits and vegetables carefully before packing and use clean utensils when preparing foods.

Shop for Lunch Foods with Safety in Mind

Check "use by" dates when you buy processed meats and poultry. This will help you choose sandwich ingredients that are fresh.

If you buy presliced or shaved meats from bulk deli displays, plan to use them within 1 or 2 days. Storage times for these meats are more limited than for prepackaged lunch meats.

If you have no way to keep meat, poultry, or fish ingredients cold, buy forms that do not need refrigeration. Canned meats, meat spreads, entrees, poultry products, or fish will be safe to eat without refrigeration. Just open and use, or heat before eating. However, discard unused portions if you can't refrigerate them. Fermented and cured sausages like hard salami, pepperoni, beef sticks, and jerky are safe without refrigeration.



Natural cheeses are also safe, but may become oily on the surface. And, of course, there is no potential microbial hazard in a peanut butter sandwich!

Single serving portions may be best to buy if you can't refrigerate leftovers.

If you buy food from a refrigerator or freezer case, remember that you need to keep it cold until you eat it.

Deli salads, entrees, and sandwiches that you purchase for carried lunches should be kept cold constantly until you eat them. Check "use by" dates on foods whenever you can. Buy these foods last and get them home quickly. Take a cooler with you to the store if the weather is hot or if it takes more than an hour to get home.

Careful Preparation Prevents Illness

When you prepare food for lunch, **be sure the counter top and all tools you use are clean.** One of the ways bacteria get into food is by cross-contamination from unclean cutting boards or utensils.

Wash all raw fruits and vegetables thoroughly before using in food mixtures or packing with lunch. Bacteria from the soil and from repeated handling are on these fresh products, and can easily be removed by thorough washing in cool water.

If you make sandwiches more than a day before they are to be eaten, wrap them securely and freeze them. Most sandwich fillings freeze well. Salad fillings usually made with mayonnaise (which breaks down when thawed) should be made with salad dressing instead. Add lettuce or other vegetables just before eating. If you use mustard, mayonnaise, or salad dressing on a sandwich, remember that the acid in these products actually helps slow down bacterial growth.

When you plan to use leftover cooked meats for lunches, be sure to keep them refrigerated until you use them, and use within 3 or 4 days. For longer storage, freeze leftover meat or poultry.

Homemade soups, casseroles, or entrees carried for microwave heating at work should be packed in clean, covered containers and kept cold until reheated. These foods can be risky unless they are chilled immediately after cooking and used within a day or two. Discard any food that has been at room temperature for more than **2 hours.**

When You Can't Do It Right — Don't Do It

Sometimes it just isn't possible to carry your lunch and keep it cold and safe. If some of the foods that don't require refrigeration aren't appealing to you, it's probably a good idea to eat out, or simply take along a prepackaged snack food (crackers, cheese, snack bar, trail mix). The cost in lost time, possible medical attention, and physical discomfort is far greater than an occasional purchased lunch. But if you follow the easy suggestions offered, you can pack a lunch and enjoy it without worry, too.

For a free copy of a new publication from the US Department of Agriculture, "A Quick Consumer Guide to Food Safety," write: Safe Food, Consumer Information Center, 574-X, Pueblo, CO 81009.

Try these recipes for tasty and safe eating.

Newton Muffins

Makes 1 dozen
(Pictured)

- 1 3/4 cups all-purpose flour
- 1/4 cup sugar
- 1 tablespoon DAVIS Baking Powder
- 1/3 cup FLEISCHMANN'S Margarine, melted
- 1 egg, slightly beaten
- 3/4 cup apple juice
- 10 Fat Free FIG or APPLE NEWTONS Fruit Chewy Cookies, coarsely chopped

In medium bowl, combine flour, sugar, and baking powder. Stir in margarine, egg, and apple juice just until blended. (Batter will be lumpy.) Stir in cookies. Fill 12 greased 2 1/2-inch muffin-pan cups. Bake at 400°F for 15 to 20 minutes. Serve warm or cold.

NUTRITION INFORMATION per muffin: 200 calories, 168 mg sodium, 18 mg cholesterol, 5 gm total fat, 1 gm saturated fat, 1 gm dietary fiber.

SAFETY TIP: Be careful with eggs since they may be a source of salmonella bacteria. Use the egg as soon as it is removed from the refrigerator. In addition, wash all utensils and surfaces that come in contact with any part of the egg (including the shell). Don't forget to wash your hands thoroughly and never taste uncooked batter.

Blueberry Peach Newton Crisp

Makes 6 servings

- 2 (16-ounce) cans sliced cling peaches in extra light syrup, drained
- 1 cup fresh or frozen blueberries
- 2 teaspoons cornstarch
- 10 Fat Free FIG NEWTONS Fruit Chewy Cookies, sliced
- 1/3 cup firmly packed light brown sugar
- 1/4 cup all-purpose flour
- 2 tablespoons FLEISCHMANN'S Margarine, melted

In 9-inch pie plate, toss together peaches, blueberries, and cornstarch; set aside.

In small bowl, combine cookies, brown sugar, flour, and margarine until well combined. Sprinkle evenly over peach mixture. Bake at 350°F for 25 to 30 minutes or until hot. Serve warm.

NUTRITION INFORMATION per serving: 313 calories, 203 mg sodium, 0 mg cholesterol, 4 gm total fat, 1 gm saturated fat, 3 gm dietary fiber.

SAFETY TIP: Wash the top of cans before opening to avoid introducing unwanted contaminants into food. Also, keep your can opener clean.

Dietitian Comments:	
Today's Date:	**Follow-up Date:**

NUTRITION UPDATE
DELTA PROTOCOL

Keeping Food Safe in the Kitchen

Each year, thousands of people become ill because of food mishandling in the home. It can happen anytime, but the risks are somewhat higher in the summer, when warmer temperatures favor bacterial growth and when more foods are eaten at cookouts and picnics.

We can protect ourselves against the risks of illness from several bacterial pathogens that cause the most cases of foodborne diseases. We can do this with some easy, common-sense food preparation and serving practices in the kitchen and when we cook outdoors or picnic.

Cold Facts About Safety

Refrigerator temperatures should be at 40°F or lower. Some of the bacteria that cause illness can grow at temperatures found in many home refrigerators. While most grow rapidly between 70 and 120°F, some grow at temperatures between 40 and 50°F. Surveys of actual temperatures in home refrigerators find many that are above 40°F. Check your refrigerator's temperature with a thermometer.

Thaw frozen foods in the refrigerator, in cold water in waterproof wrapping, or in the microwave. Once the food is thawed, cook it right away or refrigerate it. Frozen pizzas, entrees, and vegetables should be cooked without thawing. It's risky to let frozen foods stand on the counter to thaw, because bacteria can grow on the outer surfaces before the interior thaws. Keep a pan or dish under thawing meat, fish, or poultry to prevent juices from dripping onto other foods and contaminating them.

Keeping Food Safe in the Kitchen

To protect against the risks of foodborne illness:

- ☐ Keep your refrigerator at 40°F or lower.
- ☐ Thaw frozen foods in the refrigerator, in cold water, or in the microwave.
- ☐ Keep all perishables cold until you are ready to use them.
- ☐ Wash hands, kitchen surfaces, and utensils before and after food preparation — especially when using eggs, poultry, and meat.

Chicken Marsala

Keep meat, fish, poultry, and eggs refrigerated until you cook them. Old recipes may suggest allowing eggs to reach room temperature before you beat the whites, make omelets, or use them in recipes. This can be hazardous, since bacteria like salmonella, which can be present in or on raw eggs, grow rapidly at room temperature. Similarly, don't let steaks or other meat warm to room temperature before you broil or grill them.

Clean Cooking Strategies

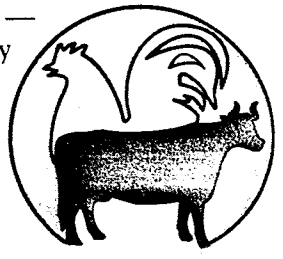
It's easy to get careless in the familiar surroundings of your own kitchen and feel overconfident about personal habits. To get a fresh perspective on your kitchen practices, pretend that you are "inspecting" the kitchen and those who work in it. Here are some important ideas about clean work habits:

Counter tops, utensils, and the sink should be clean before you start food preparation. Store any clean dishes that are drying, and wash any soiled ones before you start food preparation. Then clean off the counter top with hot soapy water and dry it. This will help reduce the chance of contaminating fresh foods as you work with them, and will make sink space available for washing fruits, vegetables, and other foods as you prepare them.

Wash your hands before — and during — food preparation. Use hot water and soap. Be especially sure to wash after handling raw meat or poultry and before you handle any foods that won't be cooked.

Wash cutting boards, knives, and any other utensils immediately after using them to cut or trim fresh meat, fish, or poultry. All of these fresh foods may have bacteria on their surfaces or in their juices. Cooking will make these foods safe to eat, but bacteria could be transferred to other foods that might not be cooked if you forget to wash tools completely. Use plastic cutting boards rather than wooden ones where bacteria can be trapped in the grooves. Wash cutting boards, utensils, and counter tops in hot soapy water and rinse in hot water before using them for other foods.

If you are ill with a diarrheal illness, don't prepare food. Many cases of foodborne illness are caused by ill food preparers. There is always some alternative to cooking if you are ill — other family members can prepare simple foods or you can eat ready-to-serve foods.



Preparing Raw Ingredients Safely

Wash all fresh fruits and vegetables with cool water (without detergent) before eating or using in food preparation. Bacteria are likely to be present on the outer surfaces of these foods, since they are found in the soil and may be introduced through handling during harvest, storage, or distribution. Thorough washing will also remove pesticide residues that might be on the surface.

Rinse raw poultry before cooking. Red meat does not need washing. Fish can be rinsed in cold water, if desired, but should not stand in water.

Marinate meats in the refrigerator and if you cook with the marinade, cook it thoroughly. Do not reuse a marinade.

Don't taste raw or partially cooked meat, poultry, egg mixtures, fish, or shellfish.

Careful Cooking Counts

One guideline is more important than any other for safe eating — cook foods thoroughly. Here are some more specific suggestions for particular foods:

Cook red meat to an internal temperature of at least 160°F. Use a meat thermometer to check the temperature of thicker cuts of meat. For thinner cuts, look at the color — meat at this temperature is gray or brown with no pink juices. Don't eat rare ground beef — contamination is more likely in ground meats, and partial cooking may not destroy bacteria that could be present. Rare steaks or roast beef are not quite as risky since bacteria would be on the cut surfaces, not in the interior muscle, and would be destroyed by hot cooking surfaces or broiling.

DELTA PROTOCOL

Cook poultry to 180°F for whole birds or dark meat, to 170°F for breast meat. At this temperature, juices run clear and flesh is tender.

Use oven temperatures of at least 325°F for safe baking or roasting. Low oven temperatures can permit bacteria growth. Don't use recipes that suggest a high temperature for an hour or so followed by standing without heat for several hours.

Fish should be cooked until the flesh is opaque and flakes with a fork. Do not eat raw fish or shellfish.

Eggs should be cooked until the yolk and white are firm, not runny. Recipes where eggs remain uncooked in the final product, as in mousse, can be risky since fresh, unbroken eggs may contain salmonella.

If you cook meat or poultry in the microwave, use roasting bags or covered containers. This keeps steam in contact with the meat and ensures more even cooking. Allow a standing time of 5 to 10 minutes before serving.

If you put poultry in the microwave to precook it before grilling, do this immediately before placing it on the grill. Partial cooking may not destroy all bacteria, so it is important to finish cooking immediately.

Cook in one continuous cooking period. Cooking a casserole, roast, or any other food partially at one time and holding it to finish later may provide conditions that encourage bacterial growth. Since some bacteria produce toxins as they grow, reheating the food later would not make it safe to eat. If a food must be cooked ahead, cook it completely, then chill it quickly.

If you prepare a food mixture to hold for several hours before serving (like a casserole), assemble the ingredients without heating and refrigerate it. Do all the cooking just before serving.

For a free copy of a new publication from the US Department of Agriculture, "A Quick Consumer Guide to Food Safety," write: Safe Food, Consumer Information Center, 574-X, Pueblo, CO 81009.

Try these recipes for tasty and safe eating.

Chicken Marsala

Makes 4 servings
(Pictured)

- 4 (3 1/2-ounce) boneless, skinless chicken breast halves
- 3 tablespoons EGG BEATERS 99% Real Egg Product
- 1/2 cup cracker meal
- 2 cloves garlic, minced
- 2 tablespoons FLEISCHMANN'S Margarine
- 1/4 cup Marsala wine
- 1 packet low-sodium chicken bouillon
- 2 tablespoons chopped parsley
- dash ground black pepper

Pound chicken breasts to 1/4 inch thickness. Dip in egg product; then coat with cracker meal. Set aside.

In skillet, over medium-high heat, cook garlic in margarine for 1 to 2 minutes. Add chicken; cook for 6 minutes or until done, turning once. Remove to serving platter; keep warm. In same skillet, add wine, undiluted chicken bouillon, pepper, and parsley; cook and stir until mixture begins to boil. Spoon over chicken. Serve immediately.

NUTRITION INFORMATION **per serving:** 233 calories, 136 mg sodium, 58 mg cholesterol, 7 gm total fat, 1 gm saturated fat, 0 gm dietary fiber.

SAFETY TIP: To be sure that cuts of meat or poultry that are less than 2 inches thick are fully cooked, look for juices to run clear when meat is cut into.

Spiced Fruit Cobbler

- 4 cups sliced unpeeled apples and/or pears
- 1 1/2 cups all-purpose flour
- 1/2 cup firmly packed light brown sugar
- 1 1/2 teaspoons DAVIS Baking Powder
- 1 teaspoon ground cinnamon
- 3/4 cup skim milk
- 1/4 cup BRER RABBIT Light Molasses
- 1/4 cup EGG BEATERS 99% Real Egg Product
- 2 tablespoons FLEISCHMANN'S Margarine, melted
- nonfat vanilla yogurt, optional

In 8 x 8 x 2-inch baking dish, arrange fruit; set aside. In medium bowl, combine flour, sugar, baking powder, and cinnamon. Stir in milk, molasses, egg product, and margarine just until blended. (Batter will be lumpy.) Pour batter evenly over fruit. Bake at 375° for 30 to 35 minutes or until toothpick inserted in center comes out clean. Serve warm topped with yogurt if desired.

NUTRITION INFORMATION **per serving:** (without yogurt): 218 calories, 104 mg sodium, 1 mg cholesterol, 3 gm total fat, 1 gm saturated fat, 0 gm dietary fiber.

SAFETY TIP: Wash fresh fruits and vegetables with tap water to remove surface pesticide residues and other impurities, such as soil particles. Thick-skinned produce may be scrubbed with a brush or peeled.

Dietitian Comments:
Today's Date: **Follow-up Date:**

NUTRITION UPDATE
DELTA PROTOCOL

Storing Perishable Food Safely

How long will perishable foods maintain good quality and be safe to eat? There's no exact answer, because the storage life of foods in the refrigerator is affected by:

- ❒ the freshness of the food when it reaches the supermarket
- ❒ the length of time it was in the store before you purchased it and the temperature at which it was held
- ❒ the way the food was processed and the type of packaging
- ❒ the temperature of your refrigerator
- ❒ the humidity level in the refrigerator
- ❒ the characteristics of the product itself

These conditions affect the safety of foods and the rate at which perishable foods deteriorate. In fact, all foods deteriorate in quality from the time they are harvested, slaughtered, or manufactured until they are consumed. This is because of biological, chemical, and physical changes that take place in the foods. Processing and storage practices can slow the rate of deterioration, but cannot completely prevent spoilage.

Foods can spoil without becoming unsafe to eat, or they may become both unsafe and lower in quality. Spoilage organisms usually cause changes in the odor and appearance of food, but we cannot see evidence of the growth of organisms that cause foodborne illness. From a practical standpoint, we can assume that foods with "off" odors, visible mold, or other changes in appearance should not be tasted or eaten. But for many foods, we need to follow recommendations for storage times and temperatures to avoid the risk of consuming food that may be unsafe even though it looks all right.

The storage practices suggested in this fact sheet will help you keep perishable foods safe as well as maintain other factors of quality.

Storing Perishable Food Safely

Refrigerated Foods:

- ❒ Keep refrigerator at 34 to 40°F.
- ❒ Remove spoiled foods and clean refrigerator surfaces regularly.
- ❒ Separate cooked and raw foods.
- ❒ Use raw meat promptly and pay attention to the "sell by" dates on other fresh foods.
- ❒ Refrigerate leftovers immediately.

Frozen Foods:

- ❒ Keep freezer at 0°F or lower.
- ❒ Wrap foods appropriately.
- ❒ Reheat frozen cooked foods without thawing.

Chicken Vegetable Soup

Refrigerator Storage Tips

Keep the refrigerator temperature between 34 and 40°F. This range allows the maximum storage time for most foods. Meat, fish, poultry, eggs, and milk should be stored in the coolest area of the refrigerator. Fruits and vegetables can be stored in the higher temperature zones. Use a thermometer to check the temperature at various location.

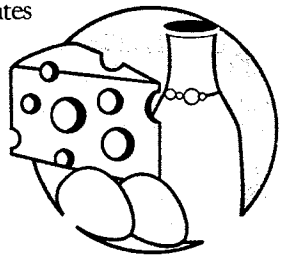
Keep the refrigerator clean. Do a quick check for "over-age" foods every day and discard food as appropriate. Clean shelves and other surfaces with warm soapy water every week or two, and wipe up spills immediately. A weak bleach solution (1 teaspoon of chlorine bleach per quart of water) can be used after washing to sanitize the refrigerator or containers where foods have spoiled.

Raw meat and poultry should be wrapped securely or placed on a tray so that they do not leak and contaminate other foods or surfaces. This is especially important if you have a meat storage drawer in the refrigerator. Don't store raw meats in it along with ready-to-eat meat unless you have the raw meat in a leak-proof package or container.

Use fresh meat, fish, or poultry within a day or two of purchase. Detailed storage recommendations are available from the US Department of Agriculture, but in most cases recommended storage times for fresh meat products are short. If you want to keep these foods longer, put them in the freezer.

Hot dogs and lunch meats are processed to last longer than many other meat and poultry products, but must be refrigerated. They will keep in the original vacuum-sealed pouch for 2 weeks, and can be kept up to 1 week after the "sell by" date. Freeze them if you cannot use within that time. Once a package of hot dogs is opened, use within a week. Lunch meat should be used 3 to 5 days after opening. If there is any cloudy liquid in the package around hot dogs, discard them.

Refrigerate all products marked "keep refrigerated." While some meat products (like canned hams) are clearly marked, there are newer food forms available that require careful refrigeration. New packaging techniques make it possible for food processors to offer a wide range of fully or partially processed foods in refrigerated forms. These carry a "use by" or "sell by" date on the label, but the dates assume that the product is refrigerated constantly throughout its shelf life, which may be several weeks.



Refrigerate fresh eggs until you are ready to cook them. Raw eggs should not be allowed to warm to room temperature, and egg-rich products or cooked eggs should not be kept out of the refrigerator for more than 2 hours. For best quality, eggs should be eaten within 3 weeks of purchase, but may be usable for up to 5 weeks. Avoid foods that contain raw eggs (homemade Caesar salad dressing, homemade mayonnaise, eggnog), or use egg substitutes instead. Commercial products like these use only pasteurized eggs, so they are safe.

Arrange foods in the refrigerator so that there is plenty of room for air to circulate.

Store foods in covered containers to minimize quality loss from drying out or from flavor transfer. In most cases, the containers foods are purchased in can be used for storage after opening. Leftover canned fruits or acidic vegetables should be transferred to other, clean, storage containers to avoid the development of metallic "off" flavors.

The "sell by" dates on dairy products allow a reasonable time after the date to use the product, provided you keep it cold. These dates are guides to the best product quality, and do not mean that the product will be unsafe if eaten after the date stamped on the container.

Leftover cooked foods should be refrigerated immediately after a meal, in covered shallow containers. Debone large pieces of meat or poultry and divide into smaller portions to refrigerate or freeze within 2 hours after cooking. If you have large quantities of soup, stew, or similar foods, chill rapidly by placing the cooking container in a sink with ice water in it. Stir frequently while the food cools, then refrigerate or freeze it. Date leftover foods so that you can use them in a day or two.

When you cook ahead for later reheating, always cook all the food immediately after cooking, either by placing it in the refrigerator or by using the ice-water bath just described.

Freezer Storage Tips

The freezer temperature should be 0°F or lower. If your freezer is separate from the refrigerator, check it every day or two to be sure that it is operating.

Package food in freezer wrap, freezer plastic bags, or freezer containers. These materials reduce air contact with the food and preserve quality. Date all foods so that you can use them within a reasonable time. Frozen food stored too long will be safe to eat, but may not taste or look good because of quality changes.

Frozen entrees, pizzas, dinners, vegetables, and similar foods should be cooked or reheated without thawing. Thaw frozen meat, poultry, or fish in the refrigerator, microwave, or in cold water if it is in a watertight wrap.

Partially thawed foods or those that have ice crystals remaining in them can be safely refrozen. Meat, fish, or poultry that has been thawed in the refrigerator can be refrozen within 24 hours.

For a free copy of a publication that includes food storage charts, write: Safe Food, Consumer Information Center, 574-X, Pueblo, CO 81009.

Try these recipes for tasty and safe eating.

Chicken Vegetable Soup

Makes 10 servings
(Pictured)

- 1 pound boneless chicken breast, cut into 1-inch pieces
- 1 cup chopped onion
- 2 cloves garlic, minced
- 2 tablespoons FLEISCHMANN'S Margarine
- 1 (10-ounce) package frozen sliced carrots
- 4 cups low sodium vegetable juice cocktail
- 4 cups water
- 1 1/2 cups large bow tie macaroni
- 1 tablespoon Italian seasoning
- 1 (10-ounce) package frozen chopped spinach
- 60 HARVEST CRISPS 5-Grain Crackers

In large saucepan, over medium-high heat, cook chicken, onion, and garlic in margarine until onion is tender. Add carrots, vegetable juice, water, macaroni, and Italian seasoning. Heat to a boil. Cover; reduce heat to low. Simmer for 20 minutes. Stir in spinach; cook for 5 minutes more. Serve 1 cup soup with 6 crackers.

NUTRITION INFORMATION per serving: (1 cup soup, 6 crackers): 241 calories, 255 mg sodium, 27 mg cholesterol, 5 gm total fat, 1 gm saturated fat, 2 gm dietary fiber.

SAFETY TIP: Wash hands, cutting boards, and all equipment thoroughly with hot soapy water after handling raw poultry to prevent contamination by salmonella bacteria.

Peachy Raisin Cracker Pudding

Makes 9 servings

- 1 (16-ounce) can sliced peaches in light syrup, drained and coarsely chopped
- 48 HARVEST CRISPS Oat Crackers
- 1/3 cup seedless raisins
- 1 1/2 cups skim milk, scalded
- 3/4 cup EGG BEATERS 99% Real Egg Product
- 1/3 cup sugar
- 1/2 teaspoon ground cinnamon
- vanilla frozen lowfat yogurt, optional

In 9 x 9 x 2-inch baking dish, combine peaches, crackers, and raisins; set aside.

In medium bowl, combine milk, egg product, sugar and cinnamon; pour over peach mixture. Bake at 350°F for 15 minutes; stir. Bake for 10 minutes more or until set. Cut into squares and serve warm with frozen yogurt if desired.

NUTRITION INFORMATION per serving: 148 calories, 171 mg sodium, 1 mg cholesterol, 2 gm total fat, 0 gm saturated fat, 1 gm dietary fiber.

SAFETY TIP: Purchase dated products only if the "sell by" or "use by" date has not expired. Shop for perishables last and bag refrigerated and frozen items together so they will remain cold during transport.

Turkey Chili

Makes 8 servings

- 1 pound ground turkey
- 1 cup chopped green pepper
- 1 cup chopped onion
- 2 tablespoons chili powder
- 1 clove garlic, minced
- 2 (16-ounce) cans no salt added stewed tomatoes
- 2 cups cooked kidney beans prepared without salt*
- 1 (10-ounce) package frozen corn, thawed and drained
- 48 HARVEST CRISPS Oat Crackers

In large pot, over medium-high heat, cook turkey, pepper, onion, chili powder, and garlic for 5 minutes or until vegetables are tender and meat is no longer pink. Add tomatoes, beans, and corn; heat to a boil. Reduce heat to low; simmer for 20 minutes. Serve 1 cup chili with 6 crackers.

NUTRITION INFORMATION per serving: (1 cup chili, 6 crackers): 279 calories, 255 mg sodium, 41 mg cholesterol, 7 gm total fat, 1 gm saturated fat, 3 gm dietary fiber.

*1 (16-ounce) can kidney beans, rinsed and drained, can be substituted.

SAFETY TIP: As grinding and mixing increase the likelihood of contamination by bacteria, it is important to cook ground meat and poultry thoroughly. Cook ground meats to 160°F and ground poultry to 180°F.

Dietitian Comments:

Today's Date: Follow-up Date:

APPENDIX H

PILOT STANDARDIZATION OF LIPID ANALYSES

APPENDIX I

WOMENS' HEALTH SUPPLEMENT

INTRODUCTION:

The Research on Women's Health supplement provides an unusual opportunity to extend the planned research on diet, lipoproteins and thrombogenic activity that is part of the **DELTA** study. The **DELTA** study comprises a series of discrete experimental diet trials aimed at elucidating the effect of various dietary perturbations on lipoprotein metabolism and thrombogenesis. Approximately 100 subjects (25 each of 4 field centers) will be recruited into each study which will last approximately 6 months. Key features of **DELTA** are (1) rigid control of the diet and (2) multicenter collaboration following a common protocol. This design permits samples of sufficient size with the rigor of dietary control necessary to resolve questions with respect to public health recommendations for all segments of society, especially women and minorities. One focus in the design for the first **DELTA** protocol is to characterize pre- and post-menopausal women with respect to their responses to diet.

Our proposal is to extend the coordinating center **DELTA** workscope to support the increase in data collection by the field centers for in depth studies of lipid metabolism as a function of diet and menstrual cycle in pre-menopausal women. We propose additional measurements rather than recruiting additional women because the gain in study power from a feasible recruitment of additional women would not be sufficient to compare responses of pre- and post-menopausal women. Additional measurements gives a greater scientific return for the funds available.

SPECIFIC AIMS:

The workscope of the Coordinating Center in the **DELTA** study encompasses activities associated with assaying the experimental diets, as well as those involving data collection and management of the lipid and hemostasis data. Specific aims of this proposal are confined to those involving the increased blood measurements.

1. Assist in the development of the study protocol and prepare and distribute manuals of operation, forms, and protocols.

2. Propose procedures for collecting, editing, storing and analyzing data generated by the field centers.

3. Organize and provide administrative support for meetings of the Steering Committee and subcommittees, including preparation of management and quality control statistical reports and minutes of meetings.

4. Serve as a repository for Steering Committee records, manuals, data collection instruments, research data and publications.

5. Assist in the design, management and analysis of ancillary studies.

6. Collaborate in the preparation of scientific publications and presentations.

Our estimates are based on the following assumptions:

Year 1	Protocol 1	6 extra blood draws x 60 women x 3 diet periods for lipid profiles, Apo A1, Apo B, Lp(a), fibrinogen, Factor VII, and PAI-1 Additional variables for all bloods: HDL_2, HDL_3, HDL and LDL particle size, TBARS, Oxidized LDL
	Protocol 2	Omega - 3 fatty acids parallel arm design with 6 extra blood draws for women in each arm of the study.
Year 2		Assumes two protocols similar to Year 1.

Funds requested in this supplement are based on the costs of increased data entry, management and statistical analysis incurred by 6 additional variables, and 6 additional measurements of lipid and hemostasis variables on all blood draws (including the additional blood draws). We expect to receive these data on paper forms.

DISCUSSION OF STUDY DESIGN:

The Cross-Over Treatment Design

The treatment design is a randomized 3-treatment, 3-period, 6-sequence cross-over. After a brief run-in period, each participant will be randomly assigned to one of six diet sequences: ABR, ARB, BAR, BRA, RAB, and RBA. Each diet period will be seven weeks in duration. For example, the group assigned to the ABR sequence will be fed diet A for seven weeks, then diet B for seven weeks, and finally diet R for seven weeks.

The relative advantages of this treatment design have been evaluated in terms of monetary expense and in terms of statistical power for detecting treatment effects. Monetary costs are proportional to the number of meals served. Sample size comparisons were made under the assumptions that the underlying sources of variation combine additively (rather than multiplicatively, for example.) Compared to an equally capable parallel-arm design, the selected cross-over costs less and requires many fewer subjects to obtain efficient estimates of the direct effects of treatment. This feature allows each Field Center to establish rapport with small cohorts of well motivated participants. Furthermore, the selected design has satisfactory statistical properties under reasonable assumptions in the setting of this study of serum analytes.

To avoid carryover of effects from one period to the next, an active washout interval will be used. That is, the first four weeks of each diet period will provide time for stabilization of the responses of interest. Blood draws during the last three weeks of each diet period will yield triplicates of endpoint measurements. Consequently, baseline measurements at the beginning of each period will not be obtained. We anticipate that lipoprotein responses will stabilize in four weeks for less. There is less certainty in regard to the hemostasis variables. In both cases the proposed analysis plans will provide for evaluation of degree of stabilization.

Statistical Computation and Analysis Methods

The proposed cross-over is a special kind of longitudinal design in involving correlations among the repeated measurements made on each individual in the study. This feature must be incorporated in the analyses of the data. "Data analysis" includes selecting and managing analysis strategies, interpreting the subsequent results via written reports, and writing of papers for publication. "Statistical computation" refers to the use of computer systems to produce meaningful calculations, summary tabulations, and graphic figures, as dictated by analysis strategy. A tremendous variety of statistical analysis methods exist for cross-over studies. Appropriate choices of methods depend upon the scale of the outcome variables (dichotomous, ordinal, interval, time-to-event) and upon the objectives of the study. For example, the parametric methods of linear statistical models ("general" or "generalized") can provide cross-sectional and longitudinal analyses for outcome variables in a wide variety of situations.

The General Linear Mixed Model

For the purposes of this study, analysis methods based on a general linear mixed model with linear covariance structure are preferred. This approach will allow great flexibility in estimation and testing of the expected-value effects (treatment, period, sequence) effects as well as exploration of the covariance structure. Such models are effective in cross-over studies such as this one -- with or without baseline measurements. The linear covariance structure permits use of different variance and covariance parameters for different groups, it allows one to assume that specified covariances are zero, and provides for tests of hypotheses about specified variance and covariance parameters. We will use maximum likelihood (ML) and restricted maximum likelihood (REML) estimation methods. The SAS software system will provide the necessary iterative algorithms (via the "MIXED" procedure). Furthermore, the mixed model will cope with any ignorably missing data. (See Laird and Ware (1982), Jennrich and Schlucter (1986), Ware (1985), Neter and Wasserman (1974), Harville (1977).)

This modeling strategy also allows flexibility in detecting and dealing with within-period temporal trends. In the presence of such trends, we will have the option of (a) using only (the third and most stabilized) blood sample in the diet period, or (b) using all the data to estimate the final level.

Working Assumptions

The a priori model for power analysis, planning, and for ultimately testing the primary hypotheses specifies a fairly simple linear covariance structure; namely, the constant-correlation structure. This model is a special case of the general linear mixed model. The usual interpretation of this structure is that the underlying process involves additive errors. In other words, total variance is the sum of two or more sub-components of variance. We anticipate that this model is approximately correct in spite of some sources of variation that are most likely multiplicative in nature. However, it should be noted that the additivity assumption is pivotal for many or our important conclusions. Sensitivity of results to this and other modeling assumptions will be evaluated using diagnostics methods for the mixed model.

JUSTIFICATION FOR ADDITIONAL MEASUREMENTS:

Power Analysis

Power analysis results are shown in the Appendix. Table 1 and Figure 1 present power analysis results for the first DELTA study of N=96 subjects at four field centers. Table 2 and Figure 2 present comparable results for N=120 subjects at four field centers. In both cases, we will have adequate power for detecting diet differences (diet A versus diet B) in terms of serum lipids and lipoproteins. For example, we estimate that we will have 90% power for detecting a 6.7mg/dl difference overall in a study of 38 males and 58 females. The detectible difference is 8.1mg/dl for the females alone. This means that if the true difference is 8.1mg/dl, then 90% of studies like this one would be expected to detect a difference. We use 90% power as a criterion because procedures with power smaller than this are too sensitive to slight inaccuracies in the assumed magnitudes of effects.

It has been suggested that diet-by-gender/hormonal interactions may exist. That is, the difference between responses to diets A and B in pre-menopausal women may not be the same as in post-menopausal women. Our power analyses indicate that such an interaction (difference of differences) would have to be as large as 15.1mg/dl for a study of size N=96 to have a 90% probability of detecting it. Increasing the sample size to N=120 improves this "detectible difference" only slightly: 12.8mg/dl.

We conjecture that the difference between diets A and B may be as large as 15mg/dl in pre-menopausal women. In order for an interaction of magnitude 13mg/dl to exist, the A-B difference for post-menopausal women would have to be either very small (15-13 mg/dl) or very large (15+13 mg/dl). We conclude that the detection of diet-by-gender/hormonal interactions is not within the scope of our current investigations.

In contrast, collection of additional measurements will allow us to better study the role of gender effects in metabolic responses to diet. Under the assumption of additive sources of variability, additional measurements will increase the statistical power of the study. For example, we estimate that increasing the number of repeated measures of total serum cholesterol from 3 to 4 will decrease the "detectible" A-B diet difference from 8.1mg/dl to 7.0mg.dl.

Figure 1

Total Serum Cholesterol (mg/dl)

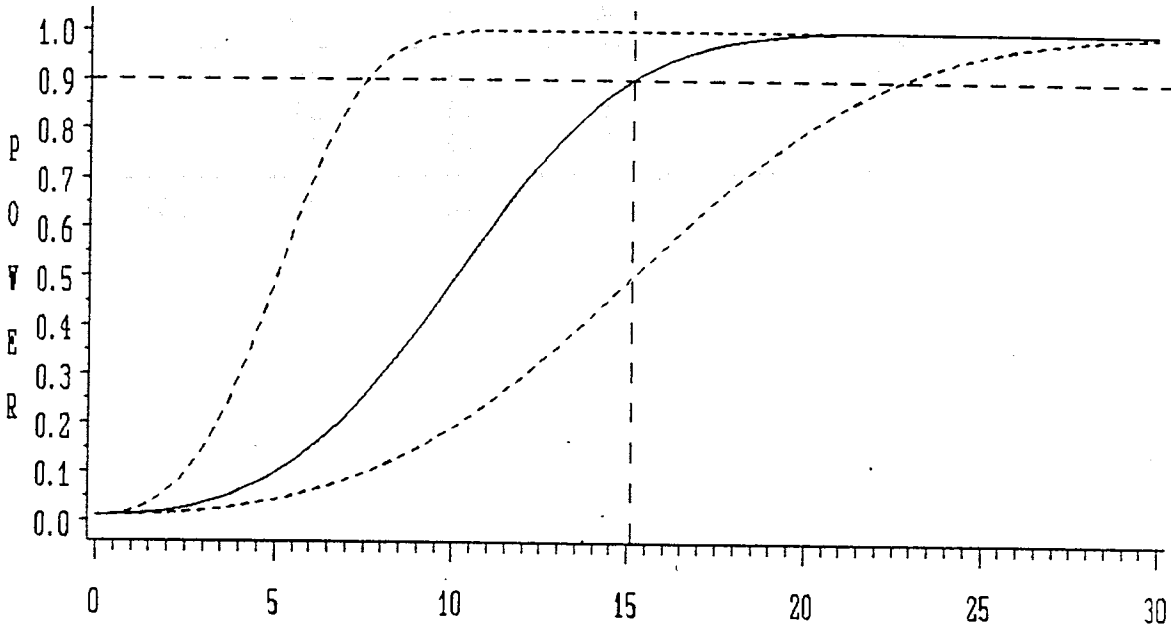
In Response to Diets A, B, and R Among,

Black(b), White/Other(w), Male(m) and Female(f) Subjects.

Comparison of Pre- versus Post-Menopausal Females

With Respect to (A-B) Diet Difference.

CENTERS=4 SUBJECTS=96 REPS=3



Significance level of test is 0.01. Upper & lower curves indicate effect of increasing or decreasing within-subject variance by 50% Within-subject variance for one blood draw is assumed to be 324.00 Results do not depend on the between-subject variance, 972.00, and are identical for (A-R) comparisons and for (B-R) comparisons.
The mixed-model assumptions are as follows:

Fixed Effects --- Gender, Race, Center, Period, Diet, Race*Diet, Gender*Diet

Random Effects --- Intercept only

Table 2

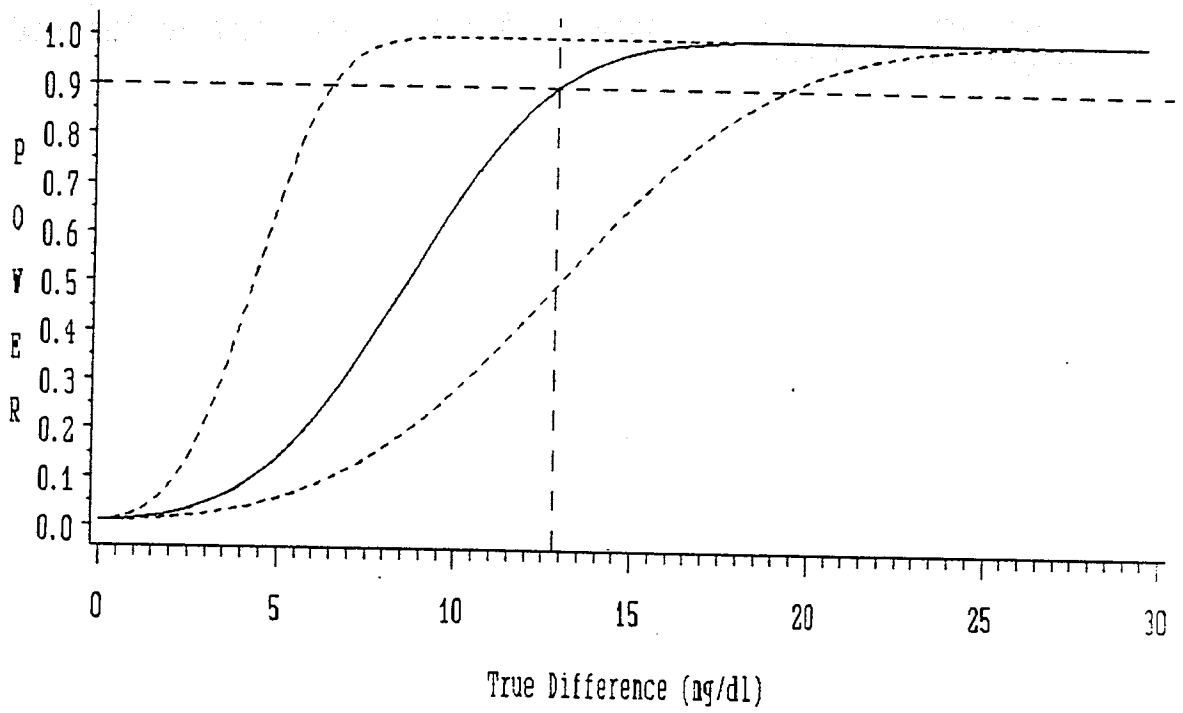
Center	Gender	Black	White	Total
Columbia U.	F+	5		10
	F-	5		10
	M	5		10
Pennington	F+	5		10
	F-	5		10
	M	5		10
U. Minn.	F+	1	9	10
	F-	1	9	10
	M	2	8	10
Penn. State	F+	2	8	10
	F-	2	8	10
	M	2	8	10
Total	F+	13	27	40
	F-	13	27	40
	M	14	26	40
		40	80	120

Figure 2

Total Serum Cholesterol (mg/dl)
In Response to Diets A, B, and R Among,
Black(b), White/Other(w), Male(m) and Female(f) Subjects.

Comparison of Pre- versus Post-Menopausal Females
With Respect to (A-B) Diet Difference.

CENTERS=4 SUBJECTS=120 REPS=3



Significance level of test is 0.01. Upper & lower curves indicate effect of increasing or decreasing within-subject variance by 50%
Within-subject variance for one blood draw is assumed to be 324.00
Results do not depend on the between-subject variance, 972.00, and are identical for (A-R) comparisons and for (B-R) comparisons.
The mixed-model assumptions are as follows:

Fixed Effects --- Gender, Race, Center, Period, Diet, Race*Diet, Gender*Diet
Random Effects --- Intercept only

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DIET RESPONSIVENESS DURING THE MENSTRUAL CYCLE

SPECIFIC AIMS

The overall goal of this project is to describe the levels of serum total cholesterol, HDL-cholesterol, triglycerides, LDL-cholesterol, fibrinogen, Factor VII, and PAI-1 levels at different phases of the menstrual cycle in healthy premenopausal women who are fed three different experimental diets (e.g., Average American Diet, a Step-One Diet, and a Very Low Saturated Fat Diet). The proposed supplemental study will provide valuable qualitative and quantitative data on the variance of these blood constituents in menstruating women. This is important because it will allow us to identify the appropriate sample size needed for premenopausal women, the optimal time of blood sampling and the samples that can be compared with those from postmenopausal women and men in future DELTA studies. To meet this overall goal, the study has several specific aims:

1. **To study, in collaboration with the LSU/Pennington Field Center, the 15 pre-menopausal women (n=7 at Penn State and n=8 at LSU/Pennington) who will be recruited into the DELTA Study. These women will have regular menstrual cycles, be willing to keep a menstrual calendar and not use oral contraceptives.**
2. **To extend the feeding of the experimental diets at Penn State and LSU/Pennington one week prior to the beginning of the first experimental diet and one week after the end of the third experimental diet in order to allow blood sampling at the same time in the menstrual cycle on all three study diets.**
3. **To measure blood levels of luteinizing hormone, progesterone, and estradiol in these 15 women to precisely classify the phase of the menstrual cycle, to examine effects of the diet, and to relate the hormone levels with other study endpoints.**
4. **To analyze the data to examine the effects of random sampling across the menstrual cycle versus sampling that has been carefully timed to a specific part of the menstrual cycle.**

BACKGROUND AND RATIONALE

DELTA Study

The DELTA Study is a multicenter, collaborative study designed to examine the effects of specific nutrients on plasma lipids, lipoproteins and hemostatic factors in men and women, and in Caucasians and African-Americans. It is the first study to examine diet effects on major risk factors for coronary heart disease in groups that typically have not been studied extensively. The DELTA Study provides a unique opportunity to address key issues related to diet, gender, ethnicity and other factors on CHD risk status that only can be studied using a relatively large sample size. The use of a multicenter study design is conducive to answering a number of important questions in a relatively short time period while using valuable resources efficiently and effectively. The DELTA Study will advance our understanding of the role of diet in modifying major CHD risk factors in target groups (women, men, and African Americans) with a high incidence of CHD.

Coronary Heart Disease and Women

Cardiovascular diseases are the leading cause of death in the United States in both men and women, but they actually account for a greater proportion of all deaths in women (52% versus 46%) (1). As Bush et al. note (2) until recently, the importance of CHD in women has been overlooked. Few investigators have studied CHD risk factors and their environmental influences in women. With the recognition of the public health importance of CHD to women, there is a heightened interest in understanding risk factor modification in women of all ages, hormonal status, and ethnicity. Whereas HDL-cholesterol is a strong, negative independent risk factor for CHD in women, and LDL-cholesterol is a less powerful predictor of CHD risk (3-5) the extent to which diet and other factors affect these lipoproteins needs to be resolved.

Diet and Plasma Lipids/Lipoproteins

A large body of evidence has demonstrated that diet markedly affects plasma lipids and lipoproteins. The majority of diet studies have been conducted primarily with men. Moreover, the commonly used equations of Keys et al. (6) and Hegsted et al. (7) to predict changes in plasma cholesterol levels in response to changes in diet were developed using data collected from studies conducted with men. While some investigators (8) have shown diet effects in women, Bush and colleagues (9) have concluded in their review that diet does not influence plasma lipids and lipoproteins in women. In a comprehensive regression analysis of data reported in the literature, we have found that women are responsive to changes in diet and that the diet response noted is statistically different from that observed for men (Yu, Derr and Kris-Etherton, unpublished data). Our data suggest that women may be more responsive to both saturated and monounsaturated fatty acids, and less responsive to polyunsaturated fatty acids than

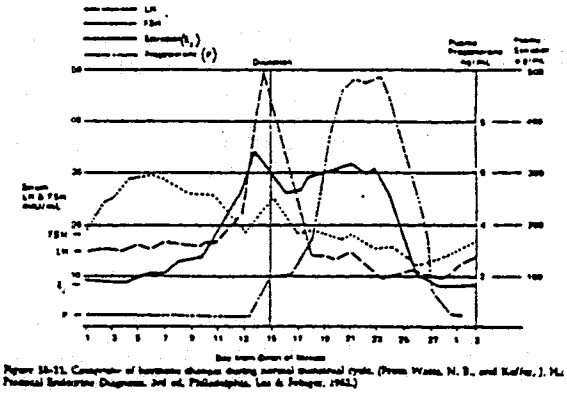
men. It is important to point out that it is difficult to interpret many of the studies conducted with women because of the small sample size used by many investigators and because the endocrine effects on plasma lipids were not controlled for. With appreciable changes in plasma total cholesterol levels and significant changes in HDL-cholesterol levels throughout the menstrual cycle (see below), it is apparent then that treatment effects could be falsely exaggerated or, more likely masked, leading to erroneous conclusions about young womens' response to diet.

A recent study (10) illustrates the importance of controlling for the phase of the menstrual cycle when studying the effects of an intervention on plasma lipids and lipoproteins. In this study, the authors were able to show a positive effect of exercise on HDL-cholesterol levels in premenopausal women, a response which previously has been difficult to establish by many other investigators. We speculate that the variation in HDL-cholesterol during the menstrual cycle masked a significant treatment effect leading many investigators to probably falsely conclude that exercise does not affect HDL-cholesterol levels in women.

The importance of the studies proposed herein is that they will provide valuable information about the most appropriate sampling times for both plasma lipids and hemostatic factors in premenopausal women. There is little information in the literature that enables us to establish the ideal sampling paradigm in intervention studies with premenopausal women.

Hormonal Variations Throughout the Menstrual Cycle

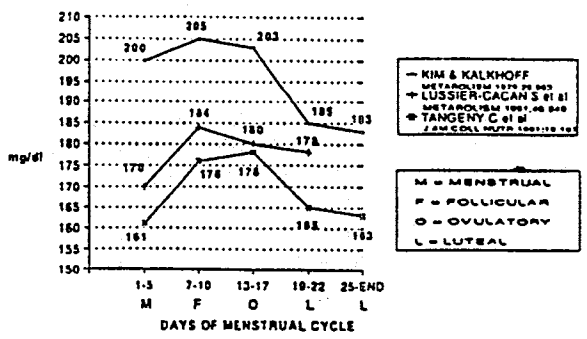
During the follicular phase, estrogen is high and progesterone is low, causing an estrogen-dominant condition (see figure below). Neither hormone is dominant during the luteal phase as the concentration of both is high (see figure below). LH and FSH peak during ovulation; both are elevated for a short period of time (i.e., 2 to 3 days).



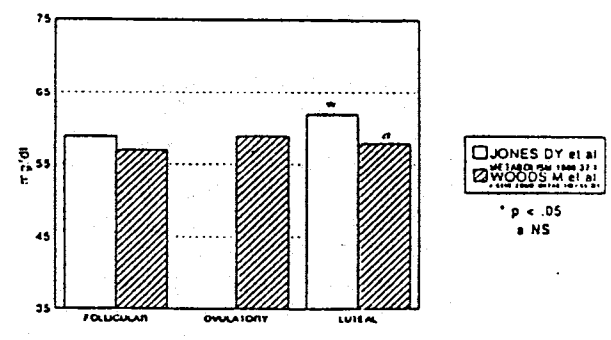
Effects of Estrogens and Progestins on Serum Lipids and Lipoproteins

The effects of exogenous estrogens and progestins on plasma lipids and lipoproteins are well established whereas the effects of endogenous hormone cyclicity are less clear (2). Several studies have reported changes in total cholesterol over the menstrual cycle (see figure below) whereas others have not observed consistent variations (11-14). HDL-cholesterol has been shown to be stable (15) or variable (see figure below). One study reported a decrease in LDL during the luteal phase (16). Thus, the effects of hormonal variations during the menstrual cycle on plasma lipids and lipoproteins are inconclusive. Bush et al. (2) note that because of serious methodological problems with these reports, including small numbers of subjects and large intra-individual variability in cycle lengths, the effects of variations in endogenous hormone levels throughout the menstrual cycle on lipids and lipoproteins are largely unknown.

TOTAL CHOLESTEROL DURING THE MENSTRUAL CYCLE



HDL-CHOLESTEROL DURING THE MENSTRUAL CYCLE



Effects of Estrogen on Thrombosis

Estrogens in pharmacologic doses (e.g., oral contraceptive agents, OCAs) are known to predispose women to thrombosis. In fact, evidence that the formulations (e.g., high dose estrogen agents) used in the 1960s and 1970s were associated with thromboembolic disease resulted in a reduction in the estrogen content of OCAs (2). Presently, "low dose" formulas are used. Several coagulation factors appear to increase with estrogens, but the effects of physiologic levels of estrogen across the menstrual cycle on these factors are poorly described.

Effects of Hormone Fluctuations on Dietary Responsiveness of Lipids, Lipoproteins, and Coagulation Factors

The effects of estrogens, progestins, and other hormones cycling with the menses on the dietary responsiveness of plasma lipids, lipoproteins, and coagulation factors are not known. Therefore, studies of the effects of diet on lipoproteins and coagulation factors require pilot data to estimate variance and optimal sampling schemes in premenopausal women. This information is essential not only for conducting diet studies with premenopausal women, but it also has relevance to virtually all other intervention studies designed to examine treatment effects on lipids, lipoproteins, and hemostatic factors in this important segment of the population.

Rationale for a Similar Study in Year 1 and Year 2

A similar study is proposed for year 2 to simply enhance statistical power and to validate our findings of the study conducted in year 1. The repository of samples from both studies will enable other DELTA Study investigators to examine hormonal effects on factors of interest in premenopausal women fed well-controlled diets.

METHODS

A. Overall Study Design

This application proposes a minor modification of the overall protocol for the DELTA study, while still promoting all specific aims of the main study, and without conflicting with the currently planned DELTA collaborative protocol. This modification proposes the addition of one week of Study Diet A prior to the originally designed first Seven-week Diet Phase, and one week of Study Diet C after the originally designed third Seven-week Diet phase, for premenopausal women only.

	Week	Week	Week	Week	Week	Week	Week	Week	Week
Diet A	0	1	2	3	4	5	6	7	
Diet B		8	9	10	11	12	13	14	
Diet C		15	16	17	18	19	20	21	22

As originally proposed in the DELTA Study I Protocol (weeks 1-21), women will be sampled at weeks 5, 6, 7, 12, 13, 14, 19, 20, 21, keeping the main DELTA protocol intact. However, the following modification has several advantages. First, it allows the effects of diets to be assessed at the same phase of the menstrual cycle, assuming a regular 28 day menstrual cycle. For example, a woman in week 3, 11, 19 of the study, should be at the same phase of the cycle, but on each different diet. Without these modifications, the only samples obtained at the same phase in the menstrual cycle would be at weeks 5, 13, and 21. Second, the entire menstrual cycle is surveyed, allowing diet responsiveness at different parts of the menstrual cycle to be assessed. Third, all samples follow three full weeks of study diets, allowing most of the dietary changes to be assessed.

We believe that this pilot study conducted for two 8-week feeding periods will provide important information to resolve our ongoing discussions about the appropriate length of feeding studies with premenopausal women. Our original decision to use a 7-week cycle for the overall study was guided by many considerations including a large sample size, carefully defined exclusion criteria, highly accurate laboratory analysis, logistical issues (e.g., lag times), compliance and study costs.

B. Study Population

Fifteen premenopausal women will be studied. Of these, 7 will be studied at the Penn State Field Center and 8 will be studied at Pennington. This represents the entire premenopausal female sample for the DELTA Study for each of these two Field Centers. A letter of commitment from Dr. Michael Lefevre from LSU/Pennington is attached.

In addition to the exclusion criteria already proposed, we will enroll only premenopausal women who have regular menstrual cycles (always 27 to 29 days), will not use oral contraceptives or other hormone preparations, agree to keep a menstrual diary and agree to participate for two additional weeks. Recruitment at both centers is not expected to be problem, as premenopausal women represent the largest population segment available for the study.

The burden of two additional weeks is not felt to be significant so as to effect retention of subjects in all phases of the study. The only additional request of these subjects is that they keep a menstrual diary.

C. Laboratory Testing

1. Blood Sampling Schedule

All laboratory tests collected as part of the original protocol (X) will be collected, plus some additional samples (Y).

	Week	Week	Week	Week	Week	Week
Diet A	3 YY	4 YY	5 XY	6 XY	7 XXY	
Diet B		11 YY	12 XY	13 XY	14 XXY	
Diet C			19 XY	20 XY	21 XXY	22 YY

The samples should be collected twice a week, spaced by three to four days (e.g., Tuesday, Friday). This constitutes approximately 750 ml of blood (1.6 units of blood) over a five month period of time, since each blood sample is 29 ml. No additional blood need be taken for hormone assays, as only 1 or 2 cryovials (0.5 ml each) are needed for all analyses, and these will be obtained from currently available aliquots.

2. Analytes

a. Endpoint Package

Each sample will consist of an endpoint package, including lipoprotein profile, apo-A1, apo-B, LP(a), fibrinogen, Factor VII, and PAI-1.

b. Hormone Assays to Assess Phase of the Menstrual Cycle

1. Progesterone

Levels of this hormone confirm ovulation and assess adequacy of the luteal phase. For the follicular phase, the reference ranges are 0.1 to 1.1 ng/ml whereas the luteal phase reference ranges are 2.5 to 28 ng/ml. Serum progesterone levels will be measured using an RIA method with ^{125}I tracer (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA). This method is a solid phase assay with a CV of 7-10%, comparing favorably with other commercial assays with CV exceeding 15%. The assay will be done in duplicate on 100 ml of serum which can be stored stably for up to six months at -70°C.

2. Estradiol

Estradiol will be able to identify early follicular and late follicular stages, and reflects the estrogen component thought to effect lipid levels. The reference ranges for the early follicular phase is 300-100 ng/ml versus 100-400 ng/ml for the late follicular phase. Estradiol levels will be measured in duplicate in serum by RIA using ^{125}I tracer (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA). This is a solid phase assay with virtually no cross reactivity with other steroid hormones. It requires 100 ml of serum which can be stored up to six months at -70°C.

3. Luteinizing Hormone (LH)

LH levels are low and constant (0-14 mIU/ml) until ovulation, rising rapidly 5- to 10-fold (20-70 mIU/ml) over a three-day period marking ovulation. Thus, samples every three days should document ovulation in almost all women. This peak is longer and higher than follicular stimulating hormone. LH will be measured by RIA using ^{125}I tracer (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA). The assay will be done in duplicate and requires 100 ml of serum which can be stably stored for six months at -70°C.

D. Data Analysis

The data analysis will include descriptive analysis of mean levels of all endpoint analytes in all 15 women at each sampling point. Then, the same data will be expressed except for the specific menstrual phase, namely follicular phase (low progestagen and low LH) defined as days 1-14, the ovulatory phase (high LH) days 15 and 16, and the luteal phase (high progesterone, low LH) days 17-28. The means and variances derived from the two ways of expressing the data will be compared. Specific variances in each phase will be examined, to identify the times in the cycle in which sampling should be avoided.

The effect of diet on endpoint analytes will be examined using both random blood sampling as well as menstrual cycle-specific sampling. The data will be applied across the cycle for each diet, and two analysis variances performed to examine the effects of menstrual cycle versus diet on endpoints.

The effects of diet on endpoints will also be compared between menstrual women at different parts of their cycle and those of postmenopausal women and men.

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Pennington Biomedical Research Center
LOUISIANA STATE UNIVERSITY

April 7, 1993

Penny M. Kris-Etherton, Ph.D.
Professor of Nutrition
Department of Nutrition
Pennsylvania State University
University Park, PA 16802

Dear Penny:

This is to confirm our willingness to collaborate with you and your co-investigators on your supplemental proposal to the DELTA Study, "Diet Responsiveness During the Menstrual Cycle."

As a Field Center for the DELTA Study, we will ask our population of pre-menopausal women subjects to participate in an extended dietary protocol consisting of two additional weeks of feeding. One additional week will be added prior to the first seven-week diet phase and the other additional week will be added immediately after the third seven-week diet phase. We will also ask these subjects to keep a menstrual diary. We anticipate that we will be able to recruit 5-8 subjects for your study. Approximately half of these subjects will be African Americans.

As part of our participation, we agree to assume the additional costs associated with meal preparation and any increase in subject compensation. While we agree to pay for any additional lipid profile determination that might be required, we understand that you will assume those additional costs associated with measurements of apolipoproteins, hemostasis variables, and circulating hormones.

Of course, our ability to collaborate is dependent upon favorable review of the revised protocol by our Institutional Review Board. However, I foresee no problems in this area.

Our DELTA Study Research Group at PBRC is looking forward to working with you on this important study.

Sincerely,

Michael Lefevre, Ph.D.
Associate Professor
Division of Nutrition and Heart Disease
Pennington Biomedical Research Center.

6400 Perkins Road, Baton Rouge, Louisiana 70808-4124 ♦ Phone: (504) 767-0880, Fax: (504) 765-2525

Introduction

In this application, we propose supplemental studies to complement our already funded studies of dietary fats on plasma lipids, lipoproteins and hemostasis. These supplemental studies have been designed to focus specifically on issues relating to women and diet, and will take advantage of the fact that our studies are being conducted as part of a collaborative multi-center trial, using standardized protocols and laboratory methodologies. This will enable us to study large numbers of females under conditions where diet control is maximized. With additional funds provided by the Office of Research on Women's Health, we propose to complement our present protocols with studies focusing on the interrelationship between the menstrual cycle and diet responsiveness, and on the effects of diet on HDL_2 and HDL_3. In this manner, we believe that we can significantly increase the information we obtain from our studies. In particular, these additional studies should allow us to determine the relevance of standard diet recommendations, which have been derived mainly from studies in men, for programs to prevent the development of coronary heart disease (CHD) in women.

1. Specific Aims

Aim 1: To carefully study the interrelationship between the menstrual cycle and the effects of diet on plasma lipids.

We propose to add additional blood samples to our planned protocols so that we can carefully determine, in our premenopausal females, the relationship of the menstrual cycle to their responses to dietary perturbation. Our approach would be to obtain two samples during the last four weeks of each diet period, with a third sample during the mid-cycle period. These additional samples would be used to measure total cholesterol and triglyceride, and HDL cholesterol concentrations.

Aim 2: To determine the effects of dietary modification on HDL subfractions.

We propose to measure, by differential precipitation methods, the effects of dietary modifications on HDL_2 and HDL_3 cholesterol concentrations. We will carry out these measurements in all our study participants: premenopausal and postmenopausal women and men.

2. Background

Although the effects that nutrient intake can have on the concentrations and metabolism of plasma lipids and lipoproteins are documented in a voluminous literature that has accumulated over many years, several key issues remain to be resolved. Among these are the efficacy of diet therapy in females. It is clear that premenopausal females have significantly less risk for developing CHD than do men. The basis of the reduced risk in women derives mostly from differences in lipid metabolism: premenopausal women have significantly higher plasma levels of high density lipoprotein (HDL) cholesterol (particularly HDL_2), lower plasma levels of triglycerides and low density lipoprotein (LDL) cholesterol, and much less

rarely demonstrate several common inherited dyslipidemic phenotypes, including Combined Hyperlipidemia and LDL Pattern A. After the menopause, HDL cholesterol levels fall, triglyceride and LDL cholesterol levels rise, and LDL Pattern B may develop.

Based on these data, several questions can be entertained regarding dietary recommendations in women. Is the response to any diet modification similar in men and women? Do premenopausal and postmenopausal women respond to diet perturbations in a similar fashion? Do HDL levels respond to higher carbohydrate and lower total fat levels similarly in men and women? Are these changes reflected by changes in HDL_2 levels in both gender groups? Does the LDL size distribution change in both men and women eating higher carbohydrate and lower total fat diets?

Very few studies have focused on the effects of diet on lipids and lipoproteins in women. One reason for the under representation of women in such studies is the concern that menstrual cycle effects on plasma lipids would confounds interpretation of the effects of any diet perturbation. It is clear from several studies that plasma total cholesterol fluctuates during the menstrual cycle, and this complicates sampling strategies and confound data analysis. As a result, most investigators have either recruited only men or postmenopausal women for their studies. Those studies that have included premenopausal women have ignored potential menstrual cycle effects. Our collaborative multicenter trials offer a unique opportunity to study the interrelationship between the menstrual cycle and diet effects on plasma lipids in a large group of premenopausal females who are consuming carefully designed and prepared diets. The data we derive from such studies will be of great benefit to investigators planning future studies in females.

Consumption of diets very low in total and saturated fats tends to be associated with reductions in HDL cholesterol concentrations. Although the effect of diet related reductions in HDL cholesterol levels on risk for developing CHD is unclear, concern has been raised, and there have been suggestions that dietary total fat be maintained by replacing saturated with monounsaturated fats. Premenopausal females have significantly higher levels of HDL cholesterol in plasma, and this is associated with increased concentrations of HDL_2 cholesterol. This difference between women and men narrows after the menopause, probably related to the loss of estrogen in the women. There is very little information concerning the effects of dietary fat modification on plasma HDL cholesterol levels in women, and none in large groups of pre- and postmenopausal women. Our collaborative multicenter study protocols provide us with a unique opportunity to address these important issues in a systematic fashion.

3. Experimental Methods and Design

Aim 1: To carefully study the interrelationship between the menstrual cycle and the effects of diet on plasma lipids.

Rationale: As described above, concern with the potentially confounding influence of the menstrual cycle on interpretation of the effects of diet perturbation on plasma lipids has

resulted in a paucity of such data in women. We propose to add additional blood samples to our planned protocols so that we can carefully determine, in our premenopausal females, the relationship of the menstrual cycle to their responses to dietary perturbation. The results should benefit greatly other investigators when they plan future diet studies involving women.

Subjects: We will study the interrelationship between the menstrual cycle and diet effects on plasma lipids in the premenopausal women recruited for our various diet protocols. All premenopausal female participants entered into the study at Columbia (n=7) will be eligible for participation in these extra blood sampling protocols.

Diets: The diets studies will be those chosen by the DELTA investigators for study in the overall protocols which will also include men and postmenopausal women. For the first study we plan to compare the effects of two diets reduced in total and saturated fat to an average American diet higher in total and saturated fat. The average American diet will contain 37% of calories as fat, with 16% saturated fat, 14% monounsaturated fat and 7% polyunsaturated fat; the Step 1 diet will have 30% of calories as fat, with 9% saturated fat, 14% monounsaturated fat and 7% polyunsaturated fat; the reduced fat diet will have 26% of calories as fat, with 5% saturated fat, 14% monounsaturated fat and 7% polyunsatured fat. All the diets will contain 250 mg cholesterol. Endpoints for the overall study will be total plasma cholesterol and triglycerides, HDL cholesterol, apoprotein B, apoprotein A-I, lipoprotein (a), and the hemostatic factors fibrinogen, factor VII and plasminogen activator inhibitor 1. ApoE genotypes will also be determined for all subjects.

A second study during the first year of protocols is being planned, and will focus on the effects of increasing omega-3 fatty acids within the context of the Step 1 diet. Plans for the second year of protocols include studies comparing the average American diet and the two diets with reduced levels of total and saturated fat in subjects with insulin resistance.

Protocol: We plan to modify our overall sampling strategy, which calls for weekly blood samples during the final three weeks of the study, with three samples in the final week. If we study the interrelationship between the menstrual cycle and diet, our approach would be to obtain two samples during the last four weeks of each diet period, with a third sample during the mid-cycle period, in each of the premenopausal women.

Laboratory: The additional samples, which would number five per subject for each 7 week diet study, or 15 per premenopausal female for the first protocol, would be processed and stored at -70°C along with all other study samples. The DELTA investigators have decided to determine plasma total cholesterol and triglycerides, along with HDL cholesterol by precipitation, in each field center laboratory. The laboratories will each be standardized by the CDC on an ongoing basis throughout the studies to insure adequate precision and accuracy.

Aim 2: To determine the effects of dietary modification on HDL subfractions.

Rationale: As noted above, the distribution of HDL cholesterol between the major subclasses, HDL_2 and HDL_3 differs significantly between men and women. We propose to measure, by differential precipitation methods, the effects of dietary modifications on HDL_2 and HDL_3 cholesterol concentrations. The results may modify significantly our conclusion concerning the effects of reductions in dietary fat intake on total HDL cholesterol levels in men and women.

Subjects: We plan to determine HDL subfractions in all participants in the diet protocols at all of the DELTA field centers.

Diets: We propose to perform measurements of HDL subfractions in each of the diet protocols described above.

Protocol: We will carry out these measurements in all study participants; premenopausal and postmenopausal women and men. The samples will be obtained at the time of scheduled blood tests during the final weeks of the study (or during the menstrual cycle in the premenopausal women).

Laboratory: Whole HDL cholesterol will be measured after precipitation of plasma apo B containing lipoproteins with 10g/L dextran sulfate and 0.5 M $MgCl_2$ (0.91 mg/ml and 0.045 M final concentrations, respectively). HDL_2 cholesterol will be determined as the difference between whole HDL cholesterol and HDL_3 cholesterol after differential precipitation of HDL_2 from the dextran sulfate/Mg++ supernatant with 10g/L dextran sulfate and 1.5 M $MgCl_2$ (0.91 mg/ml and 0.136 M final concentrations respectively).

4. **Data Analysis**

All results will be analyzed together with the main body of data collected in these protocols. Analyses will be performed as a collaborative effort between the Coordinating Center and the Field Centers of DELTA. Although we will not have adequate sample size to directly compare men and women, or pre and postmenopausal women, we will be able to determine the reponse of each of the outcome variables described above to dietary perturbations.

5. **Human Subjects:** The proposed additions to our already planned protocols will increase slightly the number of blood samples and the total volume of blood removed from participants. Only the menstrual cycle study will add significantly to the blood volume withdrawn, by about 50 mls. We will obtain additional approvals from our IRB for these proposed studies.

SPECIFIC AIMS

A growing body of evidence now suggests that the relative abundance of specific subpopulations within the major lipoprotein classes is an important determinant of CHD risk. Data suggest that both LDL and HDL subpopulation distribution can be influenced by diet. However, it is not clear if LDL and HDL distributions in men, pre-menopausal women and post-menopausal women will respond in a similar manner to identical dietary manipulations. It is believed that hormonal effects are responsible for the observed differences in LDL and HDL subpopulation distributions between men and women and between pre- and post-menopausal women. We hypothesize that these hormonal effects will also influence the pattern of change observed in LDL and HDL subpopulation distribution following changes in dietary fat content and composition such that pre-menopausal women will respond in a pattern distinct from that observed with either men or post-menopausal women.

To test this hypothesis, we propose, as a supplement to the DELTA study, the following specific aims:

1. To determine the differences in LDL and HDL subpopulation distribution between pre-menopausal women, post-menopausal women and men while consuming identical diets.
2. To identify and compare changes in LDL and HDL subpopulation distribution and size following changes in dietary fat content and composition in pre-menopausal women, post-menopausal women and men.
3. To correlate changes in LDL and HDL subpopulation distribution and size with changes in plasma lipids and lipoprotein parameters in pre-menopausal women, post-menopausal women and men.
4. To correlate changes in LDL subpopulation distribution and size with changes in HDL subpopulation distribution and size in pre-menopausal women, post-menopausal women and men.

This study was originally included as a Field Center Ancillary Study to the DELTA protocol. Funding of this application will allow us to study the entire subject population recruited across all four Field Centers. Increasing the number of subjects studied (including both pre- and post-menopausal women) by four-fold will substantially increase our statistical power to identify potential dietary effects and interactions.

BACKGROUND AND SIGNIFICANCE

Elevated levels of LDL cholesterol and reduced levels of HDL cholesterol have been clearly shown to increase one's risk for CHD. A growing body of evidence now suggests that the distribution of cholesterol among subpopulations within these major lipoprotein classes is also an important determinant of CHD risk. Indeed, the differential distribution of cholesterol among both the LDL and HDL subclasses may be partially responsible for the lower risk of premature CHD observed in women. While data

suggest that both LDL and HDL subpopulation distribution can be influenced by diet, it is not clear if men, pre-menopausal and post-menopausal women respond in a similar manner to identical dietary manipulations.

HDL Subclasses. HDL represent a complex series of interacting particles whose individual functions are only now being elucidated. In numerous reports, HDL_2 levels (as measured by ultracentrifugation or precipitation) were inversely correlated with CHD risk (1-3). Essentially the same conclusions were obtained employing nondenaturing gradient gel electrophoresis (GGE) to analyze HDL subclasses; MI survivors were characterized by having relatively less HDL_{2b} and relatively more HDL_{3c}, HDL_{3b}, and HDL_{3a} (4).

Elevated HDL_2 cholesterol levels and an HDL subpopulation distribution characterized by increased HDL_{2b} and HDL_{2a} and decreased HDL_{3b} are thought to contribute to the lower incidence of CHD in women (5). A change in menopausal status does not appear to significantly influence total HDL cholesterol levels (6). However, we could find no data regarding differences in HDL subpopulation distribution (defined by GGE) as a function of menopausal status. Such data may identify apparent changes in risk for CHD associated with post-menopausal status that may be independent of total HDL cholesterol levels. Because the dietary intake of the subjects will be controlled, the present study would provide a unique opportunity to examine effects of menopausal status on HDL subpopulation distribution without the confounding effect of differences in diet.

Few studies have specifically examining the impact of diet on individual HDL subpopulations as defined by GGE. Such studies would be of particular interest because of the growing understanding of the relationship between changes in individual HDL subpopulations, changes in the metabolism of other lipoprotein populations and changes in the risk for CHD. The recent studies of Clifton and Nestel (7) in which HDL_2 cholesterol levels were shown to increase to a greater extent in women than men in response to a high fat diet, suggests the possibility of gender-specific effects of diet. The studies on HDL subpopulation distribution proposed in this application would provide important new information regarding potential effects of gender and menopausal status on diet response.

LDL Size and LDL Phenotype. Studies suggest that the presence of small dense LDL may increase risk for CHD (8,9). Other studies have demonstrated the presence of a heritable LDL phenotype characterized by the predominance of small LDL (less than 25.5 nm in diameter on nondenaturing gradient gels) which is associated with increased risk for myocardial infarction (Phenotype B) (10). It is estimated that the allele frequency for the B phenotype is 25%. The presence of small LDL particles (or phenotype B) is associated with other known risk factors for CHD, including increased levels of plasma triglycerides and LDL cholesterol and decreased levels of HDL cholesterol and particularly, HDL_2 (9,11,12).

The presence of small LDL particles is significantly more common in men than in women (12). In women, post-menopausal status is associated with increased prevalence of small LDL (13). Similarly, in studies of LDL phenotype expression, pre-menopausal were shown to rarely express the B phenotype (14).

As with HDL, diet clearly has the capacity to influence LDL composition and size. Cross sectional population studies have suggested that a high carbohydrate diet may be associated with the presence of smaller LDL (15). Similarly, when compared to high saturated fat diets, high polyunsaturated fat diets are associated with LDL characterized by a lower cholesterol/protein ratio (16-18), consistent with the presence of smaller LDL particles. Given the strong influence of gender and menopausal status on LDL size, it is not unreasonable to speculate that the effect of changes in dietary fat content and composition will not equally affect LDL subpopulation distribution in pre-menopausal women, post-menopausal women and men.

Conclusion. Well controlled human studies have clearly demonstrated that a major effect of variations in cholesterol intake, fat intake and fat type on CHD risk is mediated through modulation of plasma lipid and lipoproteins levels. However, fewer studies have examined the effect of dietary fat modifications on LDL and HDL subpopulation distribution and none in a large population of pre- and post-menopausal women. The design of the DELTA study will provide a unique opportunity to examine the effects of specific changes in dietary fat on LDL and HDL subpopulation distribution and their interrelationships as a function of both gender and menopausal status. These data will be useful in guiding our decisions regarding diet recommendations for women throughout the life cycle.

PRELIMINARY DATA

The recent lack of availability of high-quality gradient gels from commercial sources necessitates that these gels be produced "in-house". Our laboratory has been involved in the production of in-house gradient gels for over six years (19). Gradient gels are produced with a concave gradient of 2 to 30% acrylamide in a Pharmacia GSC-8 slab gel casting apparatus. The extended length of our gels (140 mm versus 70 mm) along with the lower initial acrylamide concentration (2% versus 4%) allows for the simultaneous determination of LDL and HDL distribution on a single gel. The gels provide highly reproducible results; the intra and inter assay coefficient of variation for HDL modal diameter are between 0.5 and 1.1%.

In preliminary experiments, we have explored the effects of freeze/thawing of plasma on the distribution of LDL and HDL as determined on lipid-stained non-denaturing gradient gels. Consistent with what has been previously reported (12), we found that freeze/thawing had minimal effect of the distribution of LDL. Additionally, we found that freeze/thawing also had minimal effects on the resulting HDL distribution. These preliminary studies suggest that plasma samples can be obtained at different points in time, stored frozen, and analyzed at one time to minimize the effects of inter-assay variation.

EXPERIMENTAL DESIGN

Diet clearly has the capacity to influence LDL and HDL concentration, composition and structure. However, no study has examined the effects of diet on the coordinated changes in LDL and HDL subpopulation distribution and size as a function of gender and menopausal status. We therefore propose to examine the effects of specific changes in dietary fatty acid content and composition on LDL and HDL size distribution as assessed by non-denaturing gradient gel electrophoresis.

Subjects

Samples will be obtained form all subjects participating in the DELTA diet study. The study population will consist of normal men and women ages 25 - 65. Recruitment targets for the study are as follows: 29 pre-menopausal women; 29 post-menopausal women; and 38 men. African-Americans will make up approximately 30% of the subject population.

Diets

The first diet protocol will study the effects of substituting carbohydrate for saturated fat. Three levels of saturated fat will be considered: 16% (Average American Diet); 9% (Step 1 Diet); and 5% (similar to Step 2 Diet). The dietary variables which will be studied in subsequent DELTA protocols are still being considered. It is likely that these will include n3-fatty acids, monounsaturated fatty acids, and individual saturated fatty acids (i.e. myristic vs. palmitic vs. stearic).

Study Design

The first dietary protocol will employ a randomized, double-blinded, three-period, complete crossover design. Each dietary period will be seven weeks in length. Subsequent protocols may employ similar crossover designs or parallel arm designs.

In the first dietary protocol, fasting blood samples will be obtained during weeks 5, 6 and 7, from each subject for endpoint determinations (total cholesterol, HDL cholesterol, triglycerides, apoA-I, apoB, Lp(a), factor VII, fibrinogen, and plasminogen activator inhibitor-I). A schedule of three determinations made at 1 week intervals will allow us to estimate at what time point the measured parameters will have reached a new steady state during each dietary sequence.

For the purposes of this supplemental application, an alternate sampling schedule will be implemented in pre-menopausal women. This sampling schedule will consists of two fasting blood samples for endpoint determinations obtained during weeks 4, 5, 6 and 7. This alternate sampling schedule will be used to further define the effects of the menstrual cycle on the endpoint measurements and to assist in developing sampling strategies to be used in subsequent studies.

Analytical Methods

LDL and HDL size distribution will be determined in frozen plasma samples obtained from each of the last three weeks of each dietary period from each subject. Published data and our own preliminary results suggest that both LDL and HDL profiles remain stable after freezing at -70° C.

LDL and HDL size distribution will be determined by nondenaturing gradient gel electrophoresis as described by Musliner and Krauss (20) and Blanche et al. (21) with the exception that in-house 2-30% concave acrylamide gels will be used (19). The format of these gels is such that LDL and HDL size distribution can be determined from a single gel. All samples from a given individual will be analyzed at the same time on the same batch of gels and, to the extent possible, on the same gel. Two quality-control samples, obtained from single-use aliquots of frozen (-70° C) plasma, will be included on each gel. The quality-control plasma will be chosen to provide different LDL phenotypes and high and low HDL_2 ($HDL_{2a} + HDL_{2b}$) levels.

The gels will be stained with Sudan black B as described by McNamara et al.(12). The lipid distribution, as a function of gel migration (R_f) will be determined by densitometry at a resolution of 84 μM employing a BioRad GS-670 Imaging Densitometer. The R_f-based distribution will be converted to a particle size-based distribution employing the paradigm developed by Williams et al. (22) or by custom software available at this Field Center.

From the distribution of relative lipid-stain intensity versus particle diameter, the following parameter will be determined:

1. LDL phenotype (A, B or Intermediate) based upon the peak diameter for LDL.
2. "LDL score" as defined by McNamara et al. (12) and based upon the weighted distribution of LDL among seven size classes as described by Krauss and Burke (20).
3. Relative LDL subpopulation distribution based upon Gaussian deconvolution (PeakFit Software) of the LDL pattern. Data will be expressed as percent of total LDL lipid staining intensity distributed among the seven LDL size classes.
4. Relative HDL subpopulation distribution based upon Gaussian deconvolution of the HDL pattern. Data will be expressed as percent of total HDL lipid staining intensity distributed among five HDL size classes as defined by Blanche et al. (21)

Data Analysis

Data will be analyzed to determine the effects of diet, gender, and menopausal status plus interactions on the LDL and HDL parameters described above. The appropriate statistical methodology to be employed will be determined following consultation with the Coordinating Center.

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Influences of Gender and Menstrual Cycle on Serum Lipids and LDL Oxidation

Patricia J. Elmer

Specific Aims of this proposal are:

1) to evaluate the variation in lipid parameters (TC, TG, HDL, LDL) during the menstrual cycle in healthy women participating in a controlled feeding trial of fat and fatty acid modifications, and

2) to evaluate the effects of dietary modifications (of total fat, monounsaturates, polyunsaturates and omega-3) on LDL susceptibility to oxidation in both women and men.

Dietary Effects on Lipoprotein and Thrombogenic Activity (DELTA) is a multicenter feeding study evaluating the effects of dietary modifications (total fat, fatty acids, polyunsaturates, monounsaturates, saturates, omega-3) on lipids and hemostasis. Four field centers have been selected to conduct DELTA. They are The University of Minnesota (Patricia J. Elmer, PI); Louisiana State University (Paul Roheim, PI); Pennsylvania State University (Penny Kris-Etherton, PI); Columbia University (Henry Ginsberg, PI). The Coordinating Center is located at The University of North Carolina, Chapel Hill, (Barbara Dennis, PI). Approximately 100 participants (24/field center) will be randomized for each diet protocol. The study is completing its planning phase, and has piloted menu protocols. Recruitment for the first protocol will begin in August, 1993. The first diet protocol will study 3 diets differing in total and saturated fat content. The diets will consist of:

1) A "typical American diet" with 37% of calories from fat, 16% from SFA, 14% from MUFA, and 7% from PUFA, and 300 mg cholesterol;
2) A Step One AHA diet with 30% of calories from fat, 9% from SFA, 14% from MUFA, and 7% from PUFA, and 300 mg cholesterol; and
3) A Very Low SFA diet with 26% of calories from fat, 5% from SFA, 14% from MUFA, and 7% from PUFA, and 300 mg cholesterol.

The second diet protocol for DELTA will study the effects of omega-3 fatty acids and the subsequent protocols (covered by year two of this supplement) will evaluate the effects of these diets in individuals at high risk for CVD (hypercholesterolemia and insulin resistance). The primary endpoints for the main study are: total cholesterol and triglycerides, LDL-C, HDL-C, Apo B, Apo A-1, and Lp(a). Additionally, Apo E genotype will be determined for each participant. The primary hemostatic endpoints will be plasma levels of fibrinogen, factor VII, and PAI-1. Several ancillary studies may be carried out at individual field centers to study a variety of secondary endpoints. The questions regarding menstrual cycle influences on lipid and hemostatic responses and the effect of dietary modification on LDL oxidation were originally proposed as ancillary studies for this DELTA project. However, funds were not available from the original grant award to carry out these studies. This supplement will allow evaluation of these two questions related to women's health and CVD within the DELTA study. Utilizing measures from participants in all four DELTA centers provides a large sample size not available in other feeding studies, and will provide an efficient mechanism for studying these questions in a controlled feeding study where the fat content and fatty acid composition of the diets are altered.

Background

The field of research concerning diet and lipids has generated considerable knowledge about the effects of dietary fatty acids on the concentrations of plasma total, LDL and HDL cholesterol. However there is a considerable gap in our knowledge about how gender, menopausal and hormonal status might potentiate the effects of dietary fatty acids on lipoprotein concentrations. Although there is a considerable body of literature regarding blood lipid changes in response to dietary manipulations, the vast majority of studies have been conducted in young healthy men. Often women have not been studied within this type of research design because of the lack of information about the effects of menstrual cycle on the metabolic parameters being studied and the large cost associated with multiple measurements needed to assess this variation. Some studies which included women have failed to consider menstrual cycle variation when obtaining physiologic measures, which in turn has complicated interpretation of the results.

During the development phase of DELTA, one of the specific goals of our protocol design was the inclusion of women in these feeding studies in order to provide much needed data on the effect of dietary manipulations in both pre- and post-menopausal women. There is some indication that plasma total cholesterol concentrations rise through the menstrual phase, peaking in the ovulatory phase. HDL-C has been shown to increase at ovulation or remain constant across phases. Apo A-1 has been shown to be significantly higher in the follicular, ovulatory and luteal phases. Triglyceride levels appear to increase at ovulation or not change throughout the cycle (1, 2). Some studies

have not shown significant changes in lipids or lipoproteins during the menstrual phases. Differences in methods, time of sampling and limitations of sample size and individual variability are possible explanations for the inconsistencies. This supplemental proposal to be carried out within DELTA provides a unique opportunity to evaluate questions regarding hormonal influences on lipid and hemostatic parameters during the menstrual cycle. The study also will examine differences in variability of diet responsiveness in pre- and post-menopausal women. This information will be useful in the design of future cardiovascular disease observational and intervention studies by providing information on the optimal measurement schedule. It will also provide important information for interpreting studies, and explaining gender differences in lipid and hemostatic parameters and responses to dietary intervention.

Similarly, little is known about the effects of changes in dietary fatty acids on other factors related to cardiovascular diseases, such as hemostatic factors, apoprotein concentrations, lipoprotein subfractions, lipoprotein composition and LDL oxidation. It is now well established that hypercholesterolemia, and a specifically high LDL cholesterol, is an important cause of coronary heart disease (CHD), and clinical intervention studies have demonstrated the therapeutic value of correcting hypercholesterolemia. We know that high cholesterol levels are by no means the only causative factor and at any given level of hypercholesterolemia there is considerable variation in the clinical expression of the disease. One basis for such variation undoubtedly lies in the biologic responses of cells in the artery wall in the presence of a given level of plasma cholesterol. Recent advances in understanding of the metabolism of lipoproteins by the artery wall have yielded new insights into the factors that may be involved in the arterial response. Specifically, certain oxidative modifications in the structure of lipoproteins appear to affect their atherogenic potential (3-5).

There is a growing body of evidence to suggest that oxidative modification of low density lipoproteins (OX-LDL) plays an important role in the pathophysiology of atherogenesis. In recent years, a number of molecular mechanisms of different oxidation pathways leading to modification of LDL-C have been proposed (3). It is now clear that the earliest step in the generation of OX-LDL is peroxidation of its polyunsaturated fatty acids (PUFA) (5, 6). LDL particles entering subendothelial sanctuaries of the artery wall can become trapped and exposed to oxidative stresses (4). LDL oxidation has been shown to foster recruitment of macrophages, and by binding to scavenger receptors on the surface of macrophages, oxidized LDL can ultimately generate foam cells (4, 5). Oxidized LDL also is directly cytotoxic, particularly to vascular endothelial cells (6, 7). Such damage would presumably exacerbate atheroma formation both by allowing LDL to freely enter the artery wall and by promoting platelet adherence and growth factor liberation (8, 9-11).

Contravening this deleterious process are several types of lipid soluble antioxidants contained in LDL particles which protect LDL polyunsaturated fatty acids from oxidation (12). These include alpha and gamma tocopherol, ubiquinol-10 and several members of the carotenoid family: primarily alpha- and beta-carotene and lycopene. Except for alpha tocopherol, these antioxidants are usually found in LDL at concentrations that are less than 1 antioxidant molecule per LDL particle and can explain at best only half of the variability in LDL susceptibility to oxidation between people on average American diets. The LDL lipid and fatty acid composition probably explains additional variability between persons. Diets high in monounsaturated oleic acid increase LDL resistance to oxidation in rabbits (13) and in humans (14, 15) compared to diets high in polyunsaturated linoleic acid. Gender and hormonal influences have also been hypothesized as factors which may account for variation between men and women. It has recently been hypothesized that estrogen itself may act as a weak antioxidant (16). In addition, it has recently been hypothesized that the lower iron levels in premenopausal women may in part explain the differences in atherosclerosis and CVD rates between men and women, possibly through affects on LDL oxidation (17).

One of the promising approaches for reducing CHD risk is to alter plasma lipid composition by manipulating dietary fatty acid composition. As indicated, the enrichment of diets with monounsaturated fatty acids (MUFA) results in LDL that are resistant to oxidation. Thus, this study will test the hypothesis that dietary manipulation can reduce the oxidative potential of the LDL fraction. Oxidation of LDL may be especially important in individuals at high risk for development of atherosclerosis. The second year of this proposal will focus specifically on the effects of dietary manipulation on LDL oxidation in high risk individuals (hypercholesterolemics, insulin-resistant).

Design

The questions to be evaluated in this study will utilize the design of the DELTA study - a series of controlled crossover feeding studies in healthy men and women, age 25-65. Participants undergo baseline screening to

determine eligibility (based on medical criteria) and then enter into the feeding phase which employs three, 7-week feeding periods (for 21 weeks of feeding). The diets alter total fat and fatty acid composition by reducing saturated fat, and increasing monounsaturated fatty acids or polyunsaturated fatty acids or omega-3 polyunsaturated (as described above). Each participant is randomized to a diet sequence and the participant is blinded to the sequence. Currently, blood samples are obtained at the end of weeks 5, 6, and 7 for endpoint measurement (hemostasis and lipids). For specific aim 1, additional blood samples will be obtained at weeks 4, 5, 6, and 7. For specific aim 2, an aliquot of blood from samples already obtained at weeks 5, 6, and 7 will be used for the LDL susceptibility to oxidation analyses. Blood samples will be obtained from participants in all centers and will be shipped to the University of Minnesota for biochemical analyses. Samples will be obtained during 4 diet protocols, covering the two year period of this proposal. Data analyses will be conducted by the DELTA Coordinating Center at the University of North Carolina, Chapel Hill. The protocol for the main DELTA study has been approved by the University of Minnesota IRB. For this project, additional blood samples will be required. The University of Minnesota IRB has been provided with information regarding changes to the original protocol needed to conduct this study and we are awaiting approval of these changes and collection of the additional samples.

Methods

Specific Aim 1. Blood samples in addition to those collected in the main DELTA protocol will be obtained during weeks 4-7 of each diet period of the study for all pre-menopausal women. Two additional fasting blood samples will be drawn during week four, and one additional fasting blood sample will be drawn during weeks 5, 6, and 7. This will provide multiple samples throughout the menstrual cycle to assess variation and will also include samples during the midcycle.

Fasting Lipid Profile. Total cholesterol, HDL-C, LDL-C and triglyceride will be measured in the University of Minnesota BELRC Laboratory. A 5 mL EDTA Plasma in lavender top tube will be obtained at each measurement point. Total cholesterol is measured on a Beckman CX-5 autoanalyzer (Brea, CA) using the Beckman calibrator and enzymatic reagent. Cholesterol measurements are standardized by the Centers for Disease Control (CDC) Lipid Standardization Program. HDL-C is measured enzymatically after precipitation of VLDL-C and LDL-C with dextran sulfate and magnesium chloride. LDL-C is estimated by the Friedwald equation. This equation assumes a ratio of 5 for plasma triglyceride to VLDL-C. Total plasma triglyceride is measured on a Beckman CX-5 autoanalyzer using Beckman enzymatic GPO reagent and calibrator. This method gives a "true" triglyceride value which has been corrected for free glycerol concentrations. Triglyceride measurements are also standardized by the CDC.

Specific Aim 2. Fasting blood samples will be collected in each of the four centers and serum frozen at -70° until all feeding periods have been completed. Samples will be shipped frozen to Minnesota (BELRC) for analysis. Analyses will be conducted at the end of each diet protocol. Measures related to LDL-oxidation will include quantification of thiobarbituric acid reactive substances, diene conjugation and LDL resistance to oxidation in the presence of hemin.

Measurement of LDL resistance to oxidation with hemin and H_2O_2. LDL (1.019-1.063 g/ml) will be isolated from 1 ml of serum by sequential ultracentrifugation. LDL will be oxidized with hemin and H_2O_2 in 96-well Immulon I microtiter plates (Dynatech, Chantilly, VA). The oxidation of LDL will be monitored by measuring the decreasing absorbance of hemin at 405 nm. The decrease in hemin absorbance parallels the increase in thiobarbituric acid reactive substances (TBARS) and conjugated dienes. TBARS will also be measured. The final assay concentrations for the microtiter assay will be 40 µg/ml LDL protein, 2.5 µM hemin and 50 µM H_2O_2 in HEPES-NaCl (10-150 mM) pH 7.4 buffer in a final assay volume of 0.15 ml. The assay will be started by the addition of H_2O_2. Each LDL sample will be assayed in quadruplicate. After the addition of hydrogen peroxide, the plate will be read at 43 second intervals for 4 hours in a Vmax kinetic microtiter plate reader (Molecular Devices, Menlo Park CA). The resistance of LDL to oxidation will be measured as the time required for LDL oxidation to reach maximum velocity, i.e. time to Vmax. The time to Vmax will be computed by computer software linked to the plate reader (Molecular Devices, Menlo Park, CA). The correlation (r) between lag time and time to Vmax in 56 samples was 0.992, however time to Vmax was approximately 10 minutes longer than lag time. The analytic and analytic plus biologic coefficients of variation for the method were 5.9 and 9.6 percent, respectively.

The plasma LDL fraction will be isolated by single spin density ultracentrifugation. The peroxidation potential of LDL will be quantified by both malonaldehyde formation and diene conjugation. Approximately 150-200 µg LDL protein will be incubated with ascorbate-ADP-Fe^{+2}. Thiobarbituric acid reactive substances will be quantified at

expressed as malonaldehyde equivalent content (nmoles/mg protein). For the determination of conjugated dienes, the above reaction will be terminated after 15 minutes by the addition of 6 N HCL. Oxidized fatty acids will be extracted and separated. The organic phase will be evaporated, redissolved in methanol, and the conjugated dienes will be quantified by measuring absorbance at 234 nm.

Previous Work. Dr. Belcher is a co-investigator in the DELTA collaborative dietary feeding study and a member of the study's laboratory subcommittee. He will oversee the measurement of LDL susceptibility to oxidation. These measurements will be performed in the Biochemical Epidemiology and Lipid Research Core located in the Division of Epidemiology, School of Public Health at the University of Minnesota. Dr. Belcher, an Assistant Professor in the Division of Epidemiology, has been the director of this laboratory since 1986. His laboratory is studying the relationships between LDL oxidation and coronary heart disease.

In previously reported *in vitro* studies (7), Dr. Belcher and his colleagues found that heme, a physiologically widespread hydrophobic iron compound, can rapidly generate oxidized low density lipoprotein (LDL) which becomes cytotoxic to cultured vascular endothelial cells; both LDL oxidation and endothelial cytotoxicity were inhibited by incubation with exogenous alpha tocopherol (vitamin E) or ascorbic acid (vitamin C). Seeking relevance to *in vivo* conditions, additional studies were performed in which 10 human volunteers were given daily antioxidant supplements of 800 I.U. of dl-alpha tocopherol acetate for two weeks (18). This study demonstrated that oral vitamin E supplementation increases LDL alpha tocopherol content, lowers LDL susceptibility to oxidation and dramatically reduces the cytotoxicity of heme-conditioned LDL to cultured endothelial cells.

Dr. Belcher is also co-author of a manuscript submitted for publication by Dr. Jukka Salonen entitled "Increase in Resistance to Oxidation of Atherogenic Serum Lipoproteins Due to Lowering of Body Iron Stores by Blood Letting." This study indicated that the reduction of body iron stores by venesection can increase the oxidation resistance of serum VLDL/LDL in regularly smoking men. Currently Dr. Belcher's laboratory is measuring LDL susceptibility to oxidation and plasma antioxidants in a nested case-control study within the Atherosclerosis Risk in Communities (ARIC) cohort. Cases have been selected for this study according to their carotid artery wall thickness assessed by B-mode ultrasound.

Budget Justification

Funds for this administrative supplement will be used to cover costs of biochemical analyses of lipoproteins and LDL susceptibility to oxidation. Lipid measurement costs include four additional blood draws per premenopausal woman (in the Minnesota Center) per diet period, plus a replicate sample drawn for each 10 samples for quality control purposes and supplies and shipping for these samples. LDL oxidation costs include one sample per subject from all four DELTA centers per diet period, plus one replicate sample drawn for each 10 samples for quality control. Cost for additional blood draw supplies, storage, aliquot vials and sample shipping for the LDL oxidation studies are requested. Dr. Patricia Elmer is the Principal Investigator and Dr. John Belcher is the Co-Principal Investigator. No salary support is requested, rather salary support is provided by the main DELTA project. Samples will be analyzed in the University of Minnesota BELRC (Biochemical Epidemiology and Lipid Research Core Lab), Division of Epidemiology under the direction of Dr. Belcher. LDL oxidation (as determined by TBARS thiobarbituric acid and LDL oxidation heme method) will be analyzed by our laboratory on samples from all four of the DELTA centers. Funds are requested for two years. Samples will be obtained during this two year period on during the 4 feeding protocols employed by the DELTA study in those years. Analyses will be conducted for menstrual cycle effects and evaluation of LDL oxidation. Year two includes a 4% inflationary adjustment in cost per sample.

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