DELTA PROTOCOL 2

DRAFT

5/5/94

Dietary Effects on Lipoproteins and
Thrombogenic Activity

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CHAPTER 1

Protocol Introduction and Rationale

Chapter 1: PROTOCOL INTRODUCTIONS AND RATIONALE

Numerous clinical and epidemiologic studies have associated hypercholesterolemia, particularly elevations of LDL cholesterol, with increased risk for developing coronary artery disease (1-3). These data, together with many studies, including the classic studies of Hegsted et al (4) and Keys et al. (5), have led to dietary recommendations that focus on reducing total and saturated fat in the diets of all Americans (3,6,7). Total fat intake of less than 30% of calories and saturated fatty acid intake of less than 8-10% were recommended in the recent National Cholesterol Education Program update of its guidelines (3). Some groups, citing epidemiologic links between particular types of cancer and dietary fat intake are calling for further lowering of dietary fat intake.

Low fat diets are higher in carbohydrates, and this has alarmed some scientists who believe that high carbohydrates produce undesirable effects on plasma lipids that may more than negate the positive effects of lowering saturated fatty acid intake. Increased intake of simple carbohydrates was shown many years ago to increase plasma triglyceride concentrations (8-10), and more recently, to lower HDL cholesterol levels (11-13). The role of hypertriglyceridemia as a risk factor for coronary artery disease continues to be debated (14-16). However, this consequence of high carbohydrate diets, along with the reduction in HDL cholesterol, which is more widely accepted as an undesirable outcome, has caused several investigators to propose alternatives. In particular, the maintenance of dietary fat intake by replacing saturated fatty acids with monounsaturated fatty acids has been suggested (17-19).

The debate regarding the optimum replacement for dietary saturated fatty acids is particularly relevant to individuals with non-insulin dependant diabetes mellitus. Indeed, in a study of diabetics by Garg et al. (20), a diet high in monounsaturated fatty acids achieved similar LDL lowering compared with the standard NCEP Step 1 diet. Use of the high monounsaturated fat diet was, however, associated with lower triglyceride and higher HDL cholesterol levels than the NCEP Step 1 diet. In addition, the higher fat diet appeared to result in better diabetes control. Similar findings, albeit with smaller differences, were seen in a recent multi-center trial (21) in which a diet containing 55% of calories as carbohydrate and 30% of calories as fat was compared to a diet with 40% of calories as carbohydrate and 45% as fat. Both diets had 10% of calories from saturated fat and 10% from polyunsaturated fat, but differed (10 vs. 25%) in calories from monounsaturated fat. Results such as these have generated criticism of the American Diabetes Association dietary guidelines (7).

The question of the optimum dietary replacement for saturated fat is relevant not only for individuals with diabetes, but for an even larger group of people characterized by metabolic abnormalities that include hypertriglyceridemia, low HDL cholesterol levels, glucose intolerance and insulin resistance. This clustering of metabolic disorders, together with hypertension, has been designated Syndrome X by Reaven (22). He and others believe that low fat, high carbohydrate diets will be particularly problematic in individuals with Syndrome X, exacerbating their metabolic abnormalities and increasing their risk for coronary artery disease. Opponents of the high monounsaturated fat diet point to the increased caloric density of such a diet and the likelihood, therefore, that obesity will increase in this already overweight population during chronic intake of high fat diets.

In view of the important implications of this controversy to public health, DELTA has developed a protocol to compare the effects of a diet containing 30% of calories from fat and 55% from carbohydrates with a diet containing 37% of calories from fat and 48% from carbohydrate. The increased fat content of the latter diet will be derived solely from monounsaturated fatty acids. Both diets will provide 8% of calories as saturated fat and 7% as polyunsaturated fat. Both diets will provide 300 mg cholesterol per day. Each diet will be eaten for seven weeks and both will be compared to an Average American diet that has 37% of calories from fat with 16% of calories as saturated fat. We will recruit subjects with one or more of the following characteristics: plasma triglyceride levels above the 70th%, plasma HDL cholesterol levels below the 30th%, plasma insulin levels above the 70th% for age, sex and race. We will attempt to recruit equal numbers of males and females, and approximately 15% of the subjects will be African American. Endpoints will include the fasting lipids, lipoproteins, and thrombogenic factors measured in DELTA protocol 1. In addition, we will measure parameters of glucose and insulin metabolism and postprandial lipid clearance.

We envision that the proposed study will provide valuable new information that will resolve the current controversy about which diet is preferable for patients with Syndrome X. Accordingly, the results of this study may also be of benefit in the management of diabetes and in the prevention of cardiovascular diseases.

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CHAPTER 2

Organization and Administration

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 N. Ginsberg, M.D.

University of Minnesota,
Patricia Elmer, Ph.D.

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

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

B. Coordinating Center

The Coordinating Center, located at the University of North Carolina, Chapel Hill, 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.

Principal Investigator, Barbara H. Dennis Ph.D

Food Analysis Laboratory Control Center (FALCC)

FALCC, located at Virginia Polytechnic Institute and State University, is the central laboratory for food assays and associated research. Diet samples from this protocol of the DELTA study will be received, composited, assayed and archived at the FALCC.

Co-Principal Investigator and FALCC director, Kent Stewart Ph.D.

The FALCC will undertake the following tasks:

1. Develop the necessary protocols for collecting, storing, shipping, and compositing the diet samples. Develop the necessary protocols for assaying and archiving the sample composites.

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

3. Participate in the diet menu validations as appropriate including the assay of selected diet-menu-calorie level combinations for the nutrients of concern in this protocol.

4. Participate in the diet monitoring to verify that the menus have the nutrients as planned for the particular experimental period, that the diets being fed at the different centers are virtually identical, and documenting the drift (hopefully none) in the composition of the diets over the period of the study.

   The co-principal investigator of FALCC 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 monitors performance of the food assay laboratory. 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.

Director, Gary Beecher, Ph.D.

C. **Steering Committee**

The Steering Committee has primary responsibility 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 as appointed by the Director, Division of Heart and Vascular Diseases, NHLBI, is Dr. Henry Ginsberg.

The Steering Committee meets at least quarterly in years 2 -4 of the program. It has designated 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. The Steering Committee retains the prerogative to add, combine, delete, or redefine subcommittees as the study evolves and its needs change.

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

2. Diet Subcommittee: oversees the development and testing of the study diets, determines food production protocols, identifies food composition assays, evaluates nutrient databases, reviews relevant data collection forms and manuals of operation, assists in the procurement of food and food donations.

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

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

5. Publications Subcommittee: advises 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: has drafted guidelines regarding outside activities of study investigators that represent potential conflicts of interest and will collect annual disclosure statements from investigators regarding relevant activities.

7. Ancillary Studies Subcommittee: reviews and makes recommendations regarding the merit and feasibility of ancillary studies that are proposed by study investigators or other interested parties.

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

The guidelines established by the NHLBI for obtaining third party support will be followed.

A major effort has been 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 includes cholesterol screening machines and various participant incentives.

D. External Oversight

1. Protocol Review Committee: at least five experts not otherwise affiliated with the study will be appointed by the Director of 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 other 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 progress reports on the status of the study on an ongoing basis. These reports will include data on recruitment and randomization, as well as statistical tests and special analyses requested by the committee.

Members of the Protocol Review Committee are:

Roger Illingsworth, MD, Ph.D. (Chair)
Department of Medicine
Oregon Health Sciences University
Portland, OR

Ronald Eitenmiller, Ph.D.
Department of Food Science and Technology
University of Georgia
Athens, GA

Phyllis Stumbo, Ph.D, R.D.
General Clinical Research Center
University of Iowa
Iowa City, IA

Gary Cutter, Ph.D.
Pythagoras, Inc.
Birmingham, AL

Kenneth Wu, MD.
Division of Hematology
University of Texas Medical School
Houston, TX

E. Human Subjects Protection:

1. 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.

2. 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 9). It also includes discretion on the part of field center staff and arrangements for physical privacy during interviews and examinations.

3. Participant Screening and Feedback

Information obtained at screening, before enrollment, will be available at that time. Abnormal values found at screening will be reported to the participant and also, upon request, to his or her personal physician. Data will not be available until the very end of the study, because the protocol calls for batch analysis of all samples collected throughout.

4. 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 and meet subjects' calorie needs (they will be designed specifically to prevent weight change). The experimental diets will be planned using the RDA for women and men 25-50. The experimental treatments for the DELTA study are not expected to pose any risk. Since participants of this study are at increased risk of developing coronary heart disease, we will provide group instruction on the principles of a Step-1 diet at the end of each feeding period. Subjects will be encouraged to follow this diet during the breaks between feeding periods. At the end of the study, all subjects will receive either individual or group instruction on the implementation of heart healthy dietary practices. 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).

Information about food allergies will be collected 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 procedures will be rigorously applied in order to protect participants against illness due to microbiological contamination of food. Application of specified food handling standards appropriate for their particular institution (hospital-based kitchen; other setting) will be employed. 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-Temperature Indicator Labels (TTI's) (LifeLines Technology, Morris Plains, NJ) will be enclosed with instructions not to consume certain foods if the TTI indicates that holding temperatures have not been appropriate (See also Chapter 7). The consent form will include an explanation of the participants' obligation to handle take-out food according to instructions they will be given to maintain adequate sanitary conditions.

5. Safety Oversight

Safety concerns will be addressed during protocol review by the NHLBI-appointed committee and by the individual field center IRB's. 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.). A procedure will be developed for reporting deaths to the Program office and to the Coordinating Center.

6. 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

Hypotheses and Endpoints

Chapter 3: PROTOCOL 2 HYPOTHESES AND ENDPOINTS

A. Objectives

The purpose of this study is to determine the effects of two diets, both low in saturated fatty acids but differing in carbohydrate and monounsaturated fatty acid content, on plasma lipids and lipoproteins, and on hemostatic factors. In particular, we are interested in determining whether carbohydrate or monounsaturated fatty acids are the best replacement for saturated fat in people with high plasma triglycerides and/or low HDL cholesterol and/or high insulin levels.

B. Specific Aims

1. To compare the effects of three diets differing in total fat, saturated and monounsaturated fatty acids, and total carbohydrate, on fasting plasma lipids and lipoproteins in men and women at increased risk to develop atherosclerotic cardiovascular disease and diabetes.

2. To compare the effects of these diets on plasma hemostatic factors in these men and women.

3. To determine the effects of these diets on postprandial lipids and lipoproteins, and on plasma glucose and insulin levels.

C. Study Outcomes

1. The primary lipid and lipoprotein endpoints will be plasma concentrations of total cholesterol and triglyceride, low density lipoprotein cholesterol, low density lipoprotein size, high density lipoprotein cholesterol and apolipoproteins B and A-I.

2. A second group of lipid and lipoprotein endpoints will include apo E genotypes, HDL subfractions, lipoprotein (a) and very low density lipoprotein cholesterol.

3. The primary hemostatic endpoints will be factor VII, plasminogen activator inhibitor I, and beta thromboglobulin.

4. A second group of hemostatic factors may include fibrinogen and platelet aggregation by flow cytometry.

6. Fasting plasma insulin and glucose levels will also be primary endpoints.

7. Other secondary endpoints may include postprandial lipids, lipoproteins, insulin and glucose levels.

8. Several ancillary studies may be carried out to test sub-hypotheses.

D. Rationale for endpoints:

The rationale for the study design derives from controversy concerning the optimum diet for individuals with insulin resistance/hypertriglyceridemia/low HDL cholesterol (Syndrome X by Reaven). In particular, although diets low in saturated fat will reduce LDL cholesterol levels, replacement of saturated fat by carbohydrate has been shown, in some populations (particularly diabetics), to increase plasma triglycerides and lower HDL cholesterol levels. In addition, plasma insulin and glucose levels may be higher on high carbohydrate diets. There is little or no information about the effects of these diets on hemostatic factors or lipoprotein (a). Postprandial studies may provide information concerning the effects of these diets on chylomicron remnant metabolism as well as on the daily integrated lipid, glucose and insulin levels on high carbohydrate vs. high monounsaturated fat diets.

CHAPTER 4

Statistical Methods

Chapter 4: STATISTICAL METHODS

A: 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, *D* and *E*, and a reference diet, *F*, 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: *DEF*, *DFE*, *EDF*, *EFD*, *FDE*, and *FED*. Each diet period will be 7 weeks in duration. For example, the group assigned to the *DEF* sequence will be fed diet *D* for 7 weeks, then diet *E* for 7 weeks, and finally diet *F* for 7 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 cohort of well motivated participants. Furthermore, the selected design has satisfactory statistical properties under reasonable assumptions in the setting of this study.

To avoid carryover of effects from one period to the next, an active washout interval will be used. That is, the first 4 weeks of each diet period will provide time for stabilization of the responses of interest. Blood draws during the last 3 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 4 weeks or 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.

B. STATISTICAL ANALYSIS METHODS

"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. Many of the fine details of the analysis strategy will be formulated by small collaborative writing groups. However, some fundamental aspects of the analysis strategy are given below.

A tremendous variety of statistical analysis methods exist for cross-over studies. Appropriate choices of statistical models, estimation procedures, and inferential procedures 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 continuous and discrete outcome variables in a wide variety of cross-over design settings.

Generally speaking, non-parametric methods, simple descriptive methods, and graphical methods will also be used as appropriate to complement and support parametric inferences.

Longitudinal analysis methods are appropriate for the proposed study. The cross-over design is a special kind of longitudinal design yielding a series of correlated measurements on each individual studied. The longitudinal nature of the study is both a scientific advantage and an analytic complication. The serial correlations facilitate efficient comparison of the experimental diets. However, in order to take full advantage of this, the analysis of the data requires a model for the variances and covariances in addition to a regression model for mean levels. This model specifies that the variance-covariance array is well-approximated by a particular linear or non-linear function of a small number of parameters. Usually the number of parameters required is much smaller than the number variances and covariances. This represents a great savings in total number of unknown parameters that must be estimated.

The longitudinal nature of the proposed cross-over design also admits the possibility of temporal and sequential effects. These include, for example, "period effects", "subject-by-period interaction", "subject-by-diet interaction", "carry-over effects", and miscellaneous interactions thereof. Subject-by-diet interaction (i.e., subject-to-subject variation in dietary responsiveness) is well established in animal studies and has been observed in some human studies. In the proposed study, subject-by-period and subject-by-diet interactions will be completely confounded. We anticipate that carry-over effects will not be in evidence -- partly because they are hard to detect and partly because of the length of the feeding periods. On the other hand, period effects are frequently noted in dietary feeding studies. When any of the above effects exist, formulation and use of model for the variance-covariance structure is particularly advantageous. When none of the above effects exists, inferences about differences between diets can be made using very simple computations. We anticipate that period effects and subject-by-diet interaction will be observed in the proposed study. These are easily handled and pose no major problems. If absent, the appropriate simple computations are just special cases of the proposed general algorithms.

Approximately twenty endpoint measures are proposed for this study. Most of these are continuous and follow conditional distributions well-approximated by members of the normal (Gaussian), log-normal, or inverse-normal families of distributions. For these, longitudinal analysis based on the "general linear model" methods (GLM) are preferred. A few inherently non-Gaussian measures may need alternative methods. When the underlying distribution is uncertain and the relationship between mean and variance is well-established then longitudinal analysis based on "generalized linear model" methods (GLIM or GEE) are preferred. For details see Seber and Wild (1989), and McCullagh and Nelder (1989). These models are asymptotically exact for very broad family of distributions. We anticipate that the general linear model will be a particularly effective tool in the proposed cross-over study. The associated methods allow flexibility in estimation and testing of the expected-value effects (treatment, period, sequence) while easily taking into account the variance-covariance structure. The primary hypotheses will be tested within the framework of a components-of-variance model defined *a priori*. We will use maximum likelihood (ML) and restricted maximum likelihood (REML) estimation methods. In many special cases these methods are identical to Bayesian methods and least-squares methods. The SAS software system will be used to provide the necessary iterative algorithms (via the "MIXED" procedure). Furthermore, the mixed model will cope with any ignorably

missing data. For details, see Laird and Ware (1982), Jennrich and Schlucter (1986), Ware (1985), Neter and Wasserman (1974), and Harville (1977). This approach will also facilitate exploration of the variance-covariance structure. As a diagnostic tool it will be important to evaluate the sensitivity of primary analysis results to the modeling assumptions. Facility with linear and nonlinear covariance structures will also provide an opportunity for further discovery. As noted by Jones and Kenward (1990, pp 267-311), there are still many outstanding questions about variance-covariance structures in cross-over data.

C. SAMPLE DESIGN

Table 1 gives an example of possible recruitment outcome. The centers will attempt to recruit roughly equal numbers of men and women. However, we anticipate that the proportion of males may turn out to be 60 to 75 percent. The recruitment of pre- and post-menopausal women is unconstrained (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
C	F+	1	4	5
	F-	2	4	6
	M	6	8	14
L	F+	3	3	6
	F-	2	3	5
	M	7	7	14
M	F+	2	4	6
	F-	1	4	5
	M	3	11	14
P	F+	2	4	6
	F-	1	4	5
	M	3	11	14
Total	F+	8	15	23
	F-	6	15	21
	M	19	37	56
		33	67	100

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

D. RANDOMIZATION OF SUBJECTS TO TREATMENT GROUPS

At the end of the run-in period, remaining 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. Here "*DEF*", for example means Diet *D* followed by Diet *E* followed by Diet *F*.

Table 2
Proposed Allocation of Diet-Sequences to Subjects

	Center				
	C	L	M	P	Total
DEF	5	4	4	4	17
DFE	4	4	4	4	16
EDF	4	5	4	4	17
EFD	4	4	5	4	17
FDE	4	4	4	4	16
FED	4	4	4	5	17
Total	25	25	25	25	100

E. WORKING MODEL USED 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, a three-parameter components-of-variance 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 the components of variance. We anticipate that this model is approximately correct in spite of some sources of variation that may be multiplicative in nature. It should be noted that the assumptions made are pivotal for many of the conclusions about statistical power of test procedures that will be used. The following notation suppresses the details of the fixed effects of interest in order to focus on the details of the covariance structure.

$Y_{i,d,p,t}$ <-- the assay value for the i^{th} subject at time t when fed diet d in period p.

$= \alpha_d + \pi_p$ <-- the population mean for diet d in period p. These fixed-effect are of primary interest.

$+ S_i$ <-- "subject random effect". This is the amount by which subject i consistently differs from the population mean averaging over all possible diets.

$+ Z_{i,p}$ <-- "subject*period random effect". This term has an alias, "subject*diet random effect ($Z_{i,d}$)", in this model because no subject can be fed to two different diets in a single period.

$+ e_{i,d,p,t}$ <-- a residual error term. This quantity is different for each combination of i, d, p, and t. This catch-all term includes day-to-day variation, measurement error, etc., collectively called "within-subject" or "intra-subject" variation.

Total variance is constant and is the sum of three component variances:

$V[\ Y_{i,d,p,t}\ ]$ <-- total variance
$= V[\ S_i\ ]$ <-- subject variance
$+ V[\ Z_{i,p}\ ]$ <-- subject*period variance
$+ V[\ e_{i,d,p,t}\ ]$ <-- residual variance

In comparing diets D and F, simple differences such as $(Y_{i,D,p,t}-Y_{i,F,p',t})$ have variance that increases with both residual variance and subject*period variance:

$$V[\ (Y_{i,D,p,t}-Y_{i,F,p',t})\ ] = 2*(\ V[Z_{i,p}] + V[e_{i,d,p,t}]\ )$$

Serial correlation within a period is greater than serial correlation between periods:

$$\text{Corr}[\, Y_{i,D,p,1} \,,\, Y_{i,D,p,2} \,] \;=\; (\, V[S_i] + V[Z_{i,p}] \,) \,/\, V[Y_{i,d,p,t}]$$

$$\text{Corr}[\, Y_{i,D,p,t} \,,\, Y_{i,E,p',t} \,] \;=\; (\; V[S_i] \;) \,/\, V[Y_{i,d,p,t}]$$

F. WORKING MODEL PARAMETERS

Collaborative "Period 1" data from DELTA protocol 1 and previous data sets provided by the Columbia and Penn State Field Centers have been analyzed to obtain estimates of the covariance parameters of the working model. The DELTA data provided estimates of the sum (V[S_i] + V[$Z_{i,p}$]) for seven responses (TC, HDL, LDL, Trig, Fibrinogen, Factor VII, PAI-1). Columbia data (TC, HDL, Trig) provided estimates of the ratio of correlation coefficients

$$\frac{\text{Corr}[\, Y_{i,D,p,1} \,,\, Y_{i,E,p',1} \,]}{\text{Corr}[\, Y_{i,D,p,1} \,,\, Y_{i,D,p,2} \,]} \;=\; \frac{V[S_i]}{V[S_i] + V[Z_{i,p}]}.$$

This ratio is the proportion of subject-related variance accounted for by the "subject" random effect. In the data from Columbia the proportion ranged from 73% to 93%. The data from Penn State also provided evidence that this ratio varies from one analyte to another but indicated a different rank order for the analytes. This collective information was used in conjunction with the DELTA data to obtain approximate estimates of $V[S_i]$ for the seven analytes. In particular, all power computations reported here assume an approximate value of 85% for the ratio of correlation coefficients. All three data sets were used to assess evidence of constancy of variance and constancy of the two correlation coefficients. Several alternative covariance structures for the Columbia data were considered and were rejected using likelihood ratio tests. These included (1) the "compound symmetry" structure, (2) first-order autoregressive structure, and (3) power structures for spacial/temporal data.

All power analysis results reported here were based on the assumption that N=100 subjects will complete the study, 25 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.

Table 3
Estimation of Variance Components
Based on Data from Period 1 of Protocol 1

	Expressed as Variance			Expressed as Standard Deviation	
	Variance Estimate	Standard Error of Estimate	95% Confidence Interval for Variance Est.	Square-Root of Estimate	95% Confidence Interval for Std. Dev. Est.
Cholesterol					
Inter-Subject	727.23	103.54	[524.28, 930.18]	26.97	[22.90, 30.50]
Intra-Subject	106.02	7.94	[90.44, 121.60]	10.30	[9.51, 11.03]
HDL					
Inter-Subject	113.85	15.94	[82.61, 145.10]	10.67	[9.09, 12.05]
Intra-Subject	8.92	0.66	[7.61, 10.24]	2.99	[2.76, 3.20]
LDL					
Inter-Subject	663.24	94.05	[478.89, 847.59]	25.75	[21.88, 29.11]
Intra-Subject	85.41	6.40	[72.86, 97.96]	9.24	[8.54, 9.90]
Triglyceride					
Inter-Subject	1336.22	192.41	[959.10, 1713.3]	36.55	[30.97, 41.39]
Intra-Subject	267.07	20.01	[227.84, 306.30]	16.34	[15.09, 17.50]
Fibrinogen					
Inter-Subject	2353.25	343.86	[1679.29,3027.21]	48.51	[40.98, 55.02]
Intra-Subject	611.29	45.63	[521.84, 700.74]	24.72	[22.84, 26.47]
Factor VII					
Inter-Subject	178.82	25.97	[127.92, 229.73]	13.37	[11.31, 15.16]
Intra-Subject	40.61	3.05	[34.64, 46.59]	6.37	[5.89, 6.83]
PAI-1					
Inter-Subject	57.63	8.72	[40.52, 74.73]	7.59	[6.37, 8.64]
Intra-Subject	22.34	1.67	[19.06, 25.62]	4.73	[4.37, 5.06]

NOTE: These estimates were obtained by fitting components-of-variance models to the preliminary data from period 1 of protocol 1. Each such model included fixed effects for Diet, Gender-Group, Diet*Gender-Group, and Center. Under the assumptions of the working model described above, the "Intra-Subject Variances" given in this table are estimates of the sum of subject-related components ($V[S_i] + V[Z_{i,p}]$). The "Inter-Subject Variances" are estimates of the "residual variance" defined in the working model.

G. POWER ANALYSIS COMPUTATIONS AND RESULTS

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=100, (3) the sample is distributed as shown in Tables 1 and 2, (4) the endpoint is an average of three weekly blood draws ("REPS=3" in the figures), (5) variances are as shown in Table 3, (6) the variance-covariance structure for the repeated measurements has the covariance structure previously described, (7) a correlation ratio of 85% (see above discussion), (8) fixed effects for gender, race, center, age, period, diet, and gender*diet interaction, (9) no carryover effects. Computations were made using probability functions available in the IML Procedure published by SAS Institute, Cary, NC.

Figures 1-8 present a summary of the results in terms of "minimum detectable differences" when power is held fixed at 90%. This concept is illustrated in Fig. 1 (TC).

Figure 1 presents power as a nonlinear function of the overall difference between diets *D* and *E* for total cholesterol. The power curve ranges from 0.01 (the significance level or "size" of the test) up to 1.00. If the true difference between diets *D* and *E* is about 7.2mg/dl total cholesterol then the power of the intended test procedure is approximately 90%. Our point estimate of the "minimum detectable difference" is then 7.2mg/dl. We use 90% power as the criterion for "minimum 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 the variance components in the proposed multi-center study, the curve is plotted for three different values of total variance: the assumed value (108), an optimistic value (50% smaller than expected) and a pessimistic value (50% larger than expected). The upper and lower curves provide an interval estimate of "minimum detectable difference" illustrated in Fig. 1.

Figures 2-8 present a collection of such interval estimates for the primary comparisons of diets *D* and *E*. Estimates of "minimum 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).

Identical results are obtained for *D-F* and *E-F* 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.

H. RECRUITMENT FAILURES

The two design strategies indicated in Table 4 would have roughly equal statistical power for the overall comparison of two diets. The benefit of having four blood draws (relative to having 3) varies with the measure of interest. For example, tests about PAI-1 would benefit more than tests about HDL.

These equivalences indicate a criterion for resorting to more blood draws to offset recruitment failures. For example, if it appears that less than 100 subjects will complete the study, then resorting to 4 blood draws would provide compensation plus some bonus for the overall test. For HDL, the compensation would be "exact" (i.e., no bonus power) if the number of subjects had fallen to 96.

A decision to resort to 4 blood draws would have to take into account (1) the relative importance of the various outcome variables, (2) the relative importance of specific hypothesis tests, (3) design imbalances caused by recruitment failures. Based on the power computations given below, power appears to be in shortest supply for HDL. However, the benefit of four blood draws is most minimal for HDL. Therefore we anticipate that resorting to four blood draws will not be warranted.

Table 4
Roughly Equivalent Sampling Designs
(Under the Assumptions of the Working Model)

Measurement	Alternative Sampling Designs: Total Subjects	Alternative Sampling Designs: Blood Draws Per Subject	Proposed Sampling Design: Total Subjects	Proposed Sampling Design: Blood Draws Per Subject
HDL Serum Cholesterol (mg/dl)	96	4	100	3
Total Serum Cholesterol (mg/dl)	94	4	100	3
LDL Serum Cholesterol (mg/dl)	94	4	100	3
Triglyceride (mg/dl)	92	4	100	3
Factor VII (%)	92	4	100	3
Fibrinogen (mg/dl)	91	4	100	3
PAI_1 (mg/ml)	88	4	100	3

The criterion used to establish rough equivalence of designs was the equality of the simple difference between averaged values:

$$V[\ (Y_{i,D,p}-Y_{i,F,p'})\ ] \ = \ (2/N)*V[Z_{i,p}] \ + \ (2/NP)*V[e_{i,d,p,t}]$$

I. EFFECTS OF LABORATORY 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. The data entry system will facilitate detection of gross errors by providing a simple algorithm for detection of extreme assay values.

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.

J. EFFECTS OF VARIATION IN NUTRIENTS

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 within-subject and between-subject variances. Inaccuracies that are consistent across time and diet can, in theory, cancel out when within-subject diet comparisons are made. However we should not trust that such cancellation will occur. In regard to minimizing food preparation errors or ameliorating their effects, we have five objectives:

1. 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 are "within tolerances" when production begins.

2. Minimize errors via quality assurance strategies. Appropriate strategies include food-purchasing strategies, standard within-kitchen procedures for quality assurance, and assaying sample menus.

3. 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.

4. Perform ancillary research to identify and evaluate sources of variation in food preparation. Identifying these sources of error will not only be important for present and future DELTA studies, but for other well-controlled feeding studies as well. In turn, these studies will enable us to advance the field of clinical nutrition research.

5. Evaluate "Portion Control". We will estimate variances in assayed nutrient levels in this study (Protocol 2) and compare them with variances obtained from the previous cross-over trial (Protocol 1). Compared with Protocol 1, Protocol 2 will make more extensive use of "portion control" food preparation methods. We hypothesize that variances will increase only slightly with this change.

K. DESIGN FOR SAMPLING THE PREPARED MENUS

A fractional-factorial design will be used to sample the meals fed to the study participants. The factors balanced in this design are period, cycle, center, diet and calorie level. ("Cycle" refers to the 8-day menu cycle that is repeated six times during the course of each feeding period.) there are 1,080 combinations of the levels of these factors: 3 periods x 6 cycles x 4 centers x 3 diets x 5 calorie levels. The fractional design includes only 24 of these 1,080 combinations.

The work load for each field center is best quantified in terms of menu cycles within feeding periods. Each such cycle comprises 24 meals: 3 meals/day on each of eight successive days, and each period comprises 6 cycles. During each period, each field center will send a total of two 8-day menu cycles to FALCC. For example, during cycle #1 of period #1, field

center "L" will contribute the sample menus for Diet *D* at calorie level #1. During this same cycle in period #1, field center "M" will contribute the sample meals for Diet *D* at calorie level #4. Later, center "M" contributes samples again during cycle #4 or period #1. The allocation of assignments to the field centers has been randomly chosen.

Table 5
Resulting Composites to be Assayed

Composite ID Number	Feeding Period	8-Day Menu Cycle	Field Center	Diet	Calorie Level
1	1	1	L	*D*	1
2	1	1	M	*D*	4
3	1	2	P	*D*	2
4	1	3	C	*E*	2
5	1	4	L	*E*	5
6	1	4	M	*E*	3
7	1	5	P	*F*	3
8	1	6	C	*F*	1
9	2	1	C	*E*	4
10	2	2	L	*F*	4
11	2	2	M	*D*	1
12	2	3	P	*E*	1
13	2	4	C	*D*	5
14	2	5	L	*D*	3
15	2	5	M	*F*	2
16	2	6	P	*F*	5
17	3	1	P	*E*	3
18	3	2	C	*F*	3
19	3	3	L	*F*	1
20	3	3	M	*F*	4
21	3	4	P	*D*	4
22	3	5	C	*D*	2
23	3	6	L	*E*	2
24	3	6	M	*E*	5

The work load for FALCC for the monitoring of the diets includes: (1) preparation of the shipping kits for the 8 day diet samples, (2) receipt of frozen diet samples from Field Centers for a total of 192 menus, (3) preparation of 24 8-day composites, (4) assay of each of these composites for the appropriate nutrients, (5) archiving of the aliquots of each composite for possible future assay, (6) documentation of all assay QA/QC data, and (7) reporting of the assay results to the Coordinating Center.

The final statistical analysis will be based on 24 data values for each nutrient assayed. By comparison, Protocol 1 yielded 72 such data values. Thus, standard errors on estimates of mean levels in Protocol 2 will be roughly 73% larger than those obtained for Protocol 1. By design the sample of menu cycle composites should be representative of the meals actually fed to the study participants. However, because it is a sample rather than a census of the food served, some uncertainty will remain. If the sample size was any smaller this

uncertainty could become large enough to weaken the interpretability of the study. For the proposed design the half-width of confidence intervals for mean levels are as follows: Chol. $\pm$12.5mg, TF% $\pm$0.49%, MUFA% $\pm$0.19%, SFA% $\pm$0.13%, PUFA% $\pm$0.07%.

Figures 9-13 provide information about the statistical power of the design for detecting departures from the targeted nutrient levels.

L. BALANCE OF THE FRACTIONAL FACTORIAL DESIGN

The frequency tables given below indicate degree of balance attained in this fraction-factorial design. For example, the first table shows that each center contributes samples for exactly two cycles during each period.

TABLE OF CENTER BY PERIOD

	1	2	3	Total
L	2	2	2	6
C	2	2	2	6
P	2	2	2	6
M	2	2	2	6
Total	8	8	8	24

TABLE OF CENTER BY CYCLE

	1	2	3	4	5	6	Total
L	1	1	1	1	1	1	6
C	1	1	1	1	1	1	6
P	1	1	1	1	1	1	6
M	1	1	1	1	1	1	6
Total	4	4	4	4	4	4	24

TABLE OF CENTER BY DIET

	D	*E*	*F*	Total
L	2	2	2	6
C	2	2	2	6
P	2	2	2	6
M	2	2	2	6
Total	8	8	8	24

TABLE OF CENTER BY CALORIES

	1	2	3	4	5	Total
L	2	1	1	1	1	6
C	1	2	1	1	1	6
P	1	1	2	1	1	6
M	1	1	1	2	1	6
Total	5	5	5	5	4	24

TABLE OF PERIOD BY CYCLE

	1	2	3	4	5	6	Total
1	2	1	1	2	1	1	8
2	1	2	1	1	2	1	8
3	1	1	2	1	1	2	8
Total	4	4	4	4	4	4	24

TABLE OF PERIOD BY DIET

	D	*E*	*F*	Total
1	3	3	2	8
2	3	2	3	8
3	2	3	3	8
Total	8	8	8	24

TABLE OF PERIOD BY CALORIES

	1	2	3	4	5	Total
1	2	2	2	1	1	8
2	2	1	1	2	2	8
3	1	2	2	2	1	8
Total	5	5	5	5	4	24

TABLE OF DIET BY CALORIES

	1	2	3	4	5	Total
D	2	2	1	2	1	8
E	1	2	2	1	2	8
F	2	1	2	2	1	8
Total	5	5	5	5	4	24

TABLE OF CYCLE BY DIET

	D	*E*	*F*	Total
1	2	2	0	4
2	2	0	2	4
3	0	2	2	4
4	2	2	0	4
5	2	0	2	4
6	0	2	2	4
Total	8	8	8	24

TABLE OF CYCLE BY CALORIES

	1	2	3	4	5	Total
1	1	0	1	2	0	4
2	1	1	1	1	0	4
3	2	1	0	1	0	4
4	0	0	1	1	2	4
5	0	2	2	0	0	4
6	1	1	0	0	2	4
Total	5	5	5	5	4	24

M. CONTINGENCY FOR IMPROVED STATISTICAL METHODS

While we expect that established statistical methods will be adequate, it should be noted that it is 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

Study Design

Chapter 5: STUDY DESIGN

A. DESIGN

Protocol 2 will employ a randomized, double-blinded, three-period, complete crossover design to examine the effects of three diets, differing in total fat, fatty acid composition, and fiber, on selected risk factors for cardiovascular disease. The diets to be studied during this protocol are as follows: Average American Diet, "AAD" (16% SFA, 14% MUFA, 7% PUFA); NCEP Step 1 Diet, "Step 1" (8% SFA, 15% MUFA, 7% PUFA) and NCEP Step 1 Diet + MUFA, "Hi Mono" (8% SFA, 22% MUFA, 7% PUFA). An outline of the study design is presented in Figure 1. Specification of carbohydrate fractions are discussed in Chapter 6.

Potential subjects will be screened for eligibility through a combination of telephone interviews and clinic visits as described in Chapter 6. Subjects who meet the eligibility requirements will be required to participate in a diet "run-in" period to take place during the two weeks immediately prior to randomization. The diet 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. The length of the diet run-in period will be left up to the discretion of each individual Field Center but will not be less than three days nor longer than five days. The choice of diets and specific menus to be presented to the subjects during the diet run-in will likewise be left up to the discretion of the individual Field Centers. In all cases, however, the individual Field Centers will report to the Coordinating Center their specific protocol for the diet run-in period.

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 provide 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 seven weeks. The decision to decrease the length of the feeding period from eight weeks (Protocol 1) to seven weeks was based upon a number of factors as outlined: 1) Previous published data have suggested that a feeding period as short as four weeks is sufficient to achieve stability in the primary lipid endpoints. Our own analysis of preliminary data obtained from the first feeding period of Protocol 1 has confirmed that the lipid endpoints remain essentially stable during the final four weeks of the eight-week diet period. 2) For Protocol 1, little data were available regarding the length of time required to achieve stabilization for the hemostasis endpoints. Analysis

of the preliminary data from Protocol 1 provided no evidence of further diet-induced changes in the hemostasis endpoints beyond week five. 3) The choice of an eight week diet period for Protocol 1 was based, in part, on a need to gather data regarding menstrual cycle effects on lipid and hemostasis endpoints. At present, we feel that it is not necessary to again gather data across the full menstrual cycle in Protocol 2.

Subjects will be provided with breaks between diet periods 1 and 2 (from November 19 to January 5) and between diet periods 2 and 3 (from February 25 to March 30). These breaks are designed to provide subjects relief from the demands of the protocol. This is deemed particularly important during the periods immediately surrounding Christmas, New Year's Day, and Easter 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 36 weeks.

B. DIET PROTOCOL

Subjects will be provided with all foods to be consumed during each dietary period with the possible exception of one weekly self-selected "free choice meal" (if they so choose). Each center will provide a minimum 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. For Saturday dinner, we will allow the subjects to have the option of either the standard Field Center menu or a self-selected free choice meal (not provided by the Field Centers). Subjects will be provided guidelines for the selection of the free choice meal so that it will be similar in composition to the NCEP Step 1 diet. Subjects who elect to consume a free choice meal will be asked to record the foods that they consumed at this meal. The option of a free choice meal will likely enhance compliance and subject retention while minimally affecting the average composition of the diet consumed. Subjects will be advised to consume a Step 1 diet during the break period.

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). Subjects will be started on the energy level that will most closely match their estimated energy requirement. Fine tuning of the calories will be accomplished with unit foods. Body weight (without shoes, jackets or heavy sweaters) will be measured twice weekly prior to the Monday and Thursday evening meal. If a subject's weight varies beyond 1 kg of its initial value, the subject will be switched to another energy level until the weight returns to within 1 kg of the initial value.

C. SCHEDULE OF MEASUREMENTS

The schedule of measurements is shown in Figure 2. 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. Fasting blood samples will be obtained from each subject for endpoint determinations once during each of weeks 5, 6 and 7. 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.
Blood pressure will be measured in a standardized fashion by certified blood pressure technicians during weeks 5, 6 and 7.

D. STUDY CLOSE - OUT

Upon completion of the diet phase, collected samples will be forwarded to the designated Central Laboratories for assay. Laboratory assays will be carried out without knowledge of the individual subject's assigned diet sequence. All laboratory and clinic data will be forwarded to the Coordinating Center for statistical analysis. When appropriate, subjects will be notified of their individual test results. Special attention will be provided to those subjects whose baseline cholesterol values would be classified as "borderline-high" or "high" based on guidelines provided by the National Cholesterol Education Program. These subjects will be provided additional diet counseling and will be referred (along with a copy of their individual results) to their primary care physician for appropriate treatment.

FIGURE 1
STUDY DESIGN

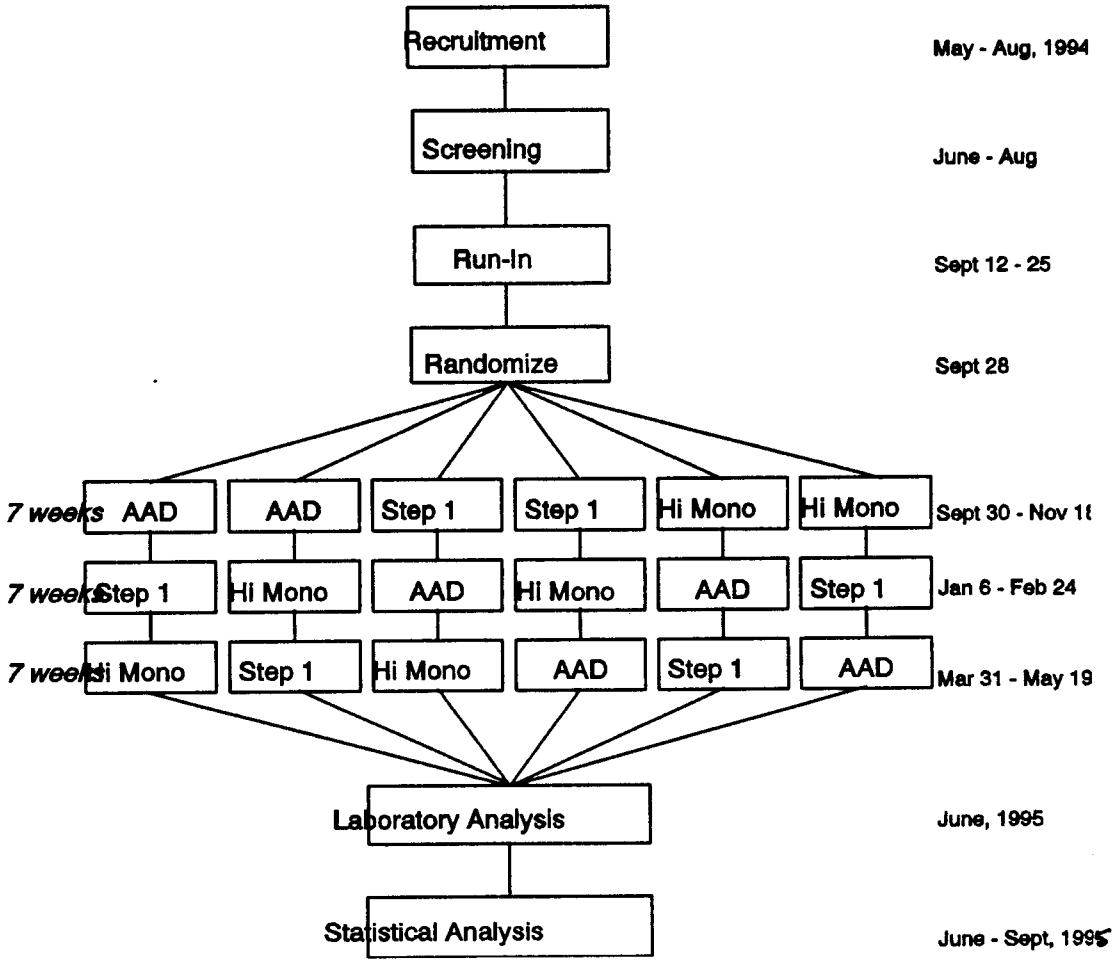


FIGURE 2

SCHEDULE OF MEASUREMENTS

WEEK	MEASUREMENTS
1	Body weight, compliance, illness, side effects
2	Body weight, compliance, illness, side effects
3	Body weight, compliance, illness, side effects
4	Body weight, compliance, illness, side effects
5	Body weight, compliance, illness, side effects **Lipid profile, apolipoproteins, hemostasis, metabolic, secondary blood pressure**
6	Body weight, compliance, illness, side effects **Lipid profile, apolipoproteins, hemostasis, metabolic, secondary blood pressure**
7	Body weight, compliance, illness, side effects **Lipid profile, apolipoproteins, hemostasis, metabolic, secondary blood pressure**

Lipid profile: plasma cholesterol, plasma triglycerides, HDL cholesterol, LDL cholesterol.

Apolipoproteins: apoA-1, apoB, Lp(a).

Hemostasis: fibrinogen, factor VII, plasminogen activator inhibitor-I, beta thromboglobulin, C-reactive protein, activated platelets.

Metabolic: glucose, insulin, uric acid.

Other: HDL subfractions, VLDL cholesterol, LDL size, postprandial lipid profile, postprandial glucose, postprandial insulin, postprandial retinyl palmitate, blood pressure.

CHAPTER 6

Population Recruitment: Screening Eligibility Criteria and Procedures

Chapter 6: POPULATION RECRUITMENT: SCREENING ELIGIBILITY CRITERIA AND PROCEDURES

The inclusion and exclusion criteria for DELTA Protocol 2 have been established in order to ensure recruitment of 1) individuals likely to have a metabolic profile characteristic of the "Insulin Resistant Syndrome"; and 2) a population that includes both a wider age range and a larger sample size of women and minorities than included in previous studies; and 3) to exclude individuals with medical conditions to insure a study population for whom it is safe to participate; 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 and completion of study requirements.

A. POPULATION INCLUSION CRITERIA

- Men and women, 21-68 years of age; the recruitment targets are 50% women and 50% men, 15% African American
- Ability and willingness to comply with all aspects and commitments of the feeding study including eating only foods provided by the study and eating 2 meals each week day at the DELTA feeding center, and with provision of informed consent.

B. LIPID AND INSULIN SELECTION CRITERIA (See Figure 1)

1) Lipid & Insulin Selection Criteria at EVI

Cholestech Fasting Finger Stick Blood

Lipid percentiles for age, gender and race will be based on NHANES III data. Insulin percentiles for age, gender and race will be based on ARIC Study and references.

Step 1 Exclusions EV1

TC < 25th% -12 mg/dl or > 90th% + 12 mg/dl

LDL > 200 mg/dl

TG < 30th% -20 mg/dl or > 600 mg/dl

HDL > 70th% + 5mg/dl

Any of these are automatic exclusions and stand alone. Individuals not excluded on the above proceed to:

Step 2a Inclusions EV1

HDL ≤ 30th% +5 mg/dl

or

TG ≥ 70th% -20 mg/dl

Step 2b May Be Eligibles for those who do not meet above Inclusions

HDL > 30th% + 5 mg/dl but ≤ 70th% + 5 mg/dl

or

TG ≥ 30th% - 20 but < 70th% - 20

These are May Be Eligibles. They proceed on to a fasting blood draw for insulin determination:

Step 3 Venous Blood

a) A fasting venous blood will be drawn for insulin determination at EV1 for all participants. However, insulins, at the time of EV1, will be measured only on May Be Eligibles. For all others blood will be stored for analysis after EV2 if needed.

b) Insulin Confirmation

Insulin ≥ 70th% - 3 μU/ml allows the May Be Eligibles from Step 2b EV1 to proceed to EV2

C) Lipid & Insulin Criteria for Final Inclusion at EV2

EV2 - Fasting Cholestech is drawn

Average of Cholestech lipids from EV1 & EV2 must meet the following:

Step 1 Exclusion Average of EV1 & EV2

TC < 25th% or > 90th% or

LDL > 190 mg/dl

TG < 30th% or > 500 mg/dl

HDL > 70th%

Step 2 Inclusion Average of EV1 & EV2

HDL ≤ 30th% or TG ≥ 70th%

Individuals who are not excluded

(HDL > 30th% ≤70th% or TG ≥30th% < 70th%) are May Be Eligibles and proceed to Step 3 for Insulin Confirmation

Step 3 Insulin for May Be Eligibles only

Average Insulin (EV1 & EV2) ≥ 70th% for age, race and gender.

C. CHEMSCREEN EXCLUSIONS CRITERIA

Participants will have a fasting blood draw at EV2 in addition to the exclusions noted in Item 2 above. Participants can be excluded with abnormal values indicating metabolic disturbances or disease on a standard Chem screen. These include abnormal: CBC, hemoglobin, TSH or creatinine (> 1.3 mg/dl), glucose >130 mg/dl.

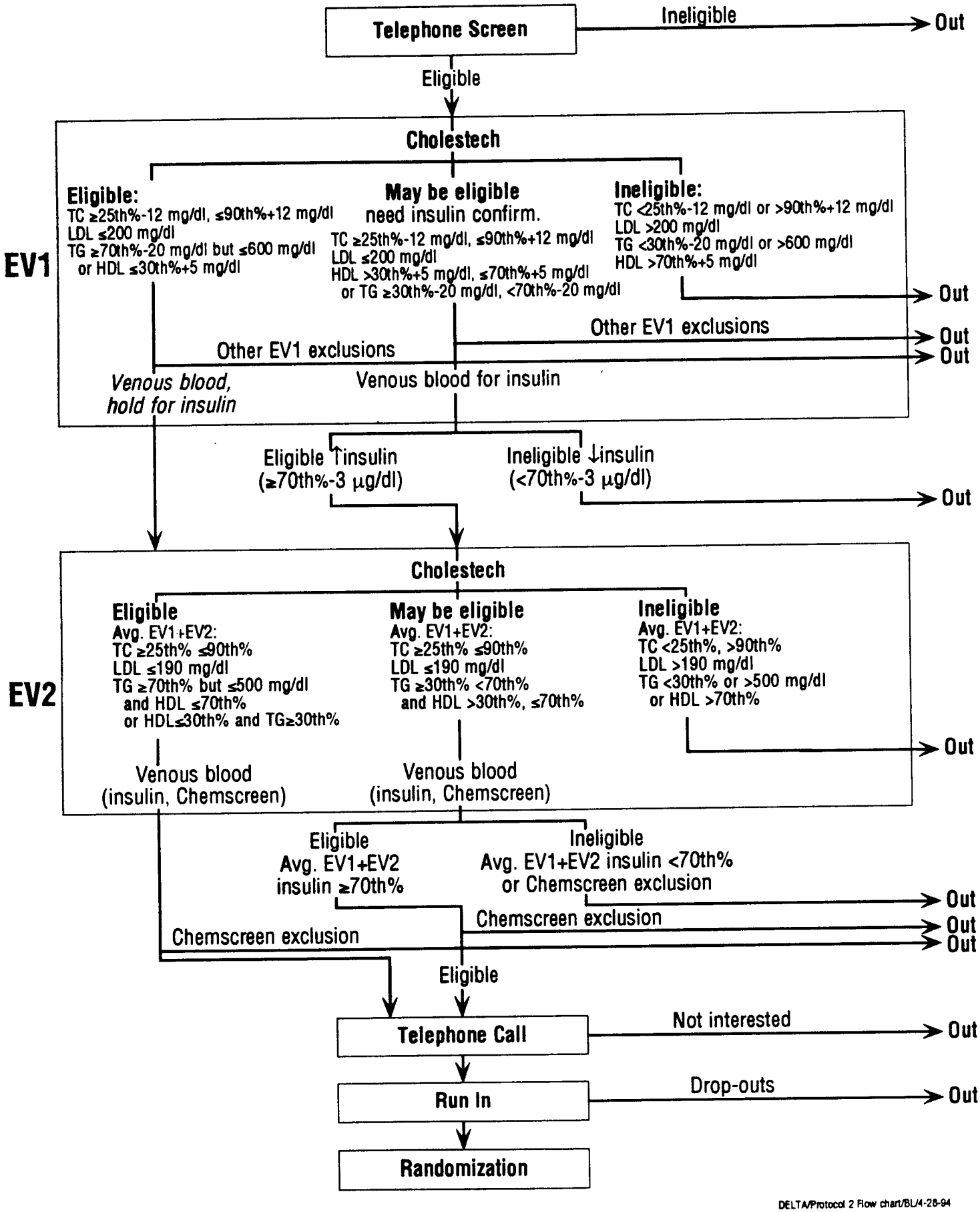
D. BLOOD PRESSURE HYPERTENSION

BP will be measured in standardized fashion at EV1 and EV2. The average of 2 BP readings at each visit will be used to determine the BP level at each visit. Individuals will be excluded in the average of their BP at EV1 and EV2 is

Systolic BP > 140 mmHg or Diastolic DBP > 90 mmHg

If the DBP ≥ 100 mmHg or SBP ≥ 160 mmHg at either Eligibility Visit 1 or Eligibility Visit 2, the applicant will be excluded. He/she will be informed of their blood pressure level and referred to their private physician or medical source.

Figure 1. DELTA Protocol 2 Eligibility Flow



E. MEDICAL OR DIETARY EXCLUSIONS

1) Medical History

- Clinical evidence of cardiovascular disease including past myocardial infarction, angina, coronary occlusion, history of coronary artery bypass and/or congestive heart failure, stroke or peripheral vascular disease
- Diabetes mellitus (use of insulin, oral hypoglycemic agents or a fasting blood sugar >130 mg/dl)
- Hypertension (use of anti-hypertensive medication or systolic pressure > 140 mmHG or diastolic pressure > 90 mmHG average on Eligibility Visits 1 and 2)
- Renal disease including history of nephritis, pyelonephritis, glomerulonephritis, and/or creatinine > 1.3
- Gastrointestinal disease including Crohn's Disease, irritable bowel syndrome, ulcerative colitis, acute ulcer, gastric resection, or any malabsorption syndrome
- Positive HIV test or Acquired Immunodeficiency Syndrome (AIDS)
- History of cancer within past 5 years
- History of blood clotting disorders or medications
- History of recent gout requiring medication (past 6 months) or history of chronic repeated gout
- History of allergies or asthma requiring use of steroid medication
- Recent history of depression or other mental illness (use of anti-depressants, tranquilizers, or involved in medical treatment which would interfere with completion of study)
- Pregnant or planning to become pregnant within next year
- Lactation current or in past 6 months
- Hematocrit >5% below gender-specific normal range
- Any illness or symptom not otherwise specified that would interfere with participation

2) Medication

- Use of lipid-lowering medication currently or within the last 6 weeks, except Probucol within the past six months
- Use of estrogen replacement medication, excluding oral contraceptives, currently or within the last 3 months.
- Use of thyroid medication
- Chronic use of laxatives or antacids

- Ongoing use of aspirin or aspirin containing medications

3) Diet

- Alcohol consumption > 12 drinks/week
- Food allergies involving foods in the study design
- Food preferences or restrictions that would interfere with protocol adherence
- 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 for the study duration

4) Body weight: BMI > 90% percentile specified by age, gender, race. Percentiles will be based on NHANES III Phase 1 data.

5) Physical activity extremes including marathon running, competition sports and/or exercise training > 7 hours each week or any training or competition activities that would interfere with completion of protocol requirements

6) Planning to leave the area prior to the end of the protocol

7) Planned out of town travel during the feeding periods

8) Daily schedule that is incompatible with feeding center hours

9) Investigator discretion for safety or compliance reasons

10) Poor compliance during Run-In

F. RATIONALE FOR INCLUSION AND EXCLUSION CRITERIA

Population Target to be Included

The DELTA study will include men and women, ages 21-68, of all socioeconomic groups and representing U.S. ethnic populations. DELTA intends to include a wide age range to obtain information on lipid and hemostatic response applicable to a broader population. Minorities will be recruited in proportion to their representation in the local populations of each center.

Medical Exclusions

DELTA will include adult men and women who are free of known clinical disease, Criteria have been established to exclude those individuals where, due to disease, there may be safety concerns related to dietary intake. Use of medications which indicate disease and laboratory parameters indicative of abnormalities or disease are used as exclusions to insure a healthy population of subjects. Prospective participants who are pregnant or are planning to become pregnant during the study duration will be excluded.

Lipid & Insulin

Lipid level ranges, as described above, have been established to recruit a population with a metabolic profile characteristic of the "insulin resistance syndrome." These values were determined based on review of the current scientific literature and review of available data on the population distribution of these parameters including NHANES III and ARIC. Individuals are excluded if their lipid or insulin levels are above the currently accepted treatment levels for hyperlipidemia (defined by NCEP - ATP II) or diabetes.

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 are also used for exclusion. Current use of prescribed medication, history of medication for a recurrent condition, and recent use of estrogen replacement medication are exclusions. Obesity above a BMI of 90%, 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 exclusions due to the known effects on lipid levels and possible effects on hemostatic factors.

Factors Which May Predict Poor Compliance or Completion of Protocol Requirements

Protection of the integrity of the study design and assurance of optimal response to the 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

have been 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 unusual dietary patterns, history of depression or other mental illness, extreme levels of physical exercise, out of town travel or a daily schedule incompatible with feeding center hours.

G. IMPLEMENTATION

Eligibility for DELTA will be determined in a sequential manner designed to exclude ineligible participants in order to minimize screening time and applicant burden (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 include 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 diet and alcohol 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. Applicants will be given directions to report to the first Eligibility Visit in a fasted state.

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 fasted lipid profile and venepuncture for insulin will be completed. 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.

Should the blood chemistry results produce an exclusion value, the person will be called to discontinue further study eligibility screening.

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 a fasted lipid profile, insulin, and 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 discontinue further study eligibility screening. 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.

Diet Run-In includes a 3 - 5 day run-in period where the participant eats meals on site and follows other protocol requirements. 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 is required. Participants who complete the run-in and remain interested must provide informed consent and sign the Participant Agreement to be randomized.

H. PROCEDURES TO BE COMPLETED AT EACH SCREENING-ELIGIBILITY VISIT INCLUDE:

Telephone Screening

Interview to obtain:

1. Sociodemographics
2. Medical History
3. Alcohol
4. Self-reported Weight
5. Special Diet
6. Study Requirements
7. Schedule EV1 for eligible candidates

EV1 Procedures

1. Cholestech (Fasting) TC, HDL, TG, LDL
2. Height, Weight, BMI Cut Points
3. Self-Administered Questionnaire
   - Medical History
   - SES
   - Food Allergies
   - Alcohol
   - Special Diet
   - Smoking Status
   - Exercise
   - Recent Weight Loss or Weight Gain
4. Clinic Interview
   - Medical Exclusion
   - Medication Use
   - Study Information
   - Participant Agreement
5. BP
6. Venepuncture for Insulin (for all who complete above and qualify to continue)
7. Schedule EV2 for eligible candidate

EV2 Procedures

1. Cholestech (Fasting) TC, LDL, HDL, TG
2. BP
3. Anthropometrics
   - Weight
   - Height
   - WHR
4. Participant Information
5. Venepuncture
   - Lipid profile (TC, LDL, HDL, TG)
   - Insulin
   - Glucose
   - TSH
   - Chem Screen (CBC, hemoglobin, creatinine, SGOT)
6. Study Information and description of study requirements regarding: Run-In, weight, packed meals, alcohol, exercise, medication, travel

Diet Run-In Procedures

1. Initial caloric requirement determined
2. 37% fat Average American Diet fed for 3-5 consecutive days
3. Compliance assessed
4. Weight monitored

I. RECRUITMENT METHODS

Columbia University

Subjects will be recruited for this protocol from the large number of employees at Columbia Presbyterian Medical Center. The overwhelming response we had with the first protocol has indicated to us that our employees will attend the DELTA Health Fair and for lipid screening.Potential subjects can also be selected from the first and second year classes at the Medical and Dental schools and the School of Nursing as has been done in the past several years for our studies.

Potential candidates will also be identified through the division of General Medicine in the Department of Medicine which provides primary care for 8000 - 10,000 patients. Sixty five percent of these patients live within 5 miles of the medical center. We also have the SCOR lipid clinic. Dr. Henry Ginsberg is the director of this clinic. Three hundred new patients are evaluated yearly. Some of these individuals may be eligible for this study.

University of Minnesota

A. Sources

Recruitment sources will include lists from previous studies, university employees, and age eligible students. Recruitment efforts will be coordinated with a University of Minnesota health clinic which serves employees in an attempt to identify individuals who are most likely to meet eligibility criteria. 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 yield participants who are likely to be retained throughout the diet periods.

B. Methods and Strategies

The University of Minnesota has successfully recruited subjects for feeding studies through informational advertising on the campus. Flyers, posters, departmental E-mail messages, employee and student letters and advertisements in the campus newspapers and newsletters will serve as a base of methods to be utilized to attract interest among the campus population. A University of Minnesota health clinic population will be surveyed to obtain lists of likely eligible individuals to whom study recruitment information will be mailed. Recruitment of the surrounding community will likely be necessary and will involve mailings to specific zip code areas, posters in community locations and advertisements and feature articles in targeted community newspapers.

A thorough description of the feeding study and the expectations and commitment necessary will be provided in the beginning phase of recruitment in an effort to minimize

otherwise eligible individuals who are not interested in the rigors of a feeding trial. This effort will reduce professional staff time and laboratory costs and will likely minimize study dropout rates.

Pennington Biomedical Research Center

A. Sources

PBRC Volunteer Data Base. Over the course of the last 3 years, the PBRC Clinic has received over 4,000 responses from individuals wishing to participate in ongoing clinical studies. In excess of 1,000 individuals have expressed specific interests in participating in dietary studies. These potential subjects will be pre-screened for eligibility and targeted for recruitment.

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.

B. Methods and Strategies

All recruiting will be coordinated through a full-time Clinical Subjects Recruiter.

Advertisements describing the study will be placed in the university and community newspaper. Publications Director Ben Phillips, a veteran journalist, 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.

Pennsylvania State University DELTA

A. Sources

Recruitment sources will include university employees and students, lists from previous studies, patients of physicians in the local and surrounding communities and the general population in and around State College. The University's Office of Health Promotion (OHP) willingly shares lists of employees who will be targeted. In addition, the Office of Minority Affairs is a source of contact to assist with the recruitment of minority (e.g.African Americans) subjects into the study. Local physicians have expressed an interest in the study; they will be contacted (via a letter followed by a telephone call) to provide assistance with recruitment efforts.

B. Methods and Strategies

Various methods have proved to be very effective in recruiting subjects for previous feeding studies at Penn State. Most notably, personalized letters sent to the University community (employees and age-eligible students) that describe the study and encourage participation in it have been highly effective. Lists and mailing addresses of employees and graduate students will be obtained from Office of Administrative Systems and the Penn State registrar. In addition, other successful recruitment approaches that have been used in previous feeding studies will be used. These include announcements to various organizations on and off campus, flyers, posters, radio announcements (public service and advertisements), newspaper advertisements and articles (campus and local newspapers), television announcements (C-Net), E-mail, electronic bulletins, and newspapers. Recruitment meetings will be scheduled for potentially interested subjects. These are held at different times to inform potential subjects about the requirements of the study. This has been an effective strategy for recruiting friends and significant others of interested individuals.

Physicians in the local community, especially those who have participated in other Penn State studies, will be informed about the study. Their participation in recruiting eligible students via word-of-mouth will be sought. In addition, flyers and posters will be placed in these physicians' offices.

Finally, lists of individuals who have participated in other nutrition studies or in programs sponsored by the OHP over the years will be collected. These persons will be contacted via mailings and informed about the DELTA Study Protocol 2.

CHAPTER 7

Participant Adherence and Retention

Chapter 7: PARTICIPANT ADHERENCE AND RETENTION

A: PARTICIPANT RETENTION

Retention of study participants begins with accurate and detailed staff training regarding the eligibility requirements and level of commitment expected from prospective participants. Ample time is taken during eligibility visits to thoroughly inform each individual of the study purpose, implementation and the very important role of the study participant. Adequate recruitment efforts are needed to produce enough prospective participants to allow for careful subject selection.

Prospective participants should demonstrate an understanding of the study. This exchange allows and promotes exchange of questions regarding basic issues and adherence specifics. Prospective participants are provided with a study menu cycle for their review followed by a discussion at a later visit regarding their willingness to consume the set menu as well as their ability to consume a controlled intake for lengthy periods of time. Food dislikes will be solicited and eating patterns evaluated in regard to the possible impact on study participation and adherence. All requirements of participation will be discussed and provided in writing for review. Each participant will be asked to sign a Participant Agreement indicating their understanding of study expectations and their willingness to comply with the stated requirements.

Following selection of eligible and interested candidates, a diet run-in period will be utilized to allow chosen individuals to experience the feeding center atmosphere, menus, and challenges. Individuals completing the run-in phase will be evaluated for understanding of the commitment involved and adherence to feeding center expectations during the run-in.

The dining facility at each DELTA center is pleasant and comfortable, accessible and furnished to encourage participant interaction. Each DELTA center will provide staff support to work closely with study participants. Participants will have access to designated staff members' telephone numbers for use at any time. Following the randomization of each participant, every effort will be made to promote cooperation and enhance commitment throughout study feeing periods. Designated study staff will be available to answer questions, reinforce protocol issues, and solve problem situations.

Study participants will benefit from the study in several ways. Participants will receive all of their food for twenty-one (21) weeks free of charge. Study menus have been calculated to provide a nutritionally balanced intake. They will gain an understanding of good nutrition practices by consuming balanced, nutritious meals. In addition, they will learn the principles of planning a heart healthy diet that they will follow during the breaks between the feeding periods. Each participant will be provided information regarding their lipid levels following study completion and data availability. They will also be invited to attend group nutrition classes to learn in detail how to plan and follow a heart healthy diet. Monetary compensation may be provided at the discretion of the individual DELTA centers.

B: PARTICIPANT ADHERENCE

Participant adherence to the diet is critical to the successful completion of the study. Participants will agree to eat all food provided by the feeding center and not eat any foods which have not been provided by the center. Two meals each day will be served at the feeding center, which will allow basic observation of participant attitudes toward the importance of compliance and will allow a tray inspection following each meal. Participants will be reminded to eat "every last bit" of the food. Individual spatulas may be provided at each meal to serve as a reminder of the need to consume all foods served and to facilitate the consumption of sauces, gravies, oils, and remnants of food(s) remaining on plates or in containers.

Body weight measurements will be taken at a minimum of twice weekly to assess the appropriateness of the calculated calorie level and to provide information regarding any gross deviation from the experimental diet. For meals and snacks eaten away from the feeding center, participants will be required to record any foods that were not completely eaten and/or any intake of additional food or beverages that were not part of the experimental diet.

Encouragement and positive feedback as well as monitoring of food intake to assess adherence will take place on an ongoing basis. Participants will be provided with written adherence guidelines for their continued reference throughout the study. Guidelines will include information on self-selected meals, non-caloric beverages and limitation of caffeinated and alcoholic drinks, restrictions on and use of medications, and instructions for fasting before scheduled study endpoint blood samples. Copies of the guidelines will be available in the feeding center area for quick reference and referral when questions or need for clarifications arise.

CHAPTER 8

Diet Specifications

Chapter 8: DIET SPECIFICATIONS

A. THE DIETS

Three experimental diets will be used to achieve the specific aims presented in Chapter 3. The nutrient specifications are as follows:

	Average American Diet	High Mono Diet	New Step One Diet
	Diet "D"	Diet "E"	Diet "F"
Total fat, % Kcal	37	37	30
SFA, % Kcal	16	8	8
MFA, % Kcal	14	22	15
PFA, % Kcal	7	7	7
Fiber g/1000 Kcal	7.5	7.5	15
CHO, % of Kcal	47	47	54
Simple CHO, % of total CHO	40	40	*
Protein, % Kcal	16	16	16
Cholesterol, mg	300	300	300

*Kept at same absolute level as "D" and "E" but with increased starch

The Average American Diet was representative of the U.S. diet during the collection of NHANES II data from 1976 to 1980. (The newly released NHANES III data indicate that total fat and saturated fat intake have decreased to 34% and 12 % of calories, respectively). Diet D is similar is Diet A used in Protocol 1. In addition to serving as a controlled reference diet, it will be possible to examine the lipid/lipoprotein and hemostatic responses to an Average American Diet in an insulin resistant population and a healthy population studied previously. Diet E is high in MFA and low in SFA. (It was designed to meet the lower range of the SFA recommendations of the Step-One Diet of the National Cholesterol Education Program Adult Treatment Panel-II Report). Because of the enrichment of this diet in MFA, the total fat content of Diet E is the same as that for Diet D. Diet F meets the nutrient specifications of the Step-One Diet. Diets E and F differ in the amount of MFA and, therefore, total fat; the contribution of SFA and PFA to the diets is the same. Diet F is higher in dietary fiber and complex carbohydrates. All experimental diets provide 300 mg of cholesterol per day to enable comparisons to be made between diets on the basis of

differences in only the amount of MFA, complex carbohydrate and dietary fiber. The design of the experimental diets will answer the question of whether diet E or F is preferable for individuals who are insulin resistant. Since total fat and MFA are lower, and dietary fiber and the amount of complex carbohydrate are both higher in Diet F, versus Diet E it will not be possible to identify the dietary factor that accounts for this. Rather, the diet design will permit comparisons between two different dietary patterns that both meet the ATP-II recommendations for SFA and cholesterol but with different amounts of total fat (e.g., a high complex carbohydrate, high fiber and lower fat diet versus a high fat and specifically, a high MFA diet).

Terms used in this protocol:

Diet:	This term is used to describe the experimental diets that will be fed to subjects. It is defined by the macronutrient composition, levels of fatty acid classes, cholesterol, dietary fiber, and proportion of complex to simple carbohydrates. Each experimental diet will meet the nutrient criteria specified but will vary in energy (to meet the different calorie needs of the subjects).
Menu:	A description of foods served at a meal. Menus refer to descriptions of a single meal or meals for one or more days.
Meal:	Food served/eaten at breakfast, lunch, dinner, or snack.
Menu Cycle:	The period of time within which a complete set of menus is served. As in DELTA Protocol 1, an 8-day menu cycle will be used. The menu cycle will be developed by modifying the menu cycle used in the Delta Protocol 1. Since the feeding periods will be 7 weeks long, there will be 6 menu cycles during each feeding period. This 8-day cycle is comprised of one 6-day weekday cycle and one 2-day weekend cycle.
Unit Food:	A food such as a muffin or a roll that is formulated to have the same ratio of control nutrients to calories. It can be eaten as desired by the subject without altering the composition of the diet. It is used as a calorie adjuster or given to subjects who wish to have a snack.
Fat Blend:	A mixture of natural fats that have a similar fatty acid profile as the experimental diets. The fat blends are used to prepare unit foods.
Portion Control:	Pre-packed individual serving size
Code:	A single meal could have a 6 digit code designating the Diet (D E F), period (1,2,3) cycle (0.1 - 6), menus (1 - 8) calorie level (15, 20, 25, 30, 35) and meal (B,L,D,S).

Figure 1 illustrates the organization of the diets.

Figure 1. Organization of the Experimental Diets

Diet

- D
- E
- F

Period (Unit of Experiment)

1.
2.
3.

Cycle of menu rotation (8 days) (Unit of compositing)

- 0.1
- 1
- 2
- 3
- 4
- 5
- 6

Calorie level

- 1500
- 2000
- 2500
- 3000
- 3500

Meal (Unit of compliance monitoring)

- Breakfast
- Lunch
- Dinner
- Snack

Menus

Menus used in DELTA Protocol 1 will be revised to meet the nutrient specifications of experimental diets D, E and F that will be fed to subjects in Protocol 2. The menus also will be revised to simplify food production efforts and to meet the food preferences of subjects in Protocol 1. All diets will be nutritionally adequate, and using as a guidance the RDA for men and women 25 to 50, and will meet the subjects' energy needs.

The guiding philosophy in the revision of the existing menus for Protocol 2 is that they meet the nutrient specifications of the defined experimental diets, that they be tasty, appetizing and culturally and regionally acceptable to all subjects at all Field Centers, and that food production efforts are simplified compared to Protocol 1. Whereas in Protocol 1, virtually all food and recipe items were gram weighed and incrementally increased or decreased for the different calorie levels, in Protocol 2 there will be maximum use of portion control items (e.g., milk, juice, bread, jelly, fat-free salad dressing, canned fruit, pudding, gelatin, and cereal). However, since portion control items are more expensive than bulk-packed items and since their presentation may be less pleasing, individual Field Centers will decide whether to use all, some or even no unit packed menu items. To simplify food production efforts, some recipes will be modified to allow batch preparation of non-fat items.

The preparation and distribution of baked products will be simplified by using a standard portion size item for each calorie level. As with Protocol 1, the weekend menus will be revised to include food items that require simple assembly (e.g., sandwiches, frozen entree menu items, cereal, among others) and not laborious food preparation methods (i.e., time consuming recipes).

Dr. Catherine Champagne from the Pennington Biomedical Research Center will develop the 2,000 and 3000 calorie menus for Protocol 2 using the ETNV Nutrient Data Base. She and her associate will conduct a training session for dietitians from each Field Center to learn techniques of menu development. Each Field Center and the Coordinating Center will receive the ETNV computer program. They will develop the 1500, 2500 and 3500 Calorie menus under the supervision of Dr. Champagne. After the draft menus have been developed using the ETNV program they will be analyzed using the University of Minnesota's NDS program by Dr. Satya Jonnalagadda. The NDS Program has carbohydrate data (sugars, starch, and fiber) which will be used to estimate the simple and complex carbohydrate content of menus for Protocol 2. Thus, eight weekday menus and 4 weekend menus will be developed and used for Protocol 2.

Following the development of the diets, all menus for 2000 calorie and 3000 calorie levels will be prepared, sent to FALCC, composited and assayed. Unacceptable menus will be discarded on the basis of the analytical data (e.g., the menus do not meet the nutrient specifications of the experimental diets), the food production efforts required, and/or the acceptability of the recipes and menus.

The menus for all diets will be similar in appearance. The fats added to the diet will be distributed throughout the day. This approach helps maintain the blinded experimental

design (no single item is extremely "oily") and to increase the palatability of the diet (i.e., a single meal does not have a disproportionate amount of fat).

Food Procurement and Preparation Procedures

To minimize nutrient variability of key nutrients of concern (i.e., fatty acids), all fats and oils that are used will be procured centrally. In addition, all beef (except top round, select grade, because it has a constant nutrient profile) for all three feeding periods will be procured centrally before the start of the first feeding period. Pork tenderloin, ham (that meet specific U.S.D.A. specifications), and sausage will be procured locally. Turkey meatballs for the weekend menus will be procured centrally. Cheese also will be procured centrally. Other (lesser) sources of fat in the diet such as fish, poultry, bread and other grain products will be procured locally according to precisely defined specifications. For example, the brand name for poultry, fish, and other products (such as bread) will be specified and all Field Centers will use the same product. Each Field Center will identify a local dairy that provides skim milk with < 0.5% fat. Whole milk will provide 3.3% fat (not 3.7% fat). Fruits and vegetables will be procured locally and centrally (e.g., frozen, canned, and sometimes fresh fruits and vegetables). Food will be purchased, prepared, and stored according to standard procedures.

Standard procedures for portioning food items will be implemented. These include weighing of all fat containing items and freeze-thaw weigh specifications for meat/fish/poultry.

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 food 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 meats, 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 with Fresh Check Time-Temperature Indicators (TTI's). Subjects will be taught how to read the TTI label. Perishable foods will be chilled 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, 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 diarrhea illness or open wound.

Handouts that describe food safety procedures will be given to all employees who handle food. 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 at the beginning of each feeding period. 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. This will be checked at the beginning of each feeding period. 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.

B. DIET ANALYSIS

The primary focus of the diet analysis component of **DELTA** is to standardize the diets across Field Centers so that the diets fed at each Field Center are sufficiently comparable to be considered identical treatments. Furthermore, since the feeding trial is a cross-over design with each subjects as his/her own control, it is necessary to ensure that the diets maintain their composition over the nine months of the total study. These aspects of diet control are critical components of the entire study. The first DELTA Protocol is the only known previous multicenter study that attempted to demonstrate that the diets fed at different Field Centers were identical, that the actual nutrient contents of each diet were as calculated, and that the actual nutrient contents of each diet were monitored over the total study. Thus the continued development of the protocols, assays, and quality control programs to accomplish these goals are, in themselves, needed research projects and this study provides unusual scientific opportunities in the area of diet composition and analyses. The research component has been divided into three areas: (1) research on methods to improve the quality and efficiency of diet assays; (2) validation of the diet menus across centers and (3) monitoring of the diets across time and across centers during the feeding trial.

RESEARCH IN METHODS

The following components will be assayed in diets for Diet Protocol 2: total fat, cholesterol, fatty acids (saturated, monounsaturated, polyunsaturated[1]), moisture, total nitrogen, ash, starch, total dietary fiber, and sugars (glucose, fructose, total disaccharides). Calories will be calculated from proximates (total fat, protein (nitrogen), ash, moisture). The protocols for the assay of total fat, cholesterol, fatty acids (saturated, monounsaturated, polyunsaturated) moisture, total protein, and ash were tested and validated as a part of DELTA Protocol 1. The accuracy and precision of these assays were found to be acceptable for the purposes of this type of study. Some of these existing methodologies have a high assay cost. Furthermore, the assay methods for starch, total dietary fiber, and sugars (glucose, fructose, total disaccharides) need to be evaluated for accuracy, precision, and analytical quality control procedures for use with composited whole diets. As part of Protocol 2, procedures will be developed, tested, and validated for the assay of sugars, starch, and total dietary fiber in composited diets. The long term gain of this research will be to provide validated protocols, assays, and quality control programs for diet validation and monitoring in multicenter diet intervention studies and to provide methodology for providing more accurate data on the carbohydrate composition of diets than is presently available.

1. **SAMPLING, STORAGE, and SHIPPING PROTOCOLS**: Protocols for sampling of the diets prepared at each Field Center, protocols for shipments of diet samples from Field Centers to the FALCC, storage of diet samples, development of reference materials and of standards at the FALCC, development of internal FALCC protocols for analytical databases, computation of results, and transmission of results to Coordinating Center were developed as part of Protocol 1.

2. **ASSAY METHODOLOGY**: The goal of this research is to develop and/or validate and/or improve methods for assays for the nutrients needed for DELTA Protocol 2. Testing, modification, and validation of analytical methods for the assay of total fat, fatty acids (saturated, monounsaturated, polyunsaturated) cholesterol, total protein, moisture, and ash were accomplished as part of Protocol 1. The methodology research for the second year will consist of development and/or validation of assays for starch, total dietary fiber, and sugars (glucose, fructose, total disaccharides), and evaluation and improvement of the efficiency (increasing sample throughput, decreasing cost) and improvement of the quality of current procedures developed in Protocol I.

   The methods included at the end of this chapter will be used for the assay of total fat, fatty acids, cholesterol, total protein, moisture, and ash. The most likely methods for the assay of starch, total dietary fiber, and sugars are listed as well. The criteria for acceptable methodology for each analyte are shown in Table 1.

Table 1: Acceptable Analytical Methodology Protocol 2

Component	Limit of Detection gm/100 g wet	Working Range gm/100 g wet	Precision (Current)
Total Fat	0.14	2.5 - 5.5	2.5%
SFA	0.058	0.5 - 2.5	5%
MUFA	0.164	1.0 - 2.0	5%
PUFA	0.088	0.5 - 1.0	5%
Cholesterol	0.0015	0.01 - 0.02	5%
Sugars	0.04	4.0 - 6.5	5%
Starch	0.06	6.0 - 10.0	5%
Total Protein (Nitrogen)	0.06	3.0 - 6.0	5%
Total Dietary Fiber	**	**	**

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 across centers and across the time of the study; and, (3) providing an ongoing quality assurance program to maintain the integrity of the dietary data.

Table 2: Cost Estimates for Assays Developed in Protocol 1

Component	Cost per sample
Total fat (grav.), SFA, MUFA,PUFA,cholesterol	$283.00
Compositing (menu)	$ 86.00
Compositing (diet cycle)	$218.00
Moisture	$ 22.00
Ash	$ 15.00
Total Protein (Nitrogen)	$ 27.00
Shipping (per 8 menus)	$172.00

RESEARCH IN DIETARY VALIDATION AND CONTROL

DELTA Protocol 1 tested the ability of a multicenter study to maintain the composition of diets across centers and across time when the amounts of the foods in different calorie levels of the diets were controlled by **proportional weighing**. In **DELTA** Protocol 2, its is planned to test the ability of a multicenter study to maintain the composition of diets across centers and across time using **portion controlled** foods across calorie levels of diets. This component of Protocol 1 will be continued as part of Protocol 2, and 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 variation over the time of the study, inter-center variation, assay variation, and calorie level.
4. How valid are current food composition databases in estimating food components of interest in this and future diet intervention studies?

Given that it is important to document that the different diets maintain their identity both between Field Centers and across the time span of the study, the data needed to answer questions 1, 2, and 3 will be collected both across all four Field Centers and across the total time duration of the diet intervention.

These research objectives will be met in both menu validation studies and on going monitoring of the diets during the course of the study. Of particular interest will be the sugar, fiber, and starch data, which are generally lacking in nutrient databases.

MENU VALIDATION

This phase of the research will be carried out in the context of validating the content of key nutrients in the experimental diets that will be used in Protocol 2. A total of twelve menus will be developed: 8 weekday menus (1-8) and 4 weekend menus (W1-W4) will be generated for each of five kcal levels (1500, 2000, 2500, 3000, and 3500 Kcal/day). A diet cycle will comprise six weekday (two weekday menus will not be used) and two weekend menus. These menus will be the same for each diet (except for added fat) to maintain

blinding. The same set of six menus will be rotated throughout the entire study and weekend menu combinations W1/W3 and W2/W4 will be alternated.

The composition of the 2000 and 3000 kcal menus will be validated for the key nutrients (total fat, saturated fat, monounsaturated fat, and polyunsaturated fat as %kcal). The assignments for the preparation of the validation menus are given in Table 3 The 2000 kcal menus will be validated first. Each center will prepare either two of the 10 menu series (1-8 and W1-W4) or one of the 10 menu series and the unit foods for all three diets. Thus there will be two samples of each menu for each menu-kcal combination (prepared at different centers) for each set of menus and unit foods. In addition, each center will prepare duplicates of the weekend menus W1-W4. Menus 1-10 for each diet will be composited and assayed individually, and weekend menu combinations W1/W3 and W2/W4 will be composited into 2-menu composites and assayed. The duplicate weekend menus (uncomposited) will be reserved for individual assay in the case that the 2-menu composites were of unacceptable composition.

Menus will be prepared at the Field Centers using the standard protocols, recipes, and centrally procured foods and specified food products procured locally that will be used during the actual feeding trials. The menus will be frozen and shipped frozen to FALCC using the materials and protocol supplied by the FALCC. At the FALCC, the samples will be composited, aliquotted, and assayed. Initially total fat, moisture, and ash will be assayed; and total fat as percent of total calories will be calculated. If the samples are within acceptable range of target values, then the composites will be assayed for fatty acids, cholesterol, and nitrogen. While the total dietary fiber, sugar and starch content will generally vary inversely with the total fat content, the exact carbohydrate composition will not be controlled or validated, due to a lack of data on these components for use in menu development. Rather, the levels of total dietary fiber, sugars and starch in the menus will be determined at a later date to document the composition of the menus/diets. A minimum of 5 aliquots of each menu composite will archived at -60°C for future studies as determined by the DELTA Steering Committee. A flow chart showing the sequence of activities and a time line is shown in Figure 2.

If discrepancies in the nutrient levels occur for a given menu, then that menu will either be discarded or reformulated and assayed. Some iterations of the reformulation process may be necessary until a total of at least 6 weekday and 4 weekend menus have been validated for each diet.

This menu validation process, in addition to standardizing the experimental diets, will provide important information on the validity of 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.

Table 3: Field Center Assignments for Preparation of Menus for Validation

PBRC	**DIET "D" AND UNIT FOODS FOR D, E, F (NO UNIT FOODS FOR THE 3000 KCAL VALIDATION)**
PSU	**DIETS "D" AND "E"**
COL	**DIETS "E" AND "F"**
MN	**DIET "F" AND UNIT FOODS FOR D, E, F (NO UNIT FOODS FOR THE 3000 KCAL VALIDATION)**

DELTA PROTOCOL 2 VALIDATION OF DIETS

TIMELINE	
02/25-03/08	CALCULATE ↓ Prepare diets 2000 and 3000 kcal

PBRC	PSU	COL	MN	
↓	↓	↓	↓	1. Save duplicate weekend menus W1,W2,W3,W4
"D" (unit foods for D,E,F)	"E" & "D"	"F" & "E"	"F" (unit foods for D,E,F)	2. No Unit Foods for 3000 kcal menu

03/31/94 2000 kcal weekday rec'd at FALCC	↓ Send to FALCC ↓ ↓
04/06/94 2000 kcal weekend rec'd at FALCC	COMPOSITE 2000 kcal and 3000 kcal menus separate weekday and composite W1 and W3 - W2 and W4 ↓ ↓
06/10/94 3000 kcal wkday & wkend rec'd at FALCC	ALIQUOT → → ARCHIVE ↓ ↓ ↓
Discard Menu←	← ← ← FAIL ← ← ← ASSAY 2000 kcal: Proximates, Fatty Acids, Cholesterol ↓
	PASS
Discard Menu←	← ← ← FAIL ← ← ← Assay Weekend menus separately ↓ ↓
	ASSAY FA and CHO Fractions: 2000 kcal ↓
06/10/94	**Results of validation 2000 kcal menus**
	ASSAY 3000 kcal: Proximates, Fatty Acids, Cholesterol ↓
	Repeat process as for 2000 kcal ↓
	Assay 2000 & 3000 kcal for CHO Fractions ↓
07/08/94	Prepare & Ship Unit Foods (PBRC and MN) ↓
_____	ASSAY Unit Foods for Proximates ↓
_____	ASSAY Unit Fat blends ↓
_____	VALIDATE Unit Foods ↓
08/31/94	**Results of Validation 3000 kcal menus**

CHARACTERIZATION OF THE STUDY DIETS

In order to obtain information on the diet composition variability by center, by calorie level and by duration of the study and to document the composition of the diets over the entire feeding trial, ongoing sampling and assay of the experimental diets will be conducted during the course of the study. The collected daily menus will be composited into 8 day menu cycles. This 8 day cycle constitutes the unit of the diet, which will be assayed. The exact sampling plan for the monitoring of the diets is given in Chapter 4.

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 the daily menus for each experimental diet from each Field Center into a **diet cycle composite**.

A minimum of five aliquots of each 8 day composite prepared throughout the study will be archived at -60°C for future studies and their dispensation will require a consensus of the DELTA Steering Committee. These archive samples will be a valuable resource, not only for this study, but for future studies as well. The archiving of diet composites samples for 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 databases for nutrients not studied in the first **DELTA** feeding study, and (5.) obtain data for designing other dietary intervention studies.

Assay program:

1. Diet quality assurance: The FALCC will determine the total weight, dry weight and total fat as % kcal of the **diet cycle composites** on an on-going basis for use in the monitoring of the experimental diets.

2. Diet documentation: For documentation of the diet composition the FALCC will also assay **diet cycle composites** for total weight, moisture, ash, total fat, total protein (nitrogen), cholesterol, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), total dietary fiber, starch, and sugars (glucose, fructose, total disaccharides). Calories will be calculated using the data for total fat, moisture content. Total fat, SFA, MUFA, PUFA, total dietary fiber, starch, and sugars will be reported on a percent dry weight basis and as calculated percent of total kcal.

Assays for DELTA Protocol 2 Nutrients

As part of DELTA Protocol 1, Standard Operating Procedures (SOPs) for all validated procedures related to the collection, shipping, and assay of samples (except starch, total dietary fiber, and sugars assays) were developed, and will be implemented in Protocol 2 as indicated. All SOPs are on file at the FALCC and are backed up on computer disks.

Compositing

Foods will be composited according to the standard procedures developed in Protocol I (FALCC SOPs #5005, #5029), and held at -60°C until assayed.

Total fat

Total lipid will be determined gravimetrically after extraction of the diet composites with chloroform/methanol (modified AOAC **983.23**, 1990; FALCC SOP #5024). Duplicate aliquots of each composite will be assayed.

Cholesterol

The gas liquid chromatography method of Thompson and Merola (*ref.: Thompson, RH, Merola, GV (1993). J. Assoc. Off. Anal. Chem. Intl., 76: 1057-68)*, with modifications, will be used to quantify cholesterol (FALCC SOP #5026). The total lipid extract (see "Total fat"above) will be assayed. Duplicate aliquots of each composite will be assayed.

Moisture

Moisture in the diet composites will be determined with a microwave moisture/solids analyzer (CEM Corp.) (FALCC SOP #5007). Moisture will be measured in triplicate aliquots of each composite and the mean value will be used to calculate total dry weight and other assay results on a dry weight basis.

Fatty acids (saturated, monounsaturated, polyunsaturated)

Fatty acid composition will be determined by gas-liquid chromatography of fatty acid methyl esters prepared from the saponified total lipid extracts (see "Total fat", above) of diet composites (*ref.: AOCS Official Method Ce 1b-89; 1991, modified*; FALCC SOP #5025). The AOCS method was modified to separate and quantify C:10 and higher fatty acid methyl esters (FAMEs). FAMEs will be reported as triacylglyerol equivalents (TAGs) according to

the classification scheme shown on page 74. Duplicate aliquots of each composite will be assayed.

Ash

Ash will be determined by heating dried composite samples at 550°C in a muffle furnace until completely ashed (FALCC SOP #5011). Duplicate aliquots of each composite will be assayed.

Protein

Protein will be determined as Kjeldahl nitrogen x 6.25 (FALCC SOP #5023). FALCC will subcontract the Kjeldahl assay to the Dept. of Human Nutrition and Foods at Virginia Tech, which has a semi-automated system. FALCC control samples (blinded) will be included in each assay batch. Duplicate aliquots of each composite will be assayed.

Starch

The total starch content of the diets will be determined enzymatically. Duplicate aliquots of diet composites will be digested with amyloglucosidase, then the supernates will be assayed for glucose using a glucose oxidase method. Glucose values will be corrected for free glucose assayed in corresponding undigested samples, and converted to starch equivalent values. The method will be validated by analyzing reference diet composite sample(s) that have been characterized independently by at least one other qualified and experienced laboratory (e.g. U.S.D.A. Nutrient Composition Laboratory, Beltsville MD), and obtaining results which agree within 5%. Since there is little reliable data on the starch content of mixed diets, acceptable precision will be determined based on attainable assay precision and the Study requirements.

Sugars

The mono- and disaccharide contents of the diets will be determined by HPLC. Duplicate aliquots of diet composites will be pre-extracted with hexane, then sugars will be extracted with aqueous methanol. The methanolic extracts will be de-ionized and filtered, brought to an aqueous solution, then assayed by high-performance liquid chromatography. Glucose, fructose, and total disaccharides will be resolved and quantified. An internal standard will be used for quantitation. The method will be validated by using the methods of standard additions to sample diets and by analyzing reference diet composite sample(s) that have been characterized independently by at least one other qualified and experienced laboratory (e.g. U.S.D.A. Nutrient Composition Laboratory, Beltsville MD), and obtaining results which agree within 5% of those results. Since there is little reliable data on the sugar content of mixed diets; acceptable precision will be determined based on attainable assay precision and the Study requirements.

Total Dietary Fiber

The AOAC total dietary fiber method is the most probable choice of methodology for total dietary fiber. The method will be validated using standard additions of the analytes, and by obtaining results for standard diet samples which agree within 5 - 8% with data from a qualified independent laboratory. SOPs for the assay will be developed and documented.

Classification of Fatty Acids

SFA	MUFA	PUFA	omega 3-FA	other
10:0	14:1	18:2*	18:3n-3 (α-linolenic)	Other FA
11:0	16:1	20:2	20:5n-3 (EPA)	Unidentified peaks
12:0*	17:1	20:3	22:6n-3 (DHA)	
13:0	18:1*	20:4		
14:0*	19:1	22:6		
15:0	20:1			
16:0*	22:1			
17:0				
18:0*				
20:0				
22:0				
24:0				

Individual concentrations of these fatty acids will also be reported (as percent of total TAGs). The values will be included in total SFA, PUFA, MUFA as well.

CHAPTER 9

Clinical Laboratory Measures

Chapter 9: CLINICAL LABORATORY MEASURES

I. Endpoint Tests Selected

The tests chosen to monitor the effects of diet on the subject population in DELTA Diet Protocol 2 are summarized in Table 1, ordered by priority. The justification for inclusion of these endpoint tests is given below.

A. Blood and urine analytes

1. Fasting lipoprotein profile with glucose

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 the inclusion of triglycerides in the formula, all samples must be collected after a 10-hour fast.

Because the population is being recruited for insulin resistance and high triglycerides, this group will include a high percentage of people with abnormal lipoprotein turnover and abnormal lipoprotein composition. For this reason, a measured VLDL cholesterol is recommended as well since the triglyceride/5 estimate of VLDL cholesterol may be inaccurate and highly variable in these subjects.

Glucose has been added to the lipid profile because of the population under study, an insulin resistant population. Fasting glucose will be an important indicator of the degree to which long term dietary changes might affect glucose metabolism in the setting of insulin resistance.

2. Insulin

Prospective studies have shown that high insulin levels are associated with an increased risk for cardiovascular disease and have been interpreted to mean that insulin resistance is associated with increased risk for atherosclerosis. Fasting insulin and postprandial insulin will be measured as indicators of the effect of diet on these markers of insulin resistance.

3. Factor VII.

Factor VII has been related to cardiovascular disease cross-sectionally in a large number of studies, although not in 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.

4. 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 positively 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 all 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.

5. 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.

6. 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,

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.

7. LDL size

Several studies suggest that the presence of small, dense LDL may increase risk for coronary heart disease. Further studies have shown that an heritable phenotype (phenotype B) characterized by the predominance of small LDL (less than 25.5 nm on nondenaturing gradient gels) is associated with increase risk for myocardial infarction. The presence of phenotype B is also associated with other known risk factors including increased levels of plasma triglycerides and decreased levels of HDL cholesterol, especially HDL_2 and insulin resistance. Expression of LDL phenotype B has a strong heritable component but environmental factors also pay a major role. Penetrance of LDL phenotype B is dependent upon age, gender, and menopausal status. Additionally, recent studies have shown that low-fat, high carbohydrate diets may also induce a phenotype B pattern in individuals who normally express a phenotype A pattern while consuming an Average American Diet. Whether this "diet inducible" phenotype B pattern confers the same risk for CHD as does the "genetic" phenotype B pattern remains to be established.

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8. Beta-thromboglobulin

There are several indications that platelet function may be an important predictor of thrombotic disease. For example, in the Caerphilly and Speedwell studies, platelet aggregation was a powerful predictor of prevalent disease. Also, in clinical trials a platelet inhibitor, aspirin, has proven effective in (1) reducing excess thrombotic risk in atrial fibrillation patients, (2) preventing re-occlusive thrombotic events following myocardial infarction, and (3) preventing progression of unstable angina. In the ARIC study (longitudinal study of healthy middle-aged adults), beta-thromboglobulin, as a marker of platelet activity, has been shown to be significantly associated with prevalent CVD. Also, there is ample evidence of aberrant platelet function in diabetes, which is greatest in those diabetics with peripheral vasculopthy and neuropathy; one may hypothesize that his is related to lipid abnormalities. While there is little information on the relationship of diet to platelet function measurements, evidence exists supporting a role of margarine oils on platelet function, and dietary fats of several types have been shown to be related to markers of platelet activity. Moreover, since platelet activation is linked to the activation of the coagulation system (i.e., thrombin is the most powerful known platelet agonist), and diet has been linked to coagulation (factor VIIa levels), there is some reason to believe that diet may be a significant modulator of platelet function.

9. VLDL-cholesterol

It is well documented that the calculated value of VLDL cholesterol varies considerably from the measured (ultracentrifugation) value, even in subjects with triglycerides in the normal range. This bias is more significant as triglyceride increases. Although estimated VLDL values are generally acceptable for calculation of LDL to classify an individual's risk, they are not likely to be adequate to detect changes in VLDL with diet, especially in a population recruited for high serum triglyceride levels.

10. HDL-subfractions

Much of the controversy around the use of low fat, high carbohydrate diet focuses on the effects of increased dietary carbohydrate on plasma triglyceride and HDL cholesterol concentrations. Plasma triglyceride levels often increase, and HDL cholesterol concentrations concomitantly decrease during periods of increase carbohydrate intake. Although our diets, with increased fiber, may not produce significant alterations in triglyceride and HDL levels, we must plan to study these potential changes in detail. HDL subfraction has been studied extensively, and it has been shown that different perturbations can affect the various subfractions differently. There is controversy concerning the meaning of changes in specific subfractions vis a vis risk for atherosclerotic cardiovascular disease, but this makes documentation of subfraction responses to our diets more important.

11. Apolipoprotein A-1 and B-100.

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.

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.

12. 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. Lipoprotein (a) has been related to the level of diabetic control in patients with frank diabetes, but its relationship to fasting insulin levels and the insulin resistance syndrome is less clear.

13. C-reactive protein

Many of the recently identified biochemical risk factors are also markers of inflammation: fibrinogen, decreased albumin, transferrin, PAI-1 and others. It may be that these blood components are markers for CVD because athero/thrombosis is inflammatory, or because they contribute to the pathophysiology, or both. An independent measure of inflammation such as c-rp may play an important role in helping to differentiate between these possibilities. In fact, a recent study by the Dutch (Haverkate, et al.) has shown the c-rp is indeed an independent, prospective risk factor for CVD. The effect of diet on inflammation is general and c-rp in particular is unknown, but recent results (RP Tracy et al. unpublished data) indicate that in multivariate analysis of 400 normal adults, obesity is a significant predictor of c-rp levels, as is HDL cholesterol levels (negative). This suggests that there may indeed be an effect of diet on c-rp.

14. Uric acid

Hyperinsulinemia syndrome includes a constellation of abnormalities, each of which is suggested to carry some risk of coronary disease and their occurrence together is suggested to aggravate the risk of coronary artery disease (See Taskinen, Current Opinion in Lipidology 1993, 4:434-443). Therefore strategies to assess risk, or to assess change of risk, need to include measurement of all abnormalities.

Hyperuricemia is part of the insulin resistance syndrome, but the extent to which serum uric acid levels can be modified by diet is unknown. Specifically, it is unclear if changes in serum uric acid levels may be related to changes in other parameters such as serum insulin levels, or are independently related to diet.

15. Microalbuminuria

An increasing body of evidence is accumulating to show that microalbuminemia is related to insulin resistance. Some authors have suggested that measurement of microalbuminemia might be used as a marker for hyperinsulinemia in hypertensives. Microalbuminemia is associated with the development of coronary disease, possible reflecting endothelial dysfunction.

The subject population for DELTA diet protocol 2 will include a high proportion of individuals with elevated insulin, hypertension, and elevated serum triglycerides. Measurement of the effect of dietary interventions on these markers of hyperinsulinemia would be complemented by measurement of urinary albumin as yet another marker of hyperinsulinemia.

B. Platelet functions

To be added. See section IIP for Methodology.

C. Post prandial studies

Two postprandial studies are proposed for the subjects. The first is feeding a standard high fat meal followed by serial blood samples to assess the rise and fall of lipids and insulin in response to a high fat load. The fat load will include retinyl palmitate to monitor chylomicron turnover. The second uses "day-long" post prandial sampling collecting blood samples from fasting subjects in the morning and just before lunch (12-1 pm) and just before supper (4-5 pm). These samples would be analyzed for insulin, glucose and triglycerides, to provide an examination of lipoprotein levels over an entire day's duration.

1. Standard Fat Load Test: The protocol will be carried out as follows: Subjects will fast at least 12 hours prior to coming to the laboratory. A fasting blood sample will be obtained using the standard protocol for fasting blood tests. Subjects will then ingest the liquid formula fat load over a period of 10-15 minutes. Post fat load samples would be taken at 4 and 8 hours. Participants would not eat any food and could drink only water or diet, non-caloric, non-caffeine drinks during the 8 hours of the test. As described below, the liquid formula would also contain carbohydrate and this may allow for meaningful measures of glucose and insulin.

Rationale: Dietary fat and cholesterol are absorbed in the small intestine and incorporated into chylomicrons. After entry into the circulation, chylomicrons interact with lipoprotein lipase on the luminal surface of endothelial cells and the chylomicron triglyceride is hydrolyzed to fatty acids and glycerol. The product of the interaction between chylomicrons and lipoprotein lipase is the chylomicron remnant. These relatively cholesterol ester-enriched particles appear to be removed by one our more receptors in the liver. The process by which dietary triglyceride and cholesterol are transported and cleared from the circulation can be affected by chronic dietary patterns, particularly dietary fat and cholesterol intake. In our proposal we will use a standard fat-load to determine the effects of difference levels of dietary fat and carbohydrate on chylomicron and chylomicron remnant metabolism. The results of these studies will help us determine the optimal diet therapy for individuals with higher triglyceride and/or lower HDL cholesterol and/or higher plasma insulin levels.

Fat formula: The formula used as the fat-test is prepared 24 hours in advance, and contains heavy whipping cream, ice cream, safflower oil, and a powdered protein source (Promod). The nutrient composition of the formula is 53 g fat/meter^2 and 300 mg cholesterol. The fat is approximately 50% saturated fat. Lactaid™ is also added to each test formula preparation as a precaution against lactose-intolerance in any of the participants. On the day of the test the appropriate volume of formula, based on the patient's surface area (calculated from height and weight) is measured and served.

2. Day long postprandial sampling: The protocol is based on having the subjects come to the center for all three meals on a single day, with bloods obtained before breakfast (8-9 am), before lunch (12-1 pm) and before dinner (4-5 pm). As always, the fasting sample follows a 12 hour fast. Breakfast is completed within 30 minutes of the fasting sample and the pre-lunch sample is obtained 3.5 hours after the end of breakfast. Lunch is completed within 30 min of the pre-lunch sample and the pre-dinner sample is obtained 4.5 hours after the end of lunch.

Rationale: In addition to determining the chronic effects of dietary constituents to the metabolism of a standard fat load, it is useful to demonstrate the immediate effects of the diet on blood levels of certain variables. In this proposal we will measure plasma lipid, glucose and insulin levels before each meal at the end of each diet period. These studies will allow us to observe the actual day-long changes in these variables while participants eat diets differing in carbohydrate and fat content.

II. Methods of Analysis

A. Local Field Center Testing

Lipid Profiles, Glucose, Uric Acid

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 SCOR at Columbia. Dr. Ginsberg is the Director of this laboratory, which is a participant in the Lipid 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 after 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). Glucose and uric acid will be determined by enzymatic methods.

2. Louisiana State University/Pennington

All routine lipid analyses (total cholesterol, triglycerides), glucose and uric acid, will be performed on the Beckman Synchron CX5 automated chemistry analyzer. HDL cholesterol is performed in the Beckman CX5 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 base's 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 cholesterol, 5% for triglycerides, 3% for HDL cholesterol). If it runs out of control, the entire run is repeated.

Interassay precision for cholesterol is 1.2% at 200 mg/dL and 0.9% at 240 mg/dL. For HDL, standard deviation is less than 1 mg/dL between 20 and 50 mg/dL and less than 1.5 mg/dL above 50 mg/dL. For triglycerides, interassay precision is 1% at 100 mg/dL and 1.8% at 200 mg/dL.

Glucose and uric acid with be determined by enzymatic methods on the Roche MIRA using Roche reagents and calibrators.

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-Kendall method is used as calibrator. Accuracy of cholesterol measurements 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 concentrations. 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.

Glucose and uric acid with be determined by enzymatic methods on the Roche FARA using Roche reagents and calibrators.

B. Insulin (MIBH)

Insulin levels for endpoints will be measured centrally, while insulin levels for screening will be done locally. The reason for central analysis of insulin is the need to measure variance and to combine data for the four centers.

Insulin will be determined by RIA using a commercially available kit (Diagnostics Products Corporation Coat-A-Count). RIA is a standard technique for determinations of insulin and although there is some evidence of cross reactivity with intact proinsulin and split proinsulin (Nagi DK et al. Diabetologia. 1990: 33, 532-7) that may lead to over estimation of circulating insulin, these products validly represent overproduction and/or over secretion of insulin. Therefore, RIA methods for quantitation will give a valid measure of insulin production and activity. Serum based calibrators are supplied by the manufacturer. All calibrators, controls and samples are run in duplicate. Analysis is carried out on a Packard Cobra system using RIAsmart software.

C. Factor VII Activity (University of Vermont)

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 VII activation.

D. 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.

E. 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 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 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 supernatant is discarded, and the resultant pellet is stored frozen. Reagents include Proteinase K (Sigma 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-MspI digest (New England Biolabs, Beverly, MA) to use as a standard marker on electrophoretic gels used to separate DNA fragments. We have prepared our own sense and antisense primers using the PCR-mate DNA synthesizer (Biorad).

G. LDL Subfractions (Louisiana State University - Pennington)

LDL 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 and Blanche et al. with the exception that in-house 2-30% concave acrylamide gels will be used. 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. 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 paradigm developed by Williams et al. 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. and based upon the weighted distribution of LDL among seven size classes as described by Krauss and Burke.

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.

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H. Beta-thromboglobulin (University of Vermont) (Stago/American Bioproducts)

B-Thromboglobulin is a specific protein of platelets that is released under the influence of stimuli that activate platelets such as ADP, collagen, immune complex, and thrombin.

In this assay (Stago/American Bioproducts, Piscataway, NJ), a plastic support coated with specific rabbit and anti-human B-TG antibodies with peroxidase binds to the remaining free antigenic determinants of the B-TG forming the sandwich. The bound enzyme peroxidase is then revealed by its activity in a predetermined time on the substrate ortho-phenylenediamine in the presence of hydrogen peroxide. After stopping the reaction with a strong acid, the intensity of the color produced bears a direct relationship with the B-TG concentration initially present in the plasma sample. CV = 5.4.

I. VLDL cholesterol (MIBH)

VLDL cholesterol will be determined on the upper one-third volume of an ultracentrifuged sample of serum. One of the concerns expressed about doing a measured VLDL is the cost and the need to do it on a fresh sample. Field centers were unable to provide the labor. There is evidence that freezing the sample has little impact on the determination of VLDL by ultracentrifugation although freezing does result in significant increase in a calculated VLDL (See Newman, Contois, Jener, McNamara and Schaefer, Clin. Chem. 36: 961, 1990). Following a procedure patterned after the micromethod of Wu, Warnick, Wu, Williams and Lalouel (Clin. Chem. 35: 1486-91, 1989), VLDL could be determined by ultracentrifugation of less than 500 ul of frozen serum (one cryovial). We propose doing two samples per subject per diet phase, which is six samples per subject. We believe that the measured VLDL will be much more useful in this setting than a calculated value.

J. HDL Subfraction (Columbia)

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 is 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).

K. Apoprotein A-1 and B-100 (Columbia)

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.

L. Lipoprotein (a) (MIBH).

Analytical method is an ELISA (Enzyme Linked Immunosorbent Assay) using the Macra Lp(a) kit manufactured by Strategic Diagnostics (Newark, NJ). 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 chromogen 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 the manufacturer in their Macro LP (a) kit. ELISA plates are read on an automated ELISA plate reader, Dynatech model MR5000 set to monitor at 492 nanometers. 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 mg per deciliter has a long-term precision (day-to-day) of 3.8% and within run of 1.8%. Samples of 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.

M. Retinyl Palmitate (Columbia)

Retinyl palmitate measurement: Plasma to be used for determination of retinyl ester levels is protected from light and stored under nitrogen at -80° C until assayed. Plasma retinol and retinyl palmitate levels are measured by reverse phase high-performance liquid chromatography. This procedure employs an internal standard technique for the calculation of retinol and retinyl ester levels. The within-assay and between-assay coefficients of variation are less than 7%. For retinoid determinations, 100 ul of serum or plasma is denatured by addition of 100 ul ethanol containing internal standard retinyl acetate, and the retinoids extracted first into hexane and then into benzene for injection onto the HPLC column. Chromatography is carried out on a 4.6 x 25 mm Beckman 5 u Ultrasphere ODS column using 70% acetonitrile-15% methanol as solvent. Flow rate is 2.0 ml/min and retinol and retinyl esters are detected by absorbance at 325 nm. Quantitation is based on peak area.

N. C-reactive Protein (Vermont)

A colorimetric competitive immunoassy, developed by R. Tracy's laboratory, used with rabbit anti-human c-reactive protein (Calbiochem, La Jolla, CA) as the capture antibody. C-reactive protein (Calbiochem, biotinylated by the method of Tijssen P. Techniques in Biochemistry and Molecular Biology, Vol. 15, p.29, 1985) competes with C-rp in the sample for the coated antibody. After washing, Avidin-Biotin complex (ABC) reagent is added. Avidin, covalently conjugated to horseradish peroxidase will bond to biotin. After washing, the enzyme substrate is added. The latter is composed of a color reagent, orthophenulene diamine, in the presence of hydrogen peroxide as substrate. The amount of color generated is inversely proportional to the amount of c-rp in the sample.

CV = 9.9%.

O. Microalbuminuria (MIBH)

The term microalbuminuria is a misnomer in that it refers to measurement of very low concentrations of albumin that is full sized albumin. A more correct term would be minimal albuminuria. The methods for measurement use antisera prepared against human serum albumin. There is no need for concern about failure of antisera to recognize a cleaved or truncated molecule.

The major concern in the analysis of these low levels of albumin is calibration in an appropriate range and recovery of the protein. The method will be a rate nephelometric method carried out on a Beckman Array rate nephelometer. Samples will be centrifuged before analysis to remove any particulate matter that would interfere with the analysis. Calibration is carried out to optimize the measurement of concentrations between 20 and 200 μg/mL, which is the concentration that reflects minimal albuminuria. Normal excretion is about 8 to 10 μg/mL.

The sample will be a first morning urine sample which has been demonstrated to be equivalent to, if not better than, the use of a 24-hr urine collection in measuring albuminuria on serial samples. (See Howey et al. *Selecting the Optimum Specimen for Assessing Slight Albuminuria* in Clin.Chem.(1987): 33, 2034-8). Since some investigators will be collecting a 24-hour urine sample, it is possible that this test could be done on a 24-hour sample, if all centers choose to collect a 24 hour urine. However, the study does not require the subjects to collect a 24-hour specimen for this analysis. Although some investigators suggest using an albumin/creatinine ratio to improve precision, the study by Howey et al., referenced above, suggests there is little benefit to using an albumin to creatinine ratio and the cost of performing this additional test seems unwarranted.

P. Platelets by Flow Cytometry

Specially prepared samples are labeled with saturating amounts of flourseceine isothiocyanate (FITC) conjugated anti-glycoprotein IIb-IIIa antibodies (AMAC Inc., Westbrook, ME) and Phycoerythrin-conjugated anti-CD62, Granule Membrane Protein (GMP-140) (Becton Dickinson, San Jose, CA). Samples are analyzed on a EPIC's Profile

Analyzer flow cytometer (Coulter Corporation). The instrument is set up to measure forward light scatter (FSC). FITC fluorescence, and PE fluorescence. All parameters were collected using four decade logarithmic amplification. FITC fluorescence was sued to trigger the instrument, and a threshold was set up so that only particles having FITC fluorescence at least equal to single platelets were measured. The instrument is calibrated using beads of known diameter, 2um and 10um (Duke Scientific, Palo Alto, CA) to calibrate the FSC parameter; and using Calibrate beads (Becton Dickinson, Cockeysville, MD) to calibrate the fluorescence parameters.

III. Sample Collection

The DELTA Study requires collection of approximately 41 ml of blood and a first morning urine sample three times at the end of each diet phase. In addition, on the days of postprandial testing two additional blood samples (approximately 20 ml per sample) will be drawn in addition to the fasting sample.

All samples will be processed according to detailed Manuals of Operations (MOA) and the samples will be stored frozen at -80° until analysis. Analysis will be performed on all samples at the end of the entire study. At that time samples will be removed from archives and either analyzed at the field center laboratory or sent to the appropriate central laboratory facility on dry ice.

Table 2 summarizes the sample collection and archiving scheme.

IV. Laboratory Organization

The laboratories involved in the protocol will consist of those affiliated with the field centers, including Columbia University (CU), Pennington Biomedical Research Center (PBRC), Pennsylvania State University/The Mary Imogene Bassett Research Institute (MIB), and the University of Minnesota (UM), as well as the central coagulation laboratory at the University of Vermont (UV) (Russell Tracy, Ph.D.).

A. Testing for entry criteria

All testing for meeting entry criteria will be done by the local field center laboratories. This will include lipid profiles, insulin, blood chemistries, TSH and hematology profile.

See **Chapter 6: Population Recruitment: Screening Eligibility Criteria and Procedures** for details of the testing to be done for determining eligibility.

B. Endpoint data

Testing for outcome will be based on endpoint data. The samples for these endpoint tests will be collected during the last three weeks of each diet phase and will be processed,

stored and analyzed as described in the following sections of this chapter. Tests for cholesterol, triglycerides, HDL-cholesterol, glucose, and uric acid will be performed by each local field center laboratory. Other endpoint tests will be performed at a designed central laboratory for that test.

1. Field center laboratories for lipid profile and glucose

The rationale for the use of field center laboratories for the analyses of fasting lipid profile 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 lipid 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) have been long term participants in this program. During DELTA Diet Protocol I, several challenges sent from CDC to all four field center laboratories demonstrated that the accuracy and precision of these laboratories is comparable and the data that would be generated from them would have no greater variance than that generated by a single lab. These results are summarized in Tables 1 and 2. The first challenge to five centers is shown with labs in lower case type. The second challenge (to MINN and MIBH) is shown in capital letters. All data are summarized in a single statistical analysis.

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.

The inclusion of glucose and uric acid at the local field laboratory is based on the ease and cost effectiveness of including them in the same analytical run on the same multichannel analyzer as is used to perform the lipid profile and the recognition that standard materials are available to calibrate for these analytes and that national proficiency testing programs (such as that operated by the College of American Pathologists) demonstrate that the various multichannel analyzers used by the field laboratories are adequately accurate and precise to assure statistical validity in endpoint analysis.

2. Central laboratories for other endpoint data

Insulin, the apolipoproteins (apo A-1, B, Lp (a)), apo-E genotypes, and coagulation factors (fibrinogen, factor VII, and PAI-1, beta-thromboglobulin) currently have no standardization programs available to provide laboratory standards. C-reactive protein and microalbuminuria require special standardization at low levels, not part of usual clinical measurement of the these analytes. Tests such as LDL size, VLDL-cholesterol, HDL-subfractions, retinyl esters, platelets by flow cytometry, involve multiple steps in preparation and analysis of samples and are best carried out at a single site to assure uniformity in handling and analysis.

Thus, the optimal way to control precision and accuracy is to use a central laboratory with recognized experience and methods for each of these tests. These laboratories were selected by the Steering Committee based on expertise and interest in performing the tests.

V. Quality Control

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. 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. The protocols for sample collection, processing, handling, storage and shipping will be similar to that for DELTA Diet Protocol I.

To assure validity of the results in this study requires rigid adherence to collection, labeling, and shipping protocols:

Training. To ensure that protocols are well understood, a training session will be held to provide training and experience before the beginning of the project. A training session will be held at each Field Center and will be coordinated by the laboratory point person for that center. (The laboratory point person is an individual who participated in a special training program before the initiation of DELTA Protocol 1 and who was designated by the Field Center as the person who would assume responsibility for training and certifying new personnel.) Since many of the endpoints are the same as for diet Protocol 1 (lipid profiles, apolipoproteins, ApoE genotyping, fibrinogen, PAI-1 and Factor VII) the details of blood drawing and processing will be the same as for Protocol 1. Several new test for Protocol 2 will require special sample handling (platelets, betathromboglobulin, microalbuminuria). A video tape will be prepared by the University of Vermont demonstrating the phlebotomy and sample processing for the testing for DELTA Protocol 2 with special emphasis on the new techniques that will be needed.

Written Protocols. Detailed instructions will be provided for each step in the collection and processing of samples. These will be compiled in the Manual of Procedures and will provide the basis for the on-site training programs.

Documentation. Forms will be provided to document each step of the blood (or urine) collection process with space for field center staff to describe any variation from the prescribed protocol. Additional forms will be used to validate the processing of the samples, including time from collection to freezing, maintenance of desired temperature during processing, and identification of any deviation from protocol during the processing and aliquoting.

Labeling. Processing and archiving includes detailed descriptions of how to set up tubes, makes provisions for assuring that correct identification labels are placed on each sample and provides a system for archiving that is redundant in identification (by label, by color code and by position in the freezer storage box).

Shipping. Shipping protocols are specific in detailing amount of dry ice and quantity of samples per box. Shipping protocols require double checking by shipper and by receiver that the packing list is correct, that the labels are attached and that there is no evidence that the sample has thawed either in storage or in transit. Sender must inform the recipient on the day of shipment that samples have been sent. If samples are not received the next day, the sender is informed and the shipping agent (Federal Express) is notified to search for the shipment.

Analysis. Specific recommendations are made for analysis of samples from each subject to assure completeness in results generated and assuring best possible analytical precision in analysis. 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 each analyte on a single run for the analyte.

Quality control. 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.

Adherence to these protocols, including periodic reviews and retraining, is the responsibility of each field center.

VI. Standards of Laboratory Practice

Laboratory work for DELTA will adhere to a designated standard of laboratory practice that assures that results are generated in a standard fashion and that original data are retained and available for review for a fixed period, should that be necessary.

The Laboratory Subcommittee is in the process of developing a document to serve as a Standard of Laboratory Practice. This document will be in the form of a checklist for evaluation of all the field and central laboratories that participate in DELTA. This standard of laboratory practice will detail the ways that data are generated for future review and will identify the extent to which the operator and supervisor will be expected to review and document their review of calibration, quality control, and subject results for each analyte, This will be similar to the "Good Laboratory Practice" standard that is usually applied to laboratories performing contract work for government and industry, but will be adapted to apply to the special situation of research work performed in research settings.

Table 1 Laboratory Tests by Priority of Assay

TEST	SITE	TEST SCHEDULE	
		tests/diet phase	timing (week #)
Obligatory Tests for Study Hypotheses			
Lipid Profile	Local Lab	3	5,6,7
Glucose	Local Lab	3	5,6,7
Insulin	MIB	3	5,6,7
Factor VII	Vermont	3	5,6,7
Fibrinogen	Vermont	3	5,6,7
PAI-1	Vermont	3	5,6,7
Tests Which Should Be Performed To Support Hypothesis Testing			
Apo E	MIB		once per subject
LDL Size	Pennington	3	5,6,7
Beta Thromboglobulin	Vermont	3	5,6,7
VLDL-C	MIB	2	6,7
HDL Subfractions	Columbia	2	6,7
Apo A-1 and Apo B-100	Columbia	3	5,6,7

Tests Which Allow Subhypotheses To Be Tested

Post-prandial standard fat load*			
Triglyceride	Local Lab	2	7; @ 4 h & 8 h
Glucose	Local Lab	2	7; @ 4 h & 8 h
Insulin	MIB	2	7; @ 4 h & 8 h
Retinyl ester	Columbia	2	7; @ 4 h & 8 h
Post-prandial post meal testing*			
Triglyceride	Local Lab	2	6; before lunch & dinner
Glucose	Local Lab	2	6; before lunch & dinner
Insulin	MIB	2	6; before lunch & dinner
Lipoprotein (a)	MIB	3	5,6,7

Tests Which Allow New Mechanisms to Be Explained

Platelets by Flow Cytometry	Vermont	1	6 or 7
C-reactive Protein	Vermont	3	5,6,7
Uric Acid	Local Lab	3	5,6,7
Microalbuminuria	MIB	3	5,6,7

**will be carried out on same day that fasting samples are collected for other testing for that week. In that way the fasting results for triglyceride, glucose and insulin will be available for comparison.*

Table 2 Endpoint Sample Collection and Processing Scheme

Once per week to give 3 sets per diet period					Once per subject	Once per diet period		
Endpoint package similar to Protocol 1 with six tubes of blood (41 mL)				Urine first AM	Buffy Coat	Platelets Special Prep	Postprandial (collection for two timed samples)	
Serum 2x10 mL	Citrated plasma 2x4.5 mL	Diatube plasma 5 mL	EDTA plasma 7 mL	Urine		Platelets special	Serum 2 x 10 mL	
14 vials	3 vials (4°) 3 vials (r.t.)	3 vials	4 vials	2 tubes	4 buffys	4 tubes	2 vial (foil wrapped) 3 vials	

Disposition of vials and tubes

	serum	citrated plasma 4°	citrated plasma r.t.	Diatube plasma	EDTA plasma	buffy	urine	platelets	postprandial
Lipid profile, glucose	2								
HDL-subfractions	2								
VLDL-chol	1								
Uric acid	1								
Apo A/B, Lp(a)	1								
Insulin	1								
Apo E						1			
Fibrinogen		1							
PAI-1		1							
Factor VII			1						
Beta thromboglobulin*				1					
CRP	1								
Platelets								2	
Microalbuminuria							1		
LDL size					2				
Postprandial									
Lipid profile, glucose									1
Insulin									1
Retinyl palmitate									1
Archive	5	1	2	2	2	3	1	2	2

*β-thromboglobulin requires filtering of the sample prior to freezing.

Table 3: DELTA PROJECT -- TOTAL CHOLESTEROL PRECISION

within laboratory

CV-total

	pool 61		pool 66		pool 67		pool 71	
lab	mean	CV	mean	CV	mean	CV	mean	CV
colu			183	0.7	220	0.9	274	0.6
loui			178	0.9	216	1.0	263	0.7
mibh			182	1.3	219	1.2	274	1.2
minn			175	1.9	210	1.6	257	1.6
stan			184	0.4	220	0.6	272	0.5
MI82	207	1.4	181	1.0			273	0.8
MIN2	206	1.3	182	1.5			267	1.4
MEAN >	207	1	181	1.1	216	0.8	269	1.0

DELTA PROJECT -- HDL PRECISION

within laboratory

CV-total

	pool 36		pool 81		pool 88	
lab	mean	CV	mean	CV	mean	CV
colu	60	0.9	40	1.3	27	1.9
loui	56	1.9	38	1.4	27	2.1
mibh	60	2.0	40	2.0	28	2.2
stan	60	1.1	41	0.8	29	0.9
MIB2	61	1.4	41	1.4	28	1.7
MIN2	58	2.2	40	2.3	28	1.4
MEAN >	58	1.6	39	1.5	27	1.6

Table 4: DELTA PROJECT--TOTAL CHOLESTEROL PRECISION

	POOL 61 VARIANCE	CV	% TOTAL	POOL 66 VARIANCE	CV	% TOTAL	POOL 67 VARIANCE	CV	% TOTAL	POOL 71 VARIANCE	CV	% TOTAL	MEAN CV	MEAN % TOTAL
TOTAL	10.2	1.5	100	12.9	2.0	100	22.2	2.2	100	48.7	3.2	100	1.9	100
LAB	0.0	0.0	0	8.1	1.6	62	15.3	1.8	69	39.8	2.9	82	1.1	43.7
RUN	8.2	1.4	81	2.4	0.9	18	4.5	1.0	20	4.0	0.9	8	1.1	39.7
REPS.	2.0	0.7	19	2.5	0.9	20	2.5	0.7	11	4.8	1.0	10	0.8	16.7
MEAN	206.7			180.6			217.1			268.7				

DELTA PROJECT--HDL PRECISION

	POOL 36 VARIANCE	CV	% TOTAL	POOL 81 VARIANCE	CV	% TOTAL	POOL 88 VARIANCE	CV	% TOTAL	MEAN CV	MEAN % TOTAL
TOTAL	9.8	5.4	100	3.6	4.9	100	2.2	5.5	100	5.2	100
LAB	8.8	5.1	90	3.2	4.6	89	2.0	5.2	89	4.9	89.3
RUN	0.2	0.8	2	0.1	0.8	3	0.1	1.0	3	0.9	2.7
REPS.	0.7	1.5	8	0.3	1.4	9	0.2	1.5	7	1.5	6.0
MEAN	58.4			39.16			27.45				

Variance components shown above are total, among labs, among runs-within labs, and among replicates.

CHAPTER 10

Data Management

Chapter 10: DATA MANAGEMENT

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. 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 recommended (the centers will make the changes) 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. Field Centers will not send paper forms to the Coordinating Center unless specifically requested. The

frequency of data transfer by the field centers to the Coordinating Center will be every other week. Central agencies and laboratories (i.e., Food Analysis and Hemostasis laboratories) also send data on diskettes to the CSCC, and all information transferred electronically must be in a format that conforms to DELTA data transfer standards to insure complete and accurate processing.

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.

APPENDIX A

List of Forms

APPENDIX A

LIST OF DELTA FORMS
1. Recruitment Log
2. Telephone Screening Form
3. Eligibility Visit 1 Screening Questionnaire
4. Eligibility Visit 2 Screening Questionnaire
5. Consent Form
6. Participant Agreement
7. Diet Compliance Forms
8. Participant Weekly Tracking Form
9. Close Out Form
10. Drop Out Form
11. Data Entry Form for Lipids and Lipoproteins
12. Data Entry Form for Hemostasis Variables
13. Clinic Visit Form for Phlebotomy and BP
14. Form to Use when Calibrating Weights for Scales

APPENDIX B

Informed Consent

DRAFT CONSENT FORM

IRB approval date _____

Approval expiration date _____

Columbia Presbyterian Medical Center

Consent to participate in a Research Study:

The purpose of this consent form is to provide you with the information you need to consider in deciding whether to participate in this research study.

Study Title: "Comparison of the efficacy of diets high in monounsaturated fat carbohydrates on lipid metabolism and hemostasis in individuals with insulin resistance and/or dyslipidemia."

IRB # 6065

Principal Investigator: Henry N. Ginsberg, Professor of Medicine

Study Purpose:

You are invited to participate in a research study of the effects of diets with differing fat and carbohydrate levels on your blood cholesterol and fat levels and on the clotting activity of your blood. Dr. Ginsberg and his associates hope to learn more about the way the dietary fat affects the amount of cholesterol and fat in your blood; and the way that fat in the diet affects the way the blood clots. All of this information is important because of the link between diet, blood cholesterol, blood clotting, and the chance of having a heart attack or stroke. You were selected as a possible participant in this study because you do not have any significant diseases, but you do have some abnormalities of fat and glucose metabolism that could increase your risk for developing atherosclerosis (hardening of the arteries) and adult-type diabetes. Specifically, although your total blood cholesterol level is within the average range for someone your age, your blood level of triglyceride (blood fat) may be higher than is considered best for your health. Additionally, your HDL cholesterol (the good cholesterol fraction) may be lower than is considered optimal. Higher triglycerides and lower HDL cholesterol may increase the risk for atherosclerosis, heart attacks and strokes. Finally, your blood insulin level (the chemical that control blood sugar levels) may not be optimal either. This could mean that you are at risk for developing diabetes.

In this study, we will be comparing the effects of diets containing different amounts of fat and carbohydrate: one diet will have the amounts of saturated fats (16% of total calories) that is usually eaten by Americans while the other two diets will have the amount recommended by the American Heart Association for all Americans (9% of total calories). The two diets that are lower in saturated fat will differ in that one will have carbohydrate replacing the saturated fat while the other will use

monounsaturated fat (the type present in olive oil) to replace the saturated fat. The diets will all contain the same amounts of protein, polyunsaturated fat and cholesterol.

The idea behind this study is that reducing the amount of saturated fat in the diet will reduce the level of cholesterol and fat in the blood, and will make the blood less likely to clot. Heart attacks are the major cause of death in the United States, and we know that both the level of cholesterol and fat in the blood as well as the likelihood that the blood will clot contribute to an individual's risk for developing coronary artery disease (narrowing of the blood vessels supplying the hear with oxygen). Coronary artery disease leads to heart attacks. However, there is a controversy among nutrition experts concerning the best way to reduce the amount of saturated fat in the diet; some want to replace the saturated fats with carbohydrates (breads, most vegetables, pasta) while others believe that monounsaturated fats would be the best replacement for the saturated fats. This study will determine which diet is best for individuals like you who may be at increased risk for heart attacks and diabetes.

Study Procedures:

If you decide to participate, you will agree to eat only foods that we provide for you (except for one dinner on the weekend) during three 7-week periods. You will come to the Bard Hall cafeteria for lunch and dinner and eat your meals there Monday through Friday. We will give you packed snacks and breakfasts to eat at home. On Friday evenings you will be given packages containing weekend breakfasts, lunches, one dinner and snacks. There will be breaks of 4-6 weeks between the first and second, and between the second and third diet periods.

We will weigh you twice weekly and you will provide the staff dieticians with information about your one weekend meal as well as alcohol intake (limited to five drinks per week) and any non-study foods you may have eaten.

We will also measure your blood pressure each week. You may have blood pressure that is slightly above the recommended levels of 140/90 mm Hg but not greater than 145/90 mm Hg. If, on two consecutive measurements, we find your blood pressure is above 145/95, we will refer you to your physician for evaluation. If your physician, at that time, determines that you require blood pressure medication, we will release you from this study.

During the final three weeks of the study (weeks 5, 6, & 7) you will come to our offices on one day of each week for blood tests. All blood samples will be obtained after a 10 hour overnight fast.

The amount of blood drawn at a single visit will be about one ounce (two tablespoons). Each visit for blood sampling should take no more that 20 minutes.

Successful completion of this study depends on the excellent cooperation of the participants. If, during the study, you cannot eat the foods provided and/or eat other foods, you will be asked to leave the study.

Study risks:

The diets to be used in these studies are eaten by significant numbers of typical Americans. Because you were recruited on the basis of abnormalities in lipid and/or glucose metabolism, the average American diet is not optimal for you. However, we do not believe that 7 weeks on the average

American diet will significantly affect your health. The other two diets that we will feed you are within the guidelines for diets to be used in people who have abnormalities in lipid and/or glucose metabolism.

You may be overweight at the start of the study. We will attempt to maintain your weight throughout the study and will weigh you twice weekly. Because it may be beneficial to your health to lose weight, we will offer you weight loss counseling at the conclusion of the study. You will not, however, be allowed to lose weight during the study. You may choose to join a weight loss program elsewhere at this time, rather than joining our diet study.

The risks involved with blood drawing include some local pain and bruising from venipuncture: we will use well trained and experienced phlebotomist. Blood sampling can also cause light-headedness and dizziness; we will watch for this and if it occurs we can alleviated symptoms by having the subject lie flat with feet raised.

Alternatives:

As noted above, you have several alternatives to entering our study. You might wish to see your physician for treatment of your elevated level of triglycerides or reduced level of HDL cholesterol. You might choose to see your physician if we have found that your blood pressure is greater than 140/90 mm Hg. You might wish to join some weight loss program rather than enter our study (during which your weight will be steady).

Compensation:

All foods, meals, snacks, fluids will be provided at no cost to participants. In some cases, subjects will be reimbursed for travel/parking fees.

Confidentiality:

Patient files and results will all be coded. All data will be locked in a file cabinet by the Principal Investigator.

Participation is Voluntary:

Your participation in this study is completely voluntary. You can refuse to participate or withdraw from the study at any time, and such a decision will not affect your medical care, employment, or student status at Columbia Presbyterian Medical Center now or in the future. As noted above, the investigators may ask you to leave the study if you cannot comply completely with diets.

Questions:

If you have any questions, please ask. In the future, should you have any questions you can reach Dr. Berglund or Dr. Ginsberg at 305-3741.

If you have any questions on your rights as a research subject you can call the Institutional Review Board (212-305-5883) for information.

Signature: _____ **Date:** _____

(Investigator)

Consent to Participate in the Study

I have discussed this study with Dr. Ginsberg (or his designated associate) to my satisfaction. I understand that my participation is voluntary and that I can withdraw from the study at any time without prejudice. I have read the above and agree to enter this research study. Signing this form does not waive any of my legal rights.

I have been informed that if I believe that I have sustained injury as a result of participating in a research study, I may contact the Principal Investigator, Henry N. Ginsberg at 305-3741, or the office of the Institutional Review Board, at 305-5883, so that I can review the matter and identify the medical resources which may be available to me.

I understand that:

a) The Presbyterian Hospital will furnish the emergency medical care determined to be necessary by the medical staff of the hospital;

b) I will be responsible for the cost of such care, either personally or through my medical insurance or other form of medical coverage;

c) No monetary compensation for wages lost as a result of injury will be paid to me by the Columbia Presbyterian Medical Center.

d) I will receive a copy of this signed consent form.

Signed _____

Date _____

Print Name _____

Witnessed _____ Date _____

The Institutional Review Board of the Columbia Presbyterian Medical Center has approved the recruitment of subjects for this study.