NATTOKINASE ATHEROTHROMBOTIC PREVENTION STUDY (NAPS)

NCT 02080520

Manual of Operations

March 10, 2014

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NATTOKINASE ATHEROTHROMBOTIC PREVENTION STUDY (NAPS)

Manual of Operations

1.0
OVERVIEW/ELIGIBILITY

1.1 Executive Summary

The potential for nattokinase to "thin" blood and to reduce blood clotting by positive antithrombotic and fibrinolytic effects presents a unique opportunity to safely study such effects on cardiovascular disease (CVD) and cognition. Unfortunately, such studies of antithrombotic and fibrinolytic pathways of prevention have been limited due to lack of safe compounds and the adverse reactions associated with current agents such as Coumadin. Nattokinase, an over-the-counter supplement used for cardiovascular health, is the most active functional constituent of natto, a fermented soy product. Natto has been consumed primarily by the Japanese for over 1000 years, a population with one of the lowest risks for CVD and dementia. CVD and dementia remain the most challenging age-related health risks of the 21st century for Americans necessitating development of further effective preemptive strategies. Whether reducing the propensity for thrombus formation and/or increasing fibrinolytic activity can prevent the progression of atherosclerosis and cognitive decline has not yet been determined.

Using nattokinase under primary prevention conditions, we propose to conduct a randomized, double-blinded, placebo-controlled trial to determine whether decreasing atherothrombotic risk can reduce the progression of atherosclerosis and cognitive decline. We propose to randomize 240 healthy non-demented women and men to nattokinase supplementation or to placebo for three years. The primary trial end points will be measurement of carotid arterial wall thickness and arterial stiffness, early changes of atherosclerosis that can be measured safely by non-invasive imaging techniques. The secondary trial end point will be ascertained through change in cognition measured by a neuropsychological battery. In addition, biochemical blood measurements and in vitro studies will be conducted to compare the effects of nattokinase relative to placebo on blood coagulation and thrombus break-down capabilities, blood flow properties, inflammation and inflammatory activation of endothelial cells that line blood vessels.

At the conclusion of this trial, we expect to have sufficient evidence as to whether reducing propensity for thrombus formation and/or increasing fibrinolytic activity can prevent atherosclerosis progression and cognitive decline. Results will provide novel and important data that will be informative concerning primary prevention through the atherothrombotic pathway. Providing evidence for a reduction in atherosclerosis progression and cognitive decline with nattokinase is likely to shift the current clinical paradigm for the prevention of these chronic age-related processes. Additionally, such evidence will serve to create a new field of discovery and opportunity for prevention of CVD and dementia.

1.2 Objectives and Hypotheses

The goal of the proposed study is to determine under randomized controlled trial conditions whether nattokinase reduces subclinical atherosclerosis and cognitive decline in healthy women and men. Our hypotheses are: 1) Compared to placebo, nattokinase will show less subclinical atherosclerosis progression and cognitive decline in healthy women and men; 2) The reduction in subclinical atherosclerosis progression and cognitive decline with nattokinase will be correlated; and, 3) The reduction in progression of subclinical atherosclerosis and cognitive decline with nattokinase will be mediated through hemostatic, fibrinolytic and hemorheological factors as well as attenuation of inflammation, monocyte activation, vascular endothelium injury and activation of vascular endothelium by circulating monocytes.

1.3 Study Overview

The Nattokinase Atherothrombotic Prevention Study (NAPS) is a randomized, double-blinded, placebo-controlled, non-invasive vascular imaging trial. A total of 240 healthy non-demented women and men without clinical evidence of CVD or diabetes mellitus will be randomized to either placebo or to active oral nattokinase 2,000 fibrinolytic units daily. Recruitment will occur over 2 years and the treatment period will be for 3 years. Candidates will be prescreened, seen for 1 screening visit to determine eligibility and then randomized at a baseline visit. Participants will then return for a follow-up visit 1 week after randomization and then will be followed monthly for the first 6 months and then every 3 months for the remainder of the trial. Rate of change in distal common carotid artery (CCA) far wall intima-media thickness (CIMT) and arterial stiffness in computer image processed B-mode ultrasonograms will be the co-primary trial end points. Cognitive decline determined with a neuropsychological battery designed to evaluate 7 cognitive domains including: attention, concentration, working memory, executive function; visuospatial/visuoconstructive skills; naming/semantic memory; and verbal and nonverbal episodic memory will be the secondary trial endpoint. Other endpoints will include blood pressure, hemostatic, fibrinolytic and hemorheological factors as well as platelet activity and biomarkers for inflammation, monocyte activation, vascular endothelium injury and activation of vascular endothelium by circulating monocytes.

1.4 Inclusion Criteria

(1) Age $\geq$55 years
(2) Male or postmenopausal female (no uterine bleeding for >6 months)

1.5 Exclusion Criteria

(1) Clinical signs, symptoms, or personal history of CVD
(2) Diabetes mellitus or fasting serum glucose >140 mg/dL
(3) Plasma triglyceride levels $\geq$500 mg/dL
(4) Uncontrolled hypertension (systolic blood pressure >160 mmHg or diastolic blood pressure >110 mmHg)*
(5) Uncontrolled tachycardia or irregular heart rates (i.e., atrial fibrillation)
(6) Thyroid disease (untreated)
(7) Renal insufficiency (defined as serum creatinine >2.0 mg/dL)
(8) Life threatening illness with prognosis <5 years
(9) Current use of lipid-lowering medication
(10) Current use of food supplements containing soy, soy protein, isoflavones or other phytoestrogens
(11) Known sensitivity or allergy to soy or nuts
(12) Regular aspirin or other antiplatelet medication use
(13) Use of anticoagulants
(14) Bleeding diatheses or tendencies

*Participants who enter screening with a systolic blood pressure >160 mmHg or diastolic blood pressure >110 mmHg will be immediately referred to their physician or to an emergency room. Once the blood pressure is controlled, the participant will become eligible for the trial. Participants who enter screening with mild to moderate hypertension (systolic blood pressure $\leq$160 mmHg or diastolic blood pressure $\leq$110 mmHg) will be notified immediately and a letter issued to the participant and to the participant's physician. Control of hypertension is a requisite to continue into the trial.

1.6 Trial Clinic Flow

Recruitment
Sources

⇓

Prescreening

- Telephone Questionnaire **[Form 1]**

⇓ - Age ≥55 years
⇓ - Male or postmenopausal female
⇓ - No cardiovascular disease
⇓ - No diabetes mellitus
⇓ - No life threatening disease
⇓ - No current use of lipid-lowering medication
⇓ - No current use of food supplements containing soy, soy protein, isoflavones or other phytoestrogens
⇓ - No known sensitivity or allergy to soy or nuts
⇓ - No regular use of aspirin or other antiplatelet medication
⇓ - No use of anticoagulants
⇓ - No bleeding diatheses or tendencies
⇓

If taking, instruct individual to stop soy, soy protein and isoflavone food supplements for 1 month prior to screening visit

⇓
⇓

Schedule screening visit appointment
8 hour fast

⇓
⇓

Screening Visit

- Assign study ID
- Informed consent and HIPAA **[Form 2]**
- Participant Information Form **[Form 3]**
- Personal Information Form **[Form 4]**
- Cardiovascular Disease History Questionnaire **[Form 5]**
- Bleeding Assessment Questionnaire **[Form 6a]**
- Smoking/ETOH Questionnaire **[Form 7a]**
- Reproductive History Questionnaire **[Form 8]**
- Medical History Questionnaire **[Form 9]**
- Rose Questionnaire **[Form 10]**
- Claudication Questionnaire **[Form 11]**
- Cardiovascular Disease Risk Factor Questionnaire **[Form 12]**
- Nonstudy Medications Record **[Forms 13,14]**
- History of Vitamin and Nutritional Supplement Use Questionnaire **[Form 15]**
- Vital signs (systolic blood pressure ≤160 mmHg and diastolic blood pressure ≤110 mmHg) **[Form 16]**
- Pulse rate **[Form 16]**
- Weight/height **[Form 16]**
- CIMT-1/arterial stiffness

- Fasting blood samples drawn for:
    - Chemistry (FSG ≤140 mg/dL; serum creatinine ≤2.0 mg/dL)
    - CBC
    - Platelet count
    - Prothrombin time (PT)
    - activated partial thromboplastin time (aPTT)
    - Lipids (triglycerides <500 mg/dL)
    - C-reactive protein (CRP)
    - Hemoglobin A1c

Draw and store at -80^{o}C (analyze only when eligible):

- Insulin
- Inflammatory markers
    - MCP-1
    - IL-8
    - TNFα
    - IL-1β
    - IL-10
    - E-Selectin
    - P-Selectin

⇓
⇓

Schedule randomization visit appointment
8 hour fast

⇓
⇓

Final Eligibility

- Final Eligibility Check List **[Form 17]**

⇓
⇓

Randomization Visit (v00)

- Nonstudy Medications Record **[Forms 13,14]**
- History of Vitamin and Nutritional Supplement Use Questionnaire **[Form 15]**
- Dietary Food Frequency **[Form 18]**
- Soy Food Questionnaire **[Form 19]**
- 7-Day Physical Activity Questionnaire **[Form 20]**
- Center for Epidemiologic Studies Depression (CES-D) Scale **[Form 21]**
- Cognitive assessment **[Form 22]**
- Vitals signs (systolic blood pressure ≤160 mmHg and diastolic blood pressure ≤110 mmHg) **[Form 16]**
- Pulse rate **[Form 16]**
- Weight **[Form 16]**
- Waist:hip measurement **[Form 23]**
- 12-lead ECG **[Form 24]**
- CIMT-2/arterial stiffness
- Fasting blood samples drawn for:
    - Fibrinogen
    - vWF antigen
    - Tissue plasminogen activator (tPA)
    - Plasminogen activator inhibitor-1 (PAI-1)
    - D-dimers
    - Platelet aggregation (ADP, collagen, epinephrine)
    - Ristocetin cofactor
    - Factor VIII

- Nattokinase
- Anti-nattokinase antibodies
- Erythrocyte sedimentation rate (ESR)
- Red blood cell aggregation (plasma)
- Red blood cell aggregability (polymer)
- Plasma viscosity
- Whole blood viscosity
- Endothelial injury in-vitro assay: VCAM-1, ICAM-1
- Monocyte activation (FACS): CD11b, CD11c, VLA-4
- Storage
    - Plasma (EDTA) generic storage
    - Plasma (citrate) generic storage
    - Serum generic storage
    - Buffy Coat
- Perform urine dipstick

- Randomization based on CIMT-1 $\leq$0.75 mm
- Active nattokinase/placebo nattokinase dispensed **[Form 25]**

⇓
⇓

Schedule week 1 appointment
8 hour fast

⇓
⇓

Week 1

- Bleeding Assessment Questionnaire **[Form 6b]**
- Fasting blood samples drawn for:
    - Fibrinogen
    - vWF antigen
    - Tissue plasminogen activator (tPA)
    - Plasminogen activator inhibitor-1 (PAI-1)
    - D-dimers
    - Ristocetin cofactor
    - Factor VIII
    - Nattokinase
    - Erythrocyte sedimentation rate (ESR)
    - Red blood cell aggregation (plasma)
    - Red blood cell aggregability (polymer)
    - Plasma viscosity
    - Whole blood viscosity
    - Storage
        - Plasma (EDTA) generic storage
        - Plasma (citrate) generic storage
        - Serum generic storage

1.7 Schedule of Visits and Laboratory Studies (See Section 7.6 (Table) for specific details)

Research Activity	Screen	Baseline	Week 1	Month 01	02	03	04	05	06	09	12	15	18	21	24	27	30	33	36
Chemistry Panel	x			x	x	x	x	x	x		x		x		x		x		x
Insulin	x					x			x		x		x		x		x		x
Complete Blood Count	x			x	x	x	x	x	x		x		x		x		x		x
Platelet Count	x			x	x	x	x	x	x		x		x		x		x		x
Prothrombin time (PT)	x			x		x			x		x		x		x		x		x
Activated Partial Thromboplastin Time (aPTT)	x			x		x			x		x		x		x		x		x
Fibrinogen		x	x	x					x		x		x		x		x		x
vWF Antigen		x	x	x					x				x				x		
Tissue Plasminogen Activator (tPA)		x	x	x					x				x				x		
Plasminogen Activator Inhitor-1 (PAI-1)		x	x	x					x				x				x		
D-Dimers		x	x	x					x				x				x		
Platelet Aggregation (ADP, Collagen, Epinephrine)		x		x					x										
Ristocetin Cofactor		x	x	x					x				x				x		
Factor VIII		x	x	x					x				x				x		
Nattokinase		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Anti-Nattokinase Antibodies		x		x					x				x				x		
Erythrocyte Sedimentation Rate (ESR)		x	x	x					x				x				x		
Red Blood Cell Aggregation (Plasma)		x	x	x					x				x				x		
Red Blood Cell Aggregability (Polymer)		x	x	x					x				x				x		
Plasma Viscosity		x	x	x					x				x				x		
Whole Blood Viscosity		x	x	x					x				x				x		
MCP-1	x				x					x				x				x	
IL-8	x				x					x				x				x	
TNFα	x				x					x				x				x	
IL-1β	x				x					x				x				x	
IL-10	x				x					x				x				x	
E-Selectin	x				x					x				x				x	
P-Selectin	x				x					x				x				x	
Endothelial Injury In-Vitro Assay: VCAM-1, ICAM-1		x			x					x				x				x	
Monocyte Activation (FACS): CD11b, CD11c, VLA-4		x			x					x				x				x	
Lipids/C-Reactive Protein (CRP)	x								x		x		x		x		x		x
Hemoglobin A1c	x					x			x		x		x		x		x		x
Storage		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Buffy Coat		x			x					x				x				x	
ApoE Genotype							x												
Urine Dipstick		x		x															

1.8 Schedule of Visits, Questionnaires and Procedures

Research Activity	Form Number	Prescreen	Screen	Baseline	Week 1	Month 01	02	03	04	05	06	09	12	15	18	21	24	27	30	33	36
Telephone Questionnaire	1	x																			
Assign Study ID			x																		
Informed Consent	2		x																		
Participant Information	3		x																		
Personal Information	4		x																		
Cardiovascular Disease History	5		x																		
Bleeding Assessment Questionnaire	6a, 6b		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Active/Passive Smoking Questionnaire	7a, 7b		x										x				x				x
Reproductive History (Female)	8		x																		
Medical History Baseline	9		x																		
Rose Questionnaire	10		x								x		x		x		x		x		x
Claudication Questionnaire	11		x								x		x		x		x		x		x
Cardiovascular Risk Factor Questionnaire	12		x																		
Non-study Medications	13,14		x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Nutraceuticals and Supplements	15		x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Blood Pressure	16		x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Pulse Rate	16		x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Weight	16		x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Final Eligibility Checklist	17			x																	
Dietary Food Frequency	18			x					x			x		x		x		x		x	
Soy Food Questionnaire	19			x					x			x		x		x		x		x	
7-Day Physical Activity Recall	20			x							x		x		x		x		x		x
CES-D*	21			x							x		x		x		x		x		x
Cognitive Assessment	22			x											x						x
Waist:Hip Circumference	23			x				x			x		x		x		x		x		x
12-Lead Electrocardiogram	24			x									x				x				x
Dispense Study Products	25			x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Randomize Participant				x																	
Compliance	26					x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Medication Termination/Dropout Form	27**																				
Adverse Events	28-38					x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Medical History On-Trial	39												x				x				x
Carotid Artery Intima-Media Thickness			x	x							x		x		x		x		x		x
Arterial Stiffness			x	x							x		x		x		x		x		x
Closeout Form	40																				x

*CES-D = Center for Epidemiologic Studies Depression Scale

**Use as needed

2.0
ORGANIZATIONAL STRUCTURE

The organizational structure is described in terms of five units according to their function in the overall study. In addition, there is a Steering Committee and an External Data and Safety Monitoring Board (EDSMB).

1) Administration

2) Clinic
    a) Physician
    b) Study coordinator
    c) Research nurses
    d) Phlebotomists
    e) Participant coordinators
    f) Recruiters
    g) Cognitive tester

3) Laboratory
    a) Core lipid laboratory – USC
    b) Hematology laboratory – USC
    c) Rheology laboratory – USC
    d) Biochemistry laboratory – USC
    e) Quest (Los Angeles)

4) Core ultrasound imaging facility

5) Data Coordinating Center (DCC)
    a) Biostatisticians
    b) Programmers
    c) Data clerks

These units interact directly under the coordination of the principal investigator. In addition, there are two oversight bodies:

1) Institutional Review Board (IRB)
2) External Data and Safety Monitoring Board (EDSMB)

The Steering Committee is comprised of the following individuals:

Howard N. Hodis, M.D., Chairman
Wendy J. Mack, Ph.D., Co-Chair
Herbert J. Meiselman, Sc.D.
Howard A. Liebman, M.D.
Vijay Kalra, Ph.D.

The External Data and Safety Monitoring Board is comprised of the following individuals:

Chairman

Christopher J. Gallagher, M.D.
Professor of Medicine
Creighton University School of Medicine

Dennis M. Black, Ph.D.
Professor in Residence
Division of Clinical Trials and Multicenter Studies
University of California, San Francisco

Kenneth A. Bauer, M.D.
Professor of Medicine
Hematology-Oncology
Harvard Medical School

3.0
RECRUITMENT/SCREENING

3.1 Recruitment

Potential participants will be recruited from multiple sources, including the University of Southern California.

We plan to target recruitment of 50% women and 30% minorities for this trial (see Table below).

	American Indian or Alaskan Native	Asian or Pacific Islander	Black, not of Hispanic Origin	Hispanic	White, not of Hispanic Origin	Total
Female	1	6	11	18	84	120
Male	1	6	11	18	84	120
Total	2	12	22	36	168	240

3.2 Screening

Potential participants will be pre-screened with a Telephone Questionnaire **[FORM 1]** that will cover major inclusion and exclusion criteria. The study will be explained to potential participants at this time.

Individuals fulfilling the Telephone Questionnaire eligibility criteria and interested in the study will be scheduled for the screening visit (SV) and instructed to stop soy, soy protein and isoflavone food supplements one month prior to SV if currently taking any of these products.

Participants will be instructed to fast for 8 hours prior to SV.

Final study eligibility will be determined over SV and the baseline visit (v00).

Telephone pre-screening ⇨ (1 week*) SV ⇨ (2 weeks) v00 (randomization)

*1 month if potential participant needs to stop soy, soy protein and isoflavone food supplement intake prior to SV.

3.3 Screening Visit (SV)

A SV appointment is contingent upon fulfilling all pre-screening eligibility criteria **[FORM 1]**. At SV, the following will be performed:

1. Assign study identification (ID) number (see Section 3.4 for procedure)
2. Explanation of study aims, protocol, benefits, risks
3. Participants sign Informed Consent and HIPPA **[FORM 2]**
4. Participants complete forms covering contact and demographic information:
   - Participant Information Form **[FORM 3]**
   - Personal Information Form **[FORM 4]**

5. Pre-screening Telephone Questionnaire **[FORM 1]** eligibility criteria reviewed with participant
6. Ensure that soy, soy protein and isoflavone food supplements have been stopped at least one month prior to SV (see Section 3.2)
7. Administer the Cardiovascular Disease History Questionnaire **[FORM 5]** and confirm that participants have no clinical manifestations of cardiovascular disease.
8. Administer the baseline Bleeding Assessment Questionnaire **[FORM 6a]** and confirm that participants have no bleeding diatheses or bleeding tendencies
9. Participants administered baseline Smoking, Passive Smoking, Alcohol, Other Substance Use Questionnaire **[FORM 7a]**
10. Administer the Reproductive History Questionnaire **[FORM 8]** and confirm that female participants have not menstruated for at least 6 months
11. Participants administered questionnaires covering health information:
    Medical History Questionnaire **[FORM 9]**
    Modified Rose Questionnaire **[FORM 10]**
    Claudication Questionnaire **[FORM 11]**
    Cardiovascular Disease Risk Factor Assessment Form **[FORM 12]**
12. Administer the Nonstudy Medications Record **[FORMS 13,14]** and confirm that participants are not taking lipid-lowering medication
13. Administer History of Vitamin and Nutritional Supplement Use Questionnaire **[FORM 15]** and confirm that participants are not taking soy, soy protein and isoflavone food supplements
14. Obtain participant's blood pressure and pulse rate **[FORM 16]** and confirm that systolic blood pressure is $\leq$160 mmHg and diastolic blood pressure is $\leq$110 mmHg
15. Obtain and record participant's weight and height **[FORM 16]**
16. Carotid artery ultrasound examination performed (first baseline - see Section 4.2 for randomization procedure)
17. Fasting blood samples drawn and analyzed for:
    - Chemistry
    - CBC
    - Platelet count
    - Prothrombin (PT)/activated partial thromboplastin time (aPTT)
    - Lipids
    - C-reactive protein
    - Hemoglobin A1c
18. Fasting blood samples drawn and not analyzed until found eligible:
    - Insulin
    - Inflammatory markers
        - MCP-1
        - IL-8
        - TNFα
        - IL-1β
        - IL-10
        - E-Selectin
        - P-Selectin

Baseline (randomization) visit (v00) is scheduled two weeks after SV to allow adequate sampling of baseline chemistry, CBC, platelet count, PT, aPTT, lipids/CRP and hemoglobin A1c. Participants will be instructed to fast for 8 hours prior to v00.

3.4 Assigning Study Identification (ID) Numbers

When a participant begins the screening process, a study ID will be assigned. This study ID will remain with the participant throughout the study, whether or not they continue into the randomization phase of the trial. All study data forms and communications with the DCC will use this study ID.

The study ID will be assigned by the ARU clinic personnel who make the SV clinic appointments by the following procedure:

When the participant appears for SV, obtain the list entitled "Nattokinase Atherothrombotic Prevention Study (NAPS) IDs."

Using the next available blank line, write in a study ID in sequence from the study ID above and the first three letters of the participant's last name and the first letter of the participant's first name and write the participant's name in the line next to the study ID.

For example, Jenny Jones is found to be potentially eligible and appears for SV at the ARU research clinic. On the " Nattokinase Atherothrombotic Prevention Study (NAPS) IDs" sheet, it is found that the last assigned study ID was 8105, assigned to Mary Smith. The blank line below would be completed as 8106JONJ and Jenny Jones written next to it. Jenny's study ID is now 8106JONJ.

Write the assigned study ID in **item 23** of the Telephone Questionnaire **[FORM 1]** and send the form to the DCC for data entry. In the example above, **item 23** of Jenny Jones' Telephone Questionnaire would be completed as 8106JONJ.

4.0
RANDOMIZATION

4.1 Visit 00 (v00)

1. Complete and review Final Eligibility Check List **[FORM 17]** and ensure that all eligibility criteria have been fulfilled before proceeding with randomization
2. Administer the Nonstudy Medications Record **[FORMS 13,14]** and confirm that participants are not taking lipid-lowering medication
3. Administer History of Vitamin and Nutritional Supplement Use Questionnaire **[FORM 15]** and confirm that participants are not taking soy, soy protein and isoflavone food supplements
4. Administer Dietary Food Frequency **[FORM 18]**
5. Administer Soy Food Questionnaire **[FORM 19]**
6. Administer 7-Day Physical Activity Recall Questionnaire **[FORM 20]**
7. Administer Center for Epidemiologic Studies Depression (CES-D) Scale **[FORM 21]**
8. Perform cognitive function assessment **[FORM 22]**
9. Obtain participants' blood pressure and pulse rate **[FORM 16]** and confirm that systolic blood pressure is $\leq$160 mmHg and diastolic blood pressure is $\leq$110 mmHg
10. Obtain and record participant's weight **[FORM 16]**
11. Measure and record waist:hip circumferences **[FORM 23]**
12. Obtain 12-lead ECG **[FORM 24]**
13. Carotid artery ultrasound examination performed (second baseline - see Section 4.2 for randomization procedure)
14. Perform urine dipstick
15. Fasting blood samples drawn and analyzed for:
    - Fibrinogen
    - vWF antigen
    - Tissue plasminogen activator (tPA)
    - Plasminogen activator inhibitor-1 (PAI-1)
    - D-dimers
    - Platelet aggregation (ADP, collagen, epinephrine)
    - Ristocetin cofactor
    - Factor VIII
    - Nattokinase
    - Anti-nattokinase antibodies
    - Erythrocyte sedimentation rate (ESR)
    - Red blood cell aggregation (plasma)
    - Red blood cell aggregability (polymer)
    - Plasma viscosity
    - Whole blood viscosity
    - Endothelial injury in-vitro assay: VCAM-1, ICAM-1
    - Monocyte activation (FACS): CD11b, CD11c, VLA-4
    - Storage
        - Plasma (EDTA) generic storage
        - Plasma (citrate) generic storage
        - Serum generic storage
        - Buffy coat
16. Active nattokinase/placebo nattokinase dispensed **[FORMS 25]**

17. Fill in the **study product ID number item** of the Eligibility Summary section on the Final Eligibility Check List **[FORM 17]** according to the randomization procedures in Section 4.2.

A follow-up clinic visit will be scheduled one week following v00. Subsequent clinic visits will be scheduled monthly (from date of randomization) for the first six months and then every three months for the remainder of the trial. Participants will be instructed to fast for 8 hours prior to each clinic visit.

4.2 Randomization Procedure

Active nattokinase and matching nattokinase placebo will be shipped directly to the DCC by Jarrow Formulas. Active nattokinase and matching nattokinase placebo will be shipped on different days separated in time by at least one week. Jarrow Formulas will clearly label the containers as active nattokinase and nattokinase placebo. Active nattokinase and matching nattokinase placebo capsules will be sealed in white plastic bottles with a quantity of 110 capsules per bottle. Each participant will be given adequate numbers of bottles of capsules for the period of time between clinic visits. The bottles of capsules will be prepared by the DCC for each randomized participant and will be dispensed at each clinic visit to each participant. The bottles of capsules will contain an attached label prepared with the following identifiers: Stratum (1,2) and a unique identification number for each bottle of capsules. The label will also contain space for the participant's name, visit number and date of dispensation to be filled in by clinic personnel when the bottle of capsules is assigned to a randomized participant.

The DCC will generate randomization lists for 2 strata with a blocking factor not identified to other study personnel:

1) Right distal common carotid arterial far wall IMT $\geq$0.75 mm
2) Right distal common carotid arterial far wall IMT <0.75 mm

As the Final Eligibility Check List **[Form 17]** is completed and a participant is found to be eligible for randomization, the clinic coordinator will do the following:

1) Using the appropriate stratum (identified in the Eligibility Summary section of the Final Eligibility Check List **[Form 17]**, refer to the study randomization log for that stratum.

2) The study randomization log contains a separate list of sequential study product ID numbers for each stratum. Note that each stratum list has a column of study product ID numbers as well as a blank column to list the participant study ID. On the study randomization log for the appropriate stratum, write the participant study ID on the next available line. Note the study product ID number.

3) Write the study product ID number in the **product ID number item** of the Eligibility Summary section on the Final Eligibility Check List **[Form 17]**.

4) From the study product storage area containing study products that have not yet been assigned to randomized participants, obtain the bottle of capsules with the same study product ID number indicated on the randomization log and written on the Final Eligibility Check List **[Form 17]**.

5) For initial randomization, the bottle of capsules will contain enough capsules to last the participant for three study months (one bottle of capsules). Write the participant's name, visit number and date of dispensation on the label of the packet to be dispensed.

6) Duplicates of the study labels will be placed on the Nattokinase/Placebo Compliance Form **[Form 25]**.

7) Send the Final Eligibility Check List **[Form 17]** to the DCC.

Upon receipt of the Final Eligibility Check List **[FORM 17]**, the DCC personnel will do the following:

1) Review form for completeness and accuracy and enter the data.

2) Run error check routine to:

   a) Confirm study eligibility.

   b) Assure that the participant has been randomized within the appropriate stratum.

   c) Inform the principal investigator and study coordinator if any of the above two protocol violations has occurred.

   d) Generate an ideal visit schedule for the participant and send it to the ARU clinic. This ideal visit schedule will include ideal clinic visits every month for the first six months and then every three months for the remainder of the trial.

   e) Generate labels for laboratory samples and distribute to clinic phlebotomist and core lipid laboratory personnel.

Only the right distal CCA far wall IMT at baseline will be used as a stratifying factor. Within each stratum, the distribution of other risk factors (specifically, LDL-C, smoking status, age and blood pressure) will be monitored across the two treatment groups. If imbalance is found across the two treatment groups, the randomization plan will be adapted to correct for this imbalance, i.e., adaptive randomization. These corrections will occur after the first 100 then 200 participants have been randomized.

4.3 Dispensing Study Products During the Trial (after Randomization)

1) At each clinic visit, record the number of capsules consumed since the last study visit on the Compliance Calculation Form **[Form 26]**. The DCC will use these data to replace the appropriate number of study products in the participant's study product storage container.

2) From the study product storage area containing study products for participants that have already been randomized, which are sorted by the participant study ID, locate the participant's stored products.

3) From the participant's storage containers, obtain the same number of bottles of capsules that were consumed since the last visit (recorded on the Compliance Calculation Form **[Form 26]**).

5.0
COMPLIANCE AND DROPOUT

5.1 Clinic Appointments

Overall compliance will be determined by the number of missed regularly scheduled clinic visits. The visit limit window will be $\pm$14 days of the actual clinic visit date. Out of window visits will also be calculated.

5.2 Medication

Compliance with active nattokinase and nattokinase placebo **[FORM 26]** will be evaluated by capsule count. Compliance will be defined as $\geq$80% of capsules consumed.

Percentage of active nattokinase and nattokinase placebo consumed will be determined by the formula:

$$\frac{TCD - TCR}{D \times A} \times 100,$$

where TCD=total number of capsules dispensed; TCR=total number of capsules returned; D=number of capsules to be taken each day; and, A=actual number of days since last visit.

5.3 Dropouts

Participants who do not want to continue to participate in the study or to take the study product will be urged to complete all clinic visits and to be followed for cardiovascular disease events as well as undergo the remainder of carotid artery ultrasound examinations and other studies including those for the secondary end points. A participant who stops taking the study product but continues to attend clinic visits and to complete ultrasound examinations and other secondary end points will be termed a "medication termination." A participant who ceases participation and does not attend clinic visits will be termed a "dropout."

All dropouts and reasons for terminating the study or terminating use of study product will be recorded on the Medication Termination/Dropout Form **[FORM 27]**.

6.0
CLINICAL EVENTS AND DEFINITIONS

6.1 Overview

Participants on trial will continue with their usual source of medical care. Laboratory abnormalities and other abnormal findings detected at the ARU Research Clinic will be immediately brought to the attention of the principal investigator and with permission from the participant, the participant's private physician will be notified by letter and/or telephone depending upon the urgency of the abnormality detected. Participants who discontinue the study product will be urged to complete all clinic visits and be followed for CVD events as well as undergo the remainder of carotid artery ultrasound scans and other studies including those for the secondary end point. All laboratory and clinical abnormalities requiring referral to or follow-up by a private physician will be recorded in the participant's clinic chart.

6.2 Cardiovascular Events

Participants who develop angina pectoris, decreased exercise tolerance, myocardial infarction, or other cardiovascular events will receive care for this from a private physician. Participants will be referred to their private physician in accordance with the standard practice of medicine but continue on the study protocol unless contraindicated. Analysis of cardiovascular events will include all randomized participants and will be based on documentation from written medical records. Documentation of myocardial infarction and unstable angina must fulfill criteria outlined below. Coronary revascularization (CABG and PTCA) will be considered a major cardiovascular clinical event. Angina pectoris will be evaluated by criteria from the Rose Questionnaire **[FORM 10]**. All cardiovascular events are reported on the Study Event Record **[FORM 28]**.

6.3 Ischemic Heart Disease Definitions

6.3.1 Myocardial Infarction

Symptoms:

Pain or discomfort occurring in the anterior chest, back, epigastrium, jaw, neck, left shoulder, elbow, forearm, or wrist, which the attending physician believes to be consistent with a myocardial infarction (MI). These symptom criteria will not apply when there is cardiovascular collapse resulting in unconsciousness or if infarction occurs under general anesthesia for non-cardiac surgery. In addition, new onset or worsening dyspnea may be a symptom of MI if it is associated with other findings of myocardial necrosis (see below).

Serum enzymes:

The following creatine kinase (CK), creatine kinase-muscle/brain (CK-MB), troponin, aspartate amino transferase (AST), or lactic acid dehydrogenase (LDH) enzyme changes are defined as diagnostic of MI if they occur independently of conditions known to be associated with elevated levels. These conditions include but are not limited to rhabdomyolysis, muscle trauma seizures and muscular dystrophy.

a) In a Q-wave MI there must be:
   1) An increase of CK two times or greater than the upper limit of normal for the laboratory where the level was determined, or
   2) A positive CK-MB fraction with a 50% rise of total CK over baseline or a 50% fall in total CK relative to peak CK of any levels occurring within 48 hours of the onset of clinical symptoms, or
   3) An increase of troponin to a level considered consistent with myocardial necrosis for the laboratory where the level is determined, or
   4) Elevations of AST or LDH three times or greater than the upper limit of normal for the laboratory where the level was determined occurring within 72 hours of the onset of clinical symptoms.

b) In a non-Q wave MI there must be:
   1) An increase of CK two times or greater than the upper limit of normal for the laboratory where the level was determined, or
   2) A positive CK-MB fraction with a 50% rise of total CK over baseline or a 50% fall in total CK relative to peak CK of any levels occurring within 48 hours of the onset of clinical symptoms.
   3) An increase of troponin to a level considered consistent with myocardial necrosis for the laboratory where the level is determined.

c) With clinical symptoms and enzyme changes only:
   1) The CK must rise two times or greater than the upper limit of normal for the laboratory where the level is determined and the CK-MB fraction must be positive, or
   2) An increase of troponin to a level considered consistent with myocardial necrosis for the laboratory where the level is determined

Electrocardiogram:

a) Q-wave MI: On a standard 12-lead ECG, new Q or QS waves with:
   a duration > 30 msec, and
   an amplitude > 0.1 mV
   demonstrated in:
   two of the three leads, II, III, or avF, or
   two adjacent leads of V1, V2, V3, V4, V5, V6, I, or avL, or growth of R waves in leads V1 or V2 with ST-T wave changes.

b) Non-Q-wave MI: On a standard 12-lead ECG, new T-wave inversions in:
   two of the three leads, II, III, avF, or
   two adjacent leads of V1, V2, V3, V4, V5, V6, I, or avL which persist for >72 hours
   Therefore, three ECGs will be required to make the diagnosis of a non-Q-wave MI:
      1) An old ECG demonstrating no T-wave inversions,
      2) An ECG showing new T-wave inversions, and
      3) An ECG taken >72 hours after development of the new T-wave inversions demonstrating persistence.

Criteria for diagnosis of MI:

A participant must have one of the following combinations of symptoms, enzyme changes and ECG changes described above to meet the criteria for the diagnosis of a MI:

a) Clinical symptoms, an ECG consistent with a Q-wave MI, and positive enzyme changes (for a Q-wave MI).
b) Clinical symptoms, an ECG consistent with a non-Q-wave MI, and positive enzyme changes (for a non-Q-wave MI).
c) With a negative ECG, clinical symptoms and positive enzyme changes (described above under serum enzymes, part c).
d) With no symptoms and no enzyme changes, a Q-wave MI is confirmed if Q or QS waves meeting the ECG criteria above appear on serial ECGs. (In the absence of symptoms and enzyme changes, a non-Q-wave MI cannot be made).
e) With no enzyme changes, clinical symptoms and Q or QS waves meeting the ECG criteria above for a Q-wave MI appear on serial ECGs.
f) With no enzyme changes, clinical symptoms and T-wave inversions meeting the ECG criteria above for a non-Q-wave MI appear on serial ECGs.

All non-fatal myocardial infarctions will be confirmed and recorded on the Confirmation of New Non-fatal Myocardial Infarction Form **[FORM 29]** as well as reported on the Study Event Record **[FORM 28]**.

6.3.2 Unstable Angina/Chest Pain

Transient ECG changes consisting of S-T segment elevation, S-T depression, T-wave changes, or a combination of these, must be documented during episodes of clinical symptoms as described above. These abnormalities must revert toward the control pattern after relief of pain and within 24 hours. There must be no ECG evidence of evolving MI (i.e., pathologic Q or QS waves). MI must be ruled out by serial serum enzyme studies.

Hospitalization for chest pain or other clinical symptoms considered to be anginal equivalents but not meeting the above criteria for unstable angina will be considered chest pain.

All unstable angina and chest pain events will be confirmed and recorded on the Confirmation of Unstable Angina/Chest Pain Form **[FORM 30]** as well as reported on the Study Event Record **[FORM 28]**.

6.4 Cerebrovascular Events

Transient ischemic attacks, reversible ischemic neurologic deficits, and cerebrovascular accidents will be documented from written medical records and must fulfill the criteria outlined below. Subjects with these cerebrovascular events will continue on the study protocol unless contraindicated. Analysis of cerebrovascular events will include all randomized subjects.

All cerebrovascular events are reported on the Study Event Record **[FORM 28]**.

6.5 Cerebrovascular Disease Definitions

Occurrence of transient ischemic attacks, reversible ischemic neurologic deficits, and cerebrovascular accidents will be documented by written medical records and must fulfill the following criteria:

6.5.1 Transient Ischemic Attack

A sudden focal neurological deficit that completely resolves within 24 hours.

All transient ischemic attack events will be confirmed and recorded on the Confirmation of Transient Ischemic Attack Form **[FORM 31]** as well as reported on the Study Event Record **[FORM 28]**.

6.5.2 Reversible Ischemic Neurologic Deficit

A focal neurological deficit that persists for more than 24 hours but in which complete neurological recovery occurs within 2 weeks.

All reversible ischemic neurologic deficit events will be confirmed and recorded on the Confirmation of Reversible Ischemic Neurologic Deficit Form **[FORM 32]** as well as reported on the Study Event Record **[FORM 28]**.

6.5.3 Cerebrovascular Accident

A focal neurological deficit that permanently persists after the acute event.

All cerebrovascular accident events will be confirmed and recorded on the Confirmation of Cerebrovascular Accident Form **[FORM 33]** as well as reported on the Study Event Record **[FORM 28]**.

6.6 Peripheral Vascular Events

Aortic aneurysms and peripheral vascular disease requiring surgical or intravascular intervention will be considered major events documented by written medical records. Claudication will be considered a symptom and evaluated by criteria defined below. Participants will continue on study protocol unless health care requirements for these events preclude this. Analysis of major peripheral vascular events will include all randomized participants.

All peripheral vascular disease events requiring surgical or intravascular intervention will be confirmed and recorded on the Confirmation of Peripheral Vascular Disease Form **[FORM 34]** as well as reported on the Study Event Record **[FORM 28]**.

6.7 Peripheral Vascular Disease Definitions

Aortic aneurysms and peripheral vascular disease requiring surgical or revascularization intervention will be considered proof of disease. Participants will be considered to have claudication if they have reproducible pain or weakness in the calf or foot after walking a fixed distance if the pain subsides within a few minutes after stopping **[FORM 11]**.

6.8 Cancer Events

The occurrence of cancer will be documented by medical records and pathology reports. Participants will continue in the study unless contraindicated. Analysis of cancer events will include all randomized participants.

All cancer events will be confirmed and recorded on the Confirmation of Cancer Form **[FORM 35]** as well as reported on the Study Event Record **[FORM 28]**.

6.9 Deep Venous Thrombotic and Pulmonary Thromboembolic Events

Occurrence of deep vein thrombosis and pulmonary thromboembolism will be documented by written medical records and must fulfill the criteria outlined below. Participants with deep venous thrombotic and pulmonary thromboembolic events will continue on the study protocol unless contraindicated. Analysis of deep venous thrombotic and pulmonary thromboembolic events will include all randomized participants.

6.9.1 Deep Vein Thrombosis Definition

Deep vein thrombosis is frequently present in the absence of the clinical signs of pain, heat, erythema, and swelling, and it is absent in 50 percent of individuals in whom clinical signs and symptoms suggest its presence. Therefore, the diagnosis of deep vein thrombosis will be based on specific diagnostic tests. These diagnostic tests include but are not limited to the following: 1) Impedance plethysmography; 2) Doppler flow; 3) Radiofibrinogen methods; 4) ^{111}Indium labeled platelets; and, 5) Ascending contrast venography.

All deep vein thrombotic events will be confirmed and recorded on the Confirmation of Deep Vein Thrombosis Form **[FORM 36]** as well as reported on the Study Event Record **[FORM 28]**.

6.9.2 Pulmonary Thromboembolism Definition

As with deep vein thrombosis, the clinical signs and symptoms of pulmonary thromboembolism are highly variable and usually are not solid enough to conclusively make a definitive diagnosis. Therefore, the diagnosis of pulmonary thromboembolism will be based on specific imaging tests, including but not limited to the following: 1) Pulmonary perfusion and ventilation scintiphotography; and, 2) Pulmonary angiography.

All pulmonary thromboembolic events will be confirmed and recorded on the Confirmation of Pulmonary Thromboembolism Form **[FORM 37]** as well as reported on the Study Event Record **[FORM 28]**.

6.10 Death

The occurrence and cause of death will be documented by written medical records, if available, and death certificates. All deaths will be confirmed and recorded on the Confirmation of Death Form **[FORM 38]** as well as reported on the Study Event Record **[FORM 28]**.

6.11 Follow-up

At annual on-trial follow-up visits (v12, v24, v36), medical history will be obtained to determine changes from baseline health status **[FORM 39]**. Any on-trial changes from baseline health status not previously reported will be reported on the Study Event Record **[FORM 28]** and other appropriate reporting forms described in the preceding sections.

Following the last scheduled study visit, participants will be enrolled into a follow-up registry for annual contact and evaluation. The evaluation will include collection of historical data on major health events.

Participants will complete the Closeout Form **[FORM 40]** at their last clinic visit.

6.12 Adverse Event Reporting

6.12.1 Reporting to the USC Institutional Review Board

The report of a serious adverse event will be submitted, along with any other relevant information to the Institutional Review Board (IRB) promptly and no later than five (5) business days following the time that it becomes known that the subject suffered the unexpected adverse event.

If an adverse event proves fatal, the IRB will be notified within 24 hours.

Any unexpected adverse event that is related or possibly related to the intervention of this trial will be reported regardless of whether or not the adverse event is serious. The Report of the Adverse Event Form will be completed by the study coordinator and the adverse event discussed with the principal investigator who will sign prior to submission. Any unrelated adverse event or non-serious adverse event judged the result of progression of the disease will not be reported.

6.12.2 Reporting to the External Data Safety Monitoring Board

The DCC will generate a safety report enumerating all clinical events and serious and non-serious adverse events for the EDSMB, both on a cumulative basis (from the start of the study to present), and new events in the past year (see Section 9.5.2.4). Events will be presented by blinded study group.

6.13 Medical Records

Medical records will be requested for confirmation of all events.

7.0
LABORATORY PROTOCOL

7.1 Blood Specimen Collection

Participants fast prior to each clinic visit for a minimum of eight hours (nothing by mouth except for water and medications). With the participant in a seated position, blood is collected by a phlebotomist from an antecubital vein using a tourniquet in a standardized manner. After the participant has been seated for 10 minutes, the tourniquet is snugly applied to the upper arm, and the blood collected into vacutainer tubes. Once blood flow commences, the tourniquet is immediately removed to avoid prolonged occlusion of the vein. Blood is collected into 10 ml and 4 ml EDTA (1.5 mg/ml wet EDTA), 5 ml Li-heparin, 3.5 ml Na-citrate and 3.5 ml dry (with separator) vacutainer tubes depending upon the blood test required (Section 7.3). Blood tubes are inverted several times to thoroughly mix with the EDTA, Li-heparin, and Na-citrate and provided as whole blood or immediately centrifuged at 4^{o}C or room temperature to separate the plasma. Serum that is collected in separator tubes sit at room temperature for 30 minutes prior to centrifugation. Plasma and serum are collected by low-speed centrifugation (1000 x g) for 10 minutes at 4^{o}C or room temperature. All tubes are labeled with the participant initials, participant identification number, visit number and visit date (Section 7.2).

In addition, 3.6 ml of extra plasma (2.4 ml of EDTA plasma and 1.2 ml of citrated plasma) and 1.2 ml of extra serum is collected at baseline and at every on-trial clinic visit, prepared as described above, and stored at -80^{o}C for future studies. The plasma and serum samples are divided into 1.2 ml aliquots and stored at -80^{o}C in Corning cryogenic vials with screw top caps. Plasma is stored for future measurements of apolipoproteins, lipoprotein particles, Lp(a), homocysteine, nitric oxide and coagulation factors. Serum is stored for future measurements of hormones (estradiol, estrone, estrone-sulfate, testosterone, progesterone, sex hormone binding globulin and prolactin) and bone markers (bone-specific alkaline phosphatase, osteocalcin, osteoprotegrin and RANKL). The buffy coat (white blood cells) is collected at baseline and four times on-trial from EDTA plasma and stored at -80^{o}C for future DNA studies. Urine for dipstick is collected at baseline and 1 month after randomization.

7.2 Labeling, Shipment and Tracking of Samples

All samples from the Atherosclerosis Research Unit Clinic are taken directly to the Atherosclerosis Research Core Lipid Laboratory on the same day they are collected from participants. The Core Lipid Laboratory serves as the central receiving point for all samples **[EXHIBIT A]**. Samples are sent to the Core Lipid Laboratory with a Laboratory Requisition Form **[EXHIBIT B]** indicating the laboratory studies to be performed as well as the samples to be stored at -80^{o}C.

Samples received by the Core Lipid Laboratory are given a laboratory sample identification number and logged into a central log book. In addition, the participant initials, participant identification number, date of sample collection, visit number, study name (NAPS) and the type of analyses to be performed are entered into the central log book. If a sample is to be stored at -80^{o}C it is written in the log book as "stored" along with the aforementioned information.

The Laboratory Requisition Form **[EXHIBIT B]** as well as the visit number and the table in Section 7.6 are used by the Core Lipid Laboratory personnel to determine tests to be performed, samples to be stored or samples to be shipped elsewhere **[EXHIBIT A]**. **Clinic staff resolves questions or discrepancies that arise concerning sample collection.**

Samples to be stored are transferred quantitatively to Corning cryogenic vials with screw top caps. Each vial is labeled with the Core Lipid Laboratory sample identification number, participant initials, participant identification number, date of sample collection, visit number, study name (NAPS) and the vial contents (Plasma + EDTA (P+E), Plasma + CITRATE (P+C), Serum (S) and Buffy Coat). The buffy coat collected at v04 is labeled as described above, identified as “Buffy Coat/APOE” and stored for determination of apolipoprotein E genotypes.

For shipment of samples to outside laboratories (Section 7.4), the same sample labeling and log procedure as described above is followed. Samples are hand-delivered or picked up by each respective laboratory. Results of laboratory tests with participant identification number, laboratory sample ID number, visit number and sample date is forwarded to the DCC by electronic transmission by each respective laboratory for data acquisition and management (Section 9.0).

7.3 Sample Collection Conditions

LAB	SAMPLE	TUBE	SIZE	CONDITION	FORM
QUEST	Chemistry Panel	GOLD	3.5 ml	RT	Serum
	Insulin	GREEN	5 ml	4°C	Plasma
	Complete Blood Count	PURPLE	4 ml	RT	Whole Blood
	Platelet Count				
	Prothrombin Time (PT)	BLUE	3.5 ml	RT	
	Activated Partial Thromboplastin Time (aPTT)				
	Fibrinogen				
HEMATOLOGY LAB	vWF Antigen	BLUE + INHIBITOR	3.5 ml	RT	Whole Blood
	Tissue Plasminogen Activator (tPA)				
	Plasminogen Activator Inhibitor-1 (PAI-1)				
	D-Dimers				
	Platelet Aggregation (ADP, Collagen, Epinephrine)	BLUE	3.5 ml	RT	
	Ristocetin Cofactor				
	Factor VIII				
	Nattokinase				
	Anti-Nattokinase Antibodies				
RHEOLOGY LAB	Erythrocyte Sedimentation Rate (ESR)	PURPLE	10 ml	RT	Whole Blood
	Red Blood Cell Aggregation (Plasma)				
	Red Blood Cell Aggregability (Polymer)				
	Plasma Viscosity				
	Whole Blood Viscosity				
BIOCHEMISTRY LAB	MCP-1	PURPLE	10 ml	4°C	Plasma
	IL-8				
	TNFα				
	IL-1β				
	IL-10				
	E-Selectin				
	P-Selectin				
	Endothelial injury in-vitro assay: VCAM-1, ICAM-1	PURPLE	10 ml	RT	Whole Blood
	Monocyte Activation (FACS): CD11b, CD11c, VLA-4				
CORE LIPID LAB	Lipids/C-Reactive Protein (CRP)	PURPLE	10 ml	4°C	Plasma
	Hemoglobin A1c	PURPLE	4 ml	RT	Whole Blood
	Storage	PURPLE	10 ml	4°C	Plasma
		BLUE	3.5 ml		Plasma
		GOLD	5 ml		Serum

7.4 Specific Sample Delivery Instructions and Protocol

7.4.1 Quest Laboratory

A. Chemistry panel, insulin, complete blood count with platelet count, prothrombin time, activated partial thromboplastin time, fibrinogen

1. 1.2 ml serum is collected in a 3.5 ml dry vacutainer tube containing a separator (gold top) at room temperature along with 4 ml of whole blood collected in a 4 ml EDTA (purple top) containing tube at room temperature.

2. 2.5 ml plasma is collected in a 5 ml vacutainer tube containing Li-heparin (green top) at 4^{o}C.

3. Along with the samples collected above, 3.5 ml, 7.0 ml or no whole blood is collected in one or two 3.5 ml Na-citrate (blue top) containing tubes, respectively at room temperature depending on the visit.

4. The gold, green, purple and blue top tubes are labeled with participant name, date of sample collection, visit number, study name (NAPS) and the laboratory test to be performed: the gold tube is labeled "Chemistry," the green top is labeled "Insulin," the EDTA (purple top) containing tube is labeled "CBC" and one 3.5 Na-citrate (blue top) containing tube is labeled "PT/aPTT" and one 3.5 Na-citrate (blue top) containing tube is labeled "Fibrinogen."

5. The Quest Laboratory Requisition forms are completed for each appropriate laboratory test.

6. The collection tubes and the Quest Laboratory Requisition forms are delivered to the Core Lipid Laboratory, assigned sample ID numbers, logged into the system and the Quest courier is notified for same day pick up of samples.

B. Delivery of samples to Quest Laboratory

Contact information:

Telephone: (818) 737-8347

7.4.2 Hematology Laboratory – Dr. Howard Liebman

A. Coagulation Factors (vWF antigen, tissue plasminogen activator, plasminogen activator inhibitor-1, D-dimers) and Heme Factors (platelet aggregation (ADP, collagen, epinephrine), ristocetin cofactor, factor VIII, plasma nattokinase levels and anti-nattokinase antibodies)

1. 14 ml of whole blood is collected in one 3.5 Na-citrate (blue top) + inhibitors containing tube (prepared by Hematology Laboratory for coagulation factors) and three 3.5 ml Na-citrate (blue top) containing tubes at room temperature.

2. The four 3.5 ml blue top tubes are labeled with participant initials, participant identification number, date of sample collection, visit number, study name (NAPS) and the panel analytes to be performed; the one 3.5 Na-citrate (blue top) + inhibitors tube is labeled "Coagulation" and the three 3.5 ml Na-citrate (blue top) containing tubes are labeled "Heme/+Platelet."

3. The four 3.5 ml blue top tubes are immediately delivered to the Hematology Laboratory under the Directorship of Dr. Howard Liebman.

B. Coagulation Factors and Heme Factors without Platelet Aggregation Studies

1. For the visits that do not require the platelet aggregation studies, 10.5 ml of whole blood is collected in one 3.5 Na-citrate (blue top) + inhibitors containing tubes (prepared by Hematology Laboratory) and two 3.5 ml Na-citrate (blue top) containing tubes at room temperature.

2. The three 3.5 ml blue top tubes are labeled with participant initials, participant identification number, date of sample collection, visit number, study name (NAPS) and the panel analytes to be performed; the one 3.5 Na-citrate (blue top) + inhibitors tube is labeled "Coagulation" and the two 3.5 ml Na-citrate (blue top) containing tubes are labeled "Heme/–Platelet."

3. The three 3.5 ml blue top tubes are immediately delivered to the Hematology Laboratory under the Directorship of Dr. Howard Liebman.

C. Nattokinase Plasma Levels (when not collected as part of the Coagulation and Heme Factors)

1. 3.5 ml of whole blood is collected in a 3.5 ml Na-citrate (blue top) containing tube at room temperature.

2. The 3.5 ml blue top tube is labeled with participant initials, participant identification number, date of sample collection, visit number, study name (NAPS) and labeled with the type of analysis to be performed "Nattokinase."

3. The 3.5 ml blue top tube is immediately delivered to the Hematology Laboratory under the Directorship of Dr. Howard Liebman.

D. Delivery of Samples to Hematology Laboratory

Laboratory address:

Howard A. Liebman, M.D.
USC/Norris Comprehensive Cancer Center and Hospital
1441 Eastlake Avenue
Topping Tower Building, Room NOR 3466
Los Angeles, CA 90033

Telephone: (323) 865-3950
Mobile: (323) 980-0820
Email: Liebman_h@med.usc.edu

Contact personnel:

Leanne Rochanda
Keck School of Medicine
1441 Eastlake Avenue
Ezralow Tower Building, Room NOR 5338A
Los Angeles, CA 90033

Telephone: (323) 865-0965
Mobile: (925) 708 1471
Email: rochanda@usc.edu

7.4.3 Rheology Laboratory – Dr. Herbert Meiselman

A. Rheology Measures (erythrocyte sedimentation rate, red blood cell aggregation, red blood cell aggregability, plasma viscosity, whole blood viscosity)

1. 10 ml of whole blood is collected in a 10 ml EDTA (purple top) containing tube at room temperature.

2. The 10 ml purple top tube is labeled with participant initials, participant identification number, date of sample collection, visit number, study name (NAPS) and labeled with the panel of analytes to be performed "Viscosity."

3. The 10 ml purple top tube is immediately delivered to the Rheology Laboratory under the Directorship of Dr. Herbert Meiselman.

B. Delivery of samples to Rheology Laboratory

Laboratory address:

Herbert J. Meiselman, Sc.D.
Keck School of Medicine
1333 San Pablo Street
Mudd Building, Room 120
Los Angeles, CA 90033

Telephone: (323) 442-1268 or (323) 442-1267
Email: hmeiselman@gmail.com or meiselman@usc.edu

Contact personnel:

Rose Wenby
Keck School of Medicine
1333 San Pablo Street
Mudd Building, Room 120
Los Angeles, CA 90033

Telephone: (323) 442-1268 or (323) 442-1267
Email: rwenby@usc.edu

7.4.4 Biochemistry Laboratory – Dr. Vijay Kalra

A. Inflammatory Markers (MCP-1, IL-8, TNFα, IL-1β, IL-10, E-selectin, P-selectin)

At screening visit:

1. 5 ml of plasma is collected in a 10 ml EDTA (purple top) containing tube at 4°C.

2. The plasma sample (5 ml) is divided into 2 aliquots. The first aliquot of 2.5 ml is transferred to a 3.0 ml screw-capped plastic cryogenic tube that can withstand the temperature of -80°C. The tube is labeled "Inflammatory." The second aliquot of 2.5 ml is used to analyze lipid levels and C-Reactive Protein (CRP) in the Core Lipid Laboratory.

At on-trial visits:

1. 5 ml of plasma is collected in a 10 ml EDTA (purple top) containing tube at 4°C.

2. The plasma sample (5 ml) is divided into 2 equal aliquots and a buffy coat collected from the EDTA sample. The first aliquot of 2.5 ml is transferred to a 3.0 ml screw-capped plastic cryogenic tube that can withstand the temperature of -80°C. The tube is labeled "Inflammatory." The second aliquot of 2.5 ml is divided equally and transferred to two 2.0 ml screw-capped plastic cryogenic tubes that can withstand the temperature of -80°C. The tubes are labeled "EDTA Plasma" and stored in the Core Lipid Laboratory. The buffy coat is collected from the EDTA plasma sample and transferred to a 2.0 ml screw-capped plastic cryogenic tube that can withstand the temperature of -80°C. The tube is labeled "Buffy Coat" and stored in the Core Lipid Laboratory.

At screening visit and on-trial visits:

3. The screw-capped plastic cryogenic tubes are labeled with participant initials, participant identification number, date of sample collection, visit number, study name (NAPS) and the panel of analytes to be performed (Inflammatory). Water-insoluble ink is used and the labels protected by wrapping them with transparent tape.

4. The screw-capped plastic cryogenic tubes are immediately frozen at -80ºC until delivery to the Biochemistry Laboratory under the Directorship of Dr. Vijay Kalra.

B. In-Vitro Studies: Endothelial Injury In-Vitro Assay: VCAM-1, ICAM-1 and Monocyte Activation (FACS): CD11b, CD11c, VLA-4

1. 10 ml of <u>whole blood</u> is collected in a 10 ml EDTA (purple top) containing tube at room temperature.

2. The 10 ml purple top tube is labeled with participant initials, participant identification number, date of sample collection, visit number, study name (NAPS) and labeled with the type of analysis to be performed "In-Vitro."

3. The 10 ml purple top tube is immediately shipped to the Biochemistry Laboratory under the Directorship of Dr. Vijay Kalra.

C. Delivery of Samples to Biochemistry Laboratory

Laboratory address:

Vijay Kalra, Ph.D.
Keck School of Medicine
2011 Zonal Avenue
Hoffman Medical Research Building (HMR), Room 611 / 613
Los Angeles, CA 90033

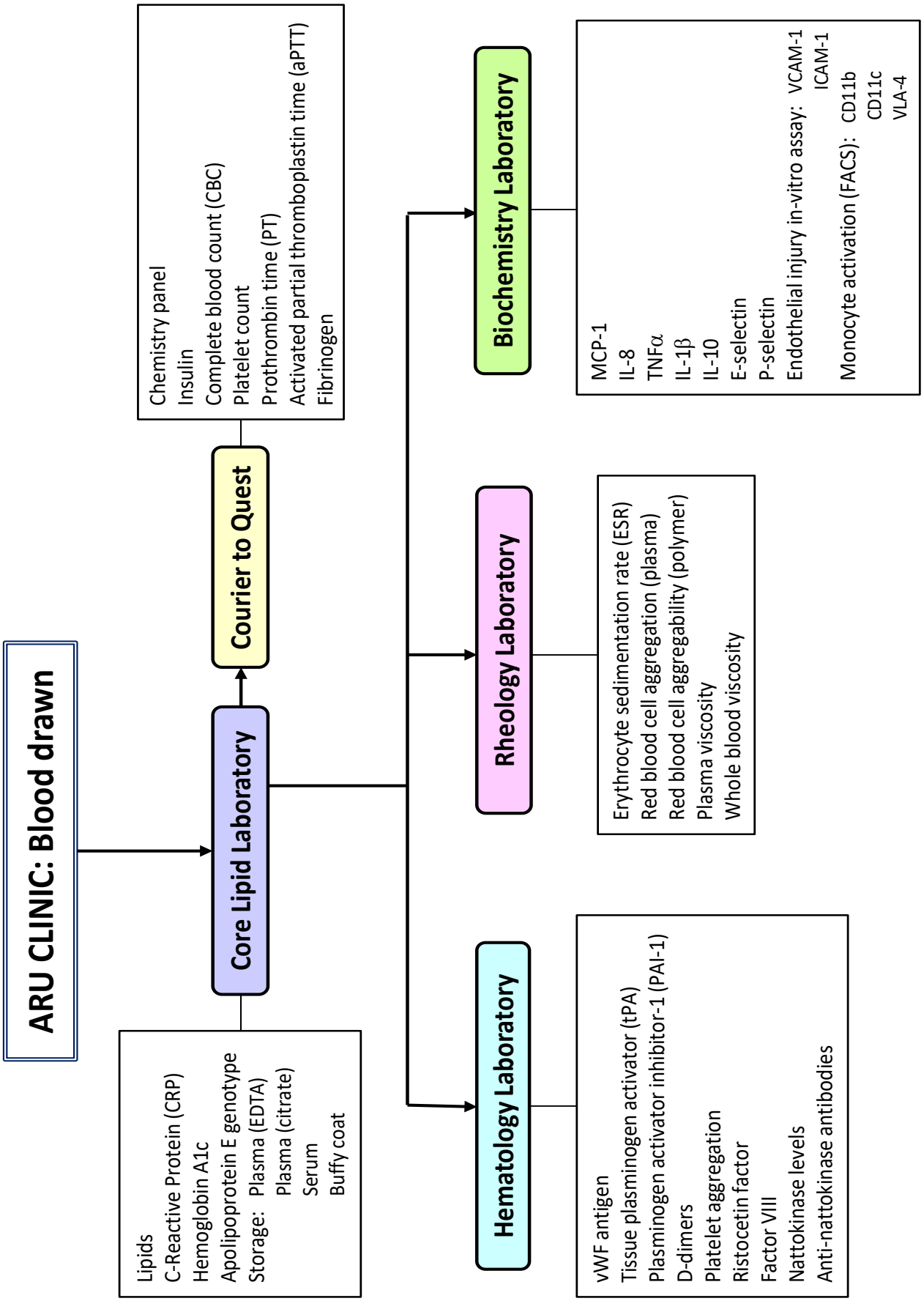
Telephone: (323) 442-1526
Laboratory: (323) 442-1528
Mobile: (626) 388-5924
Email: vkalra@usc.edu

Contact personnel:

Caryn Gonsalves, Ph.D.
Keck School of Medicine
2011 Zonal Avenue
Hoffman Medical Research Building (HMR), Room 611 / 613
Los Angeles, CA 90033

Telephone: (323) 442-1528
Mobile: (626) 353-8232

7.5 Schematic of Blood Sample Flow



7.6 Sample Flow Schedule of Biochemical Safety and Laboratory Tests

LAB	RESEARCH ACTIVITY	SCREEN	BASELINE	WEEK 1	01	02	03	04	05	06	09	12	15	18	21	24	27	30	33	36
QUEST	Chemistry Panel[1]	x			x	x	x	x	x	x		x		x		x		x		x
	Insulin[2]	x					x			x		x		x		x		x		x
	Complete Blood Count[3]	x			x	x	x	x	x	x		x		x		x		x		x
	Platelet Count[3]	x			x	x	x	x	x	x		x		x		x		x		x
	Prothrombin Time (PT)[4]	x			x		x			x		x		x		x		x		x
	Activated Partial Thromboplastin Time (aPTT)[4]	x			x		x			x		x		x		x		x		x
	Fibrinogen[4]		x	x	x					x		x		x		x		x		x
HEMATOLOGY LAB	vWF Antigen[5]		x	x	x					x				x				x		
	Tissue Plasminogen Activator (tPA)[5]		x	x	x					x				x				x		
	Plasminogen Activator Inhibitor-1 (PAI-1)[5]		x	x	x					x				x				x		
	D-Dimers[5]		x	x	x					x				x				x		
	Platelet Aggregation (ADT, Collagen, Epinephrine)[4]		x		x					x										
	Ristocetin Cofactor[4]		x	x	x					x				x				x		
	Factor VIII[4]		x	x	x					x				x				x		
	Nattokinase[4]		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Anti-Nattokinase Antibodies[4]		x		x					x				x				x		
RHEOLOGY LAB	Erythrocyte Sedimentation Rate (ESR)[3]		x	x	x					x				x				x		
	Red Blood Cell Aggregation (Plasma)[3]		x	x	x					x				x				x		
	Red Blood Cell Aggregability (Polymer)[3]		x	x	x					x				x				x		
	Plasma Viscosity[3]		x	x	x					x				x				x		
	Whole Blood Viscosity[3]		x	x	x					x				x				x		
BIOCHEMISTRY LAB	MCP-1[6]	x				x					x				x				x	
	IL-8[6]	x				x					x				x				x	
	TNFα[6]	x				x					x				x				x	
	IL-1β[6]	x				x					x				x				x	
	IL-10[6]	x				x					x				x				x	
	E-Selectin[6]	x				x					x				x				x	
	P-Selectin[6]	x				x					x				x				x	
	Endothelial injury in-vitro assay: VCAM-1, ICAM-1[3]		x			x					x				x				x	
	Monocyte Activation (FACS): CD11b, CD11c, VLA-4[3]		x			x					x				x				x	
CORE LIPID LAB	Lipids/C-Reactive Protein (CRP)[6]	x								x		x		x		x		x		x
	Hemoglobin A1c[3]	x					x			x		x		x		x		x		x
	Storage[1,6,7]		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Buffy Coat		x			x					x				x				x	
	ApoE Genotype							x												
	Urine Dipstick		x		x															

[1] Collect serum in dry gold top tubes with separator
[2] Collect plasma in Li-heparin (green top) tube
[3] Collect whole blood in EDTA (purple top) tube
[4] Collect whole blood in Na-citrate (blue top) tube
[5] Collect whole blood in special Na-citrate (blue top) tube with inhibitors supplied by Heme Lab
[6] Collect plasma in EDTA (purple top) tube
[7] Collect plasma in in Na-citrate (blue top) tube

7.7 ARU Clinic Sample Flow

Samples conducted by the ARU clinic

1. Urine is collected for urine dipstick.

Samples delivered to Core Lipid Laboratory from the ARU clinic **[EXHIBIT A]**

A. Samples prepared by ARU clinic for delivery Quest Laboratory

1. Serum for chemistry panel, plasma for insulin and whole blood for CBC and platelet count and whole blood for prothrombin (PT) and activated partial thromboplastin time (aPTT) and fibrinogen are labeled by ARU personnel with participant name, visit number, date, type of analysis to be performed and study name (NAPS).

2. The Quest laboratory requisition form is completed by ARU personnel and accompanies the samples.

B. Samples to be internally processed

Whole Blood Samples

1. Whole blood is collected in "bulk" and the collection tubes appropriately labeled by ARU personnel indicating to the Core Lipid Laboratory personnel what panel of analytes are to be determined. In addition, the labels contain participant initials, participant identification number, visit number, date and study name (NAPS).

2. The Laboratory Requisition Form **[EXHIBIT B]** accompanies the whole blood samples indicating to the Core Lipid Laboratory personnel which panel of analytes are to be determined (Section 7.5).

Plasma and Serum Samples

1. Plasma is collected in "bulk" and the tubes appropriately labeled by ARU personnel indicating to the Core Lipid Laboratory personnel that the sample is plasma + EDTA (Plasma) and plasma + citrate (Citrated Plasma). In addition, labels contain participant initials, participant identification number, visit number, date and study name (NAPS). Plasma + EDTA samples are prepared in a cold environment (4^{o}C) or room temperature; plasma + citrate samples are prepared at room temperature.

2. The Laboratory Requisition Form **[EXHIBIT B]** accompanies the plasma samples indicating to the Core Lipid Laboratory personnel which immediate determinations are to be performed with the plasma and indicating that the remaining sample is plasma storage (Section 7.5).

3. Serum is collected in "bulk" and the tubes appropriately labeled by ARU personnel indicating to the Core Lipid Laboratory personnel that the sample is SERUM. In addition, the labels contain participant initials, participant identification number, visit number, date and study name (NAPS). The serum sample is prepared at room temperature.

4. The Laboratory Requisition Form **[EXHIBIT B]** accompanies the serum samples indicating to the Core Lipid Laboratory personnel that the sample is serum storage (see Section 7.5).

7.8 Core Lipid Laboratory Sample Flow [EXHIBIT A]

1. Samples received from the ARU clinic are logged in and assigned a laboratory ID number by the Core Lipid Laboratory personnel as described in Section 7.2.

2. Serum for chemistry panel, plasma for insulin and whole blood for CBC and platelet count and whole blood for prothrombin (PT) and activated partial thromboplastin time (aPTT) and fibrinogen are accompanied by the Quest laboratory requisition form completed by ARU personnel. The samples are logged in as above, packaged in plastic bags (samples and Quest requisition form for each participant) and placed in the Quest pick up box. Samples are delivered to the Quest laboratory on a daily basis by a Quest messenger.

3. Collection tubes containing whole blood are accompanied with the Laboratory Requisition Form **[EXHIBIT B]** indicating to the Core Lipid Laboratory personnel what panel of analytes are to be determined and to which laboratory samples are to be shipped. The Core Lipid Laboratory personnel deliver samples to the appropriate laboratories (see Section 7.5).

4. Type of determinations to be performed on "bulk" samples and whether samples are for storage (plasma + EDTA, plasma + citrate, serum) is indicated on the Laboratory Requisition Form **[EXHIBIT B]**.

5. "Bulk" plasma samples drawn for lipids/CRP (plasma + EDTA) are aliquoted, labeled with participant initials, participant identification number, laboratory ID number, date, visit number, study name (NAPS) and labeled with type of analysis to be performed by the Core Lipid Laboratory personnel “Lipids/CRP.” Lipid and CRP determinations are performed by Core Lipid Laboratory personnel.

6. Collection tubes (plasma + EDTA) containing whole blood drawn for hemoglobin A1c are labeled with participant initials, participant identification number, laboratory ID number, date, visit number, study name (NAPS) and labeled with type of analysis to be performed by the Core Lipid Laboratory personnel “Hemoglobin A1c.” Hemoglobin A1c determinations are performed by Core Lipid Laboratory personnel.

7. The buffy coat is prepared by Core Lipid Laboratory personnel from EDTA plasma and transferred into a cryogenic tube, labeled with participant initials, participant identification number, visit number, date, study name (NAPS) and the words "Buffy Coat" and stored at -80^{o}C as outlined in the next section.

8. The buffy coat collected at v04 is identified for apolipoprotein E genotype and stored at -80^{o}C as outlined in the next section.

9. Plasma + EDTA, plasma + citrate and serum identified for storage at -80^{o}C follow the protocol outlined in the next section.

7.9 Sample Storage

1. The following -80^{o}C sample storage is proposed: 1) 2 cryotubes of 1.2 ml plasma + EDTA; 2) 1 cryotube of 1.2 ml plasma + citrate; 3) 1 cryotube of 1.2 ml serum; and, 4) buffy coats.
2. Plasma and serum storage samples are collected at baseline and at every clinic visit for a total of 18 collections each.

3. Buffy coats are collected at the baseline visit and at on-trial clinic visits with preparation of "bulk" plasma for sample determination of inflammatory markers and storage for a total of 5 collections.

4. Plasma + EDTA received by the Core Lipid Laboratory for storage is placed into 2 cryogenic tubes with 2 ml capacity in 1.2 ml aliquots. The cryogenic tubes are labeled by the Core Lipid Laboratory personnel with a cryogenic label containing participant initials, participant identification number, laboratory ID number, date, visit number, study name (NAPS) and type of sample "EDTA Plasma."

5. Plasma + citrate received by the Core Lipid Laboratory for storage is placed into 1 cryogenic tube with 2 ml capacity in a 1.2 ml aliquot. The cryogenic tube is labeled by the Core Lipid Laboratory personnel with a cryogenic label containing participant initials, participant identification number, laboratory ID number, date, visit number, study name (NAPS) and type of sample "EDTA Plasma."

6. Serum received by the Core Lipid Laboratory for storage is placed into 1 cryogenic tube with 2 ml capacity in a 1.2 ml aliquot. The cryogenic tube is labeled by the Core Lipid Laboratory personnel with a cryogenic label containing participant initials, participant identification number, laboratory ID number, date, visit number, study name (NAPS) and type of sample "Serum."

7. The buffy coat is labeled with participant initials, participant identification number, visit number, date, study name (NAPS) and labeled "Buffy Coat" and stored at -80^{o}C.

8. The buffy coat collected at v04 is identified for apolipoprotein E genotype, labeled with participant initials, participant identification number, laboratory ID number, date, visit number, study name (NAPS) and type of analysis to be performed (Buffy Coat/APOE). The sample is stored at -80^{o}C and delivered to Dr. Hooman Allayee's laboratory for determination of apolipoprotein E genotypes.

7.10 Quality Control Procedures

The Core Lipid Laboratory (Dr. Hwang) is a CLIA certified laboratory. The Core Lipid Laboratory is also enrolled in the Centers for Disease Control (CDC) lipid standardization program. The CDC sends reference plasma samples each quarter to the laboratory that is then analyzed for total cholesterol, total triglyceride and high density lipoprotein levels, the results of which are sent back to the CDC. The CDC then notifies the laboratory of the performance relative to the standard. A record of the results for each analyte is kept. For special laboratory determinations analyses of variance and standards have been established. Procedures for following variability and precision of laboratory determinations are given in Section 9.0.

7.11 Laboratory Determinations

7.11.1 Chemistry Panel

1) albumin
2) albumin/globulin ratio
3) alkaline phosphatase
4) ALT (SGPT)
5) AST (SGOT)
6) bicarbonate
7) BUN
8) BUN/creatinine ratio
9) calcium
12) GGTP
13) glucose
14) iron
15) LDH
16) phosphorous
17) potassium
18) sodium
19) total bilirubin
20) total protein

10) chloride
11) creatinine
21) uric acid

Blood chemistries are determined by standard analytical methods by Quest in Los Angeles California. Chemistry results are sent to the ARU clinic; clinic staff will send a photocopy of the results to the DCC for data entry.

7.11.2 Insulin

Plasma insulin levels are determined by standard analytical methods by Quest in Los Angeles California. Plasma insulin results are sent to the ARU clinic; clinic staff will send a photocopy of the results to the DCC for data entry.

7.11.3 Complete Blood Count and Platelet Count

1) WBC
2) RBC
3) hemoglobin
4) hematocrit
5) MCV
6) MCH
7) MCHC
8) RDW
9) platelet count
10) manual differential

Complete blood count along with platelet count is determined by standard analytical methods by Quest in Los Angeles California. Complete blood count results are sent to the ARU clinic; clinic staff will send a photocopy of the results to the DCC for data entry.

7.11.4 Prothrombin time (PT) and Activated Partial Thromboplastin Time (aPTT)

PT and aPTT are determined by standard analytical methods by Quest in Los Angeles California. PT and aPTT results are sent to the ARU clinic; clinic staff will send a photocopy of the results to the DCC for data entry.

7.11.5 Fibrinogen

Fibrinogen is determined by standard analytical methods by Quest in Los Angeles California. Fibrinogen results are sent to the ARU clinic; clinic staff will send a photocopy of the results to the DCC for data entry.

7.11.6 vWF Antigen

VWF antigen is measured by ELISA with a pooled normal plasma standard curve. Results are reported as a percentage of the normal pool (1,2).

7.11.7 Tissue Plasminogen Activator (tPA)

tPA in plasma is measured by ELISA (3).

7.11.8 Plasminogen Activator Inhitor-1 (PAI-1)

PA-1 in plasma is measured by ELISA (3).

7.11.9 D-Dimers

D-dimers in plasma are measured by ELISA (3,4).

7.11.10 Platelet Aggregation (ADP, Collagen, Epinephrine)

Platelet aggregation is determined with a Chrono-Log Model 700 Whole Blood/Optical Lumi Aggregometer (Havertown, PA) that measures platelet function in samples using electrical impedance in whole blood or optical density in plasma. Platelet aggregation is measured in the presence of ADP, collagen and to epinephrine (5).

7.11.11 Ristocetin Cofactor

Platelet aggregation with Ristocetin Cofactor (von Willenbrand Factor Assay) is determined in plasma with a Chrono-Log Model 700 Whole Blood/Optical Lumi Aggregometer (Havertown, PA).

7.11.12 Factor VIII

Factor VIII activity is measured by a modified aPTT assay utilizing factor VIII deficient plasma and serial dilutions of participant plasma with a pooled normal plasma standard curve. Values are reported as a percentage of normal plasma (1,2).

7.11.13 Nattokinase and Anti-Nattokinase Antibodies

Nattokinase levels and anti-nattokinase antibodies are measured in plasma by ELISA.

7.11.14 Erythrocyte Sedimentation Rate (ESR)

RBC aggregation is evaluated by the ESR method in which Westergren tubes (i.e., 3 mm ID by 200 mm high) are filled with blood and positioned vertically for one hour: the distance of RBC sedimentation reflects RBC aggregation (6).

7.11.15 Red Blood Cell Aggregation

In plasma or appropriate polymer solutions, RBCs undergo reversible aggregation into face-to-face linear or three-dimensional structures with the extent of aggregation in plasma reflecting the concentration of large proteins (e.g., fibrinogen). A Myrenne RBC Aggregometer (Myrenne GmbH, Roetgen, Germany) is used to determine the extent of aggregation at stasis and at very low shear for cells in autologous plasma and in a pro-aggregating polymer solution (e.g., 3% 70 kDa dextran in isotonic PBS). The instrument has a cone-plate shearing geometry with the blood sample placed in the gap between these surfaces. An IR source and detector measure the light transmission through the sample; greater aggregation yields increased light transmission. The aggregometer is computer-controlled with results displayed on a monitor (6,7).

7.11.16 Red Blood cell Aggregability

The cellular factors that influence RBC aggregation are termed “aggregability” to indicate that they refer to the cell and not the suspending medium, is evaluated using the Myrenne Aggregometer. In this procedure, RBCs are washed twice in isotonic PBS (pH=7.4, 290 mOsm/kg) then once in a defined pro-aggregating medium of dextran, following which the RBC-dextran suspension is adjusted to a hematocrit of 0.4 L/L. A 3% solution of 70 kDa dextran in PBS is selected for these studies since it induces moderate aggregation and represents a stable, well-defined medium. Comparisons of different RBC populations (e.g., time-based samples from a subject) will indicate any alterations of aggregability (6,8).

7.11.17 Plasma Viscosity

Plasma viscosity is measured using a small volume Couette viscometer (Contraves LS-30, Contraves AG, Zurich, Switzerland) at 37ºC. An appropriate volume of blood is centrifuged at 2,000 x g and the plasma carefully removed and saved. Viscosity testing is done either on the day the blood sample is received or stored at -80 ºC for a brief period. Results are reported as mPa.s, which is numerically equal to centipoise (9).

7.11.18 Whole Blood Viscosity

The viscosity of EDTA-treated whole blood is measured using an automated scanning capillary viscometer (Rheolog, Rheovector, Co.) at 37ºC over a shear rate range of 1 to 1,000 s^{-1}. About 2.5 ml of blood are needed per measurement. The device has a horizontal small-diameter glass capillary with vertical tubes at each end and photo detectors to sense the height of blood in each vertical tube. Initially, blood is introduced into the system so that these heights are unequal then the system is isolated and blood flows through the capillary due to the difference in heights; flow and pressure gradient (i.e., heights in both tubes) are constantly measured during flow. The resulting data are analyzed using vendor-supplied software; the results, as viscosity at each shear rate, are displayed and available for printing (6,9).

7.11.19 Inflammatory Markers

1) MCP-1
2) IL-8
3) TNFα
4) IL-1β
5) IL-10

MCP-1, IL-8, TNFα, IL-1β and IL-10 are measured in plasma using a multiplex ELISA kit by procedures previously established in our laboratory (10,11).

7.11.20 E-Selectin

Plasma levels of E-selectin and VCAM-1 (as a control) are measured by ELISA as previously described (12).

7.11.21 P-Selectin

Plasma levels of P-selectin are measured by ELISA as previously described (12).

7.11.22 Endothelial injury In-vitro Assay

1) VCAM-1
2) ICAM-1

Expression of endothelial adhesion molecules (VCAM-1 and ICAM-1) by circulating monocytes is performed in cultured human aortic endothelial cells using isolated monocytes (13)

7.11.23 Monocyte Activation (FACS)

1) CD11b
2) CD11c
3) VLA-4

Activation of monocytes are determined as the expression of the monocyte cell surface markers CD11b/CD11c and VLA-4 using fluorescence-activated cell sorting (FACS) of monocytes isolated from whole blood (14)

7.11.24 Lipids

1) calculated LDL-C
2) plasma total cholesterol
3) plasma total triglycerides
4) plasma HDL-C
5) estimated VLDL-C

Plasma total cholesterol (TC), triglyceride (TG), and HDL-cholesterol levels are determined by enzymatic assays and standardized to the CDC using the Lipid Research Clinic protocol (15). HDL-cholesterol is measured after precipitation of the apoprotein B-containing lipoproteins (LDL and VLDL) in whole plasma by heparin-manganese chloride (16). Very low density lipoprotein cholesterol (VLDL-C) is assumed to equal one-fifth of the plasma triglyceride level and LDL-C is calculated using the Freidewald equation (17):

$$LDL\text{-}C=TC-(TG/5+HDL\text{-}C)$$

If the triglyceride level exceeds 500 mg/dL, direct measurement of LDL-C is performed.

After accumulation of 20 to 24 samples, a work sheet is prepared for the samples to be analyzed for the above lipid values. The results printed from the VPSS are transferred to the worksheet and entered into a computerized Excel database and transmitted to the DCC both electronically and by hard copy for double entry key stroke verification.

7.11.25 C-Reactive Protein (CRP)

CRP is measured in plasma by a turbidimetric assay by measuring the CRP-phospholipid complex at an absorbance of 660/700 nm.

7.11.26 Hemoglobin A1c

Hemoglobin A1c is measured by turbidimetric inhibition immunoassay in hemolyzed whole blood at an absorbance of 340/659 nm.

7.11.27 Peripheral Blood Buffy Coat Preparation

General:

When a tube of anticoagulated whole blood is centrifuged at 2,200 rpm for 8 minutes in a centrifuge, it separates into three main layers:

1) Plasma
2) Buffy coat layer containing white blood cells, nucleated red blood cells and platelets
3) erythrocyte layer

Specimen Requirement:

EDTA anticoagulated whole blood

Equipment:

1) 7 ml or 10 ml EDTA tube
2) Wintrobe tube or equivalent
3) 9 inch Pasteur pipettes
4) Rubber bulbs
5) 15 ml red top tube to hold Wintrobe tube for centrifugation
6) Centrifuge

Procedure:

1) Centrifuge the EDTA (purple top) tube at 2,200 rpm for 8 minutes. If the amount of specimen is 2 ml or less, transfer into Wintrobe tube(s) and proceed to step #4 below.

2) Using a 9 inch Pasteur pipette, discard about two-thirds of the plasma.

3) Aspirate the remaining plasma, the buffy coat layer, and the packed red cells just below the buffy coat layer. Transfer the mixture to a Wintrobe tube, using as many tubes as necessary.

4) Place the Wintrobe tube into a 15 ml red top tube and seal with the top. Centrifuge the tube for 8 minutes at 2,200 rpm.

5) Aspirate the plasma, the buffy coat layer and about 1 mm of packed red blood cells directly beneath the buffy coat.

6) Transfer the mixture to a cryogenic tube placing the mixture directly on the bottom of the tube without dripping it down the sides of the tube. Label the tube (Section 7.2).

7.11.28 ApoE Genotype

Determination of the apoE2/E3/E4 alleles uses SNP genotypes derived from two SNPs, rs429358 and rs7412, as reported previously (18). Genotyping is performed using the Applied Biosystems, Inc. (ABI) TaqMan system. Briefly, for each SNP, a PCR reaction containing 2 ng of genomic DNA, amplification primers and two 20-30 bp oligonucleotides encompassing the polymorphic site is carried out according to the manufacturer's protocols.

7.12 Clinical Determinations

7.12.1 Electrocardiogram

Standard 12-lead ECG is performed by trained ARU research clinic personnel by standard technique.

7.12.2 Waist:Hip Ratio

The waist circumference is measured and recorded **[FORM 23]** in the standard manner (19) with the participant standing erect, the abdomen relaxed, the feet together and the arms to the side. An inelastic tape measure is placed directly on the skin around the subject at the narrowest part of the torso between the ribs and iliac crest as seen from the anterior aspect. The measurement is taken at the end of a normal expiration with the tape measure in a horizontal plane without compression of the skin. With tape readings to the nearest 0.1 cm as described above, the waist circumference measurement of a participant is accurate within $\pm$1 cm of the "true" waist circumference.

The hip (or buttocks) circumference is measured in the standard manner (19) with the participant standing erect and wearing nonrestrictive underwear. The level of maximum extension of the buttocks is identified and an inelastic tape measure is placed around this region in a horizontal plane without compressing the skin. Under these conditions and with tape readings to the nearest 0.1 cm, the hip measurement is accurate within $\pm$1 cm of the "true" hip circumference. The waist:hip ratio is an indicator of the pattern of subcutaneous adipose tissue distribution that has been shown to correlate with the risk of diabetes mellitus and CHD (20,21).

7.12.3 Body Weight

Height and weight is measured (in street clothes without shoes) at v00 and weight is measured at each subsequent clinic visit for the duration of the trial.

7.13 References

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8.0
CAROTID ULTRASONOGRAPHIC PROTOCOL

8.1 Summary of Carotid Artery Ultrasound Data Acquisition

a) Far wall intima-media thickness (IMT) of the right distal common carotid artery (CCA)
b) Diameter of the right distal common carotid artery

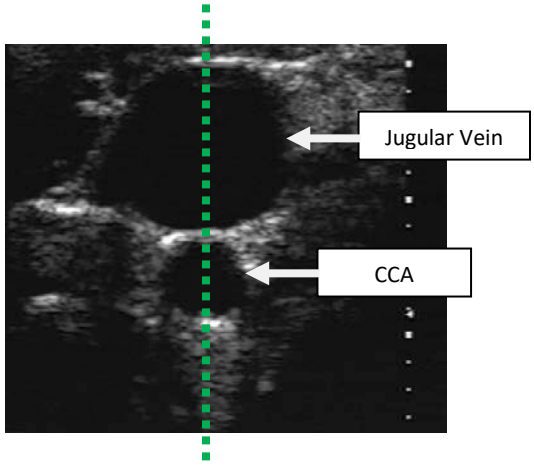
8.2 Overview of Ultrasound Image Acquisition

B-mode carotid artery images for IMT measurement are acquired with a Mindray DC 7 Digital Diagnostics Ultrasound System using a linear array L7-3 (frequency range: 3-7 MHz) transducer. The preset feature is used to enable image capture with optimized configuration of imaging parameter settings for this transducer and specific examination. Real-time dynamic clips are simultaneously captured along with a single-lead electrocardiogram (ECG) tracing and are stored as digital dynamic clips so that the ECG tracings can be viewed along with the B-mode images. The Mindray DC 7 can resolve 2 echo interfaces that are separated by 300 μm or more (in MARS (1), EPAT (2), VEAPS (3), BVAIT (4), WISH (5) and the general population (6) adult IMT is ≥400 μm).

Participant ID, visit number, scan date and study name (NAPS) are recorded at the beginning of the procedure. Five sequential blood pressure measurements are recorded at the end of the procedure using a GE-CARESCAPE V100 vital signs monitor. The average systolic and diastolic blood pressure measurements are required along with the carotid arterial diameters during systole and diastole to calculate central vascular stiffness (Section 8.6). All ultrasound examinations are performed by an experienced ultrasonographer. The following standardized image acquisition approach is conducted with each ultrasound examination.

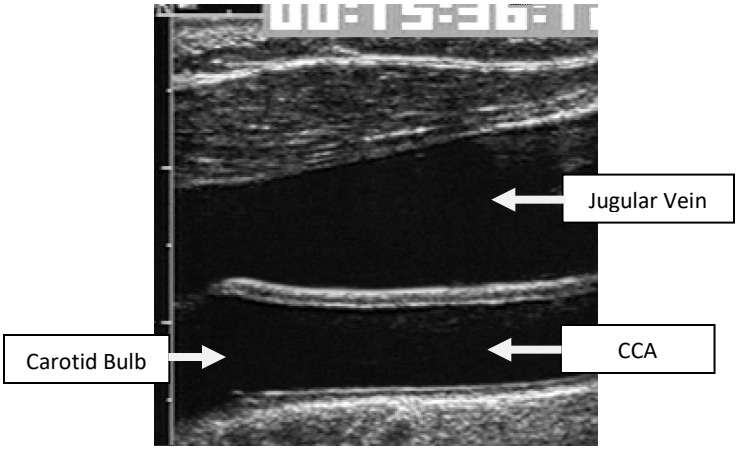
Carotid artery intima-media thickness and carotid artery diameter imaging

Participants are placed supine and positioned in a 45° molded head block with the neck extended to present the optimal angle for ultrasound examination of the right carotid artery. Using B-mode, the right CCA is imaged in cross section and the transducer moved laterally until the jugular vein and CCA are stacked with the former above the latter. In this position, the central image line passes along the common diameter of both vessels.



Stacked vessels in cross section view

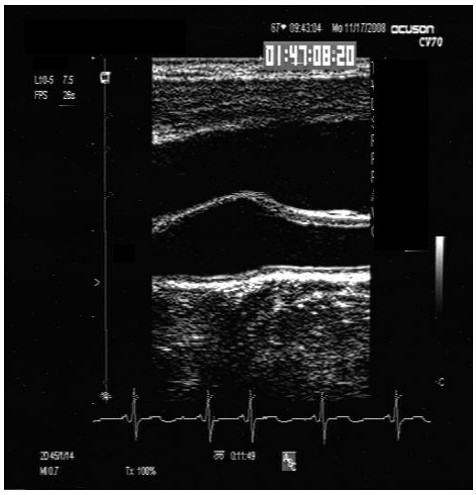
The transducer is slowly rotated 90º around the central image line of the stacked jugular vein and CCA, maintaining the jugular vein stacked above the CCA while obtaining a longitudinal image of both vessels. This will ensure that the transducer remains perpendicular to the far wall in the longitudinal view. In this view, the jugular vein and carotid artery are in horizontal orientation. Maintaining light transducer pressure, an image including the proximal portion of the carotid bulb as well as the mid- to distal-portion of the CCA is obtained. The lowest overall gain is adjusted to obtain the optimal image. Both the near and far wall CCA IMT should be clearly visible; slight transducer angle adjustment may be necessary. Emphasis is placed on optimizing visualization of the IMT. Emphasis is then placed on optimizing visualization of the distal CCA luminal diameter in systole and diastole by simultaneously capturing both the CCA near and far wall IMT in the ultrasound image.



Stacked vessels in longitudinal view

The proximal portion of the carotid bulb is included in all images as an anatomical reference point for standardization of CIMT measurements. Stacking the jugular vein and CCA determines a repeatable transducer angle that allows the same portion of the wall to be imaged at each ultrasound examination (7,8). This leads to further standardization of image acquisition and processing that in turn decreases measurement variability (7,8). Minimum gain necessary for clear visualization of structures is used. Images are acquired from the carotid bulb and internal carotid artery, but emphasis of ultrasound imaging is on the distal centimeter of the CCA because least variability occurs in this area (9). The CCA far wall IMT is used for statistical purposes since measurement of near wall thickness is less accurate (10). The distal CCA is visualized longitudinally and 4-5 video clips (preset at 6 seconds in duration for each clip) are captured using the optimal transducer angle (OTA) view. While maintaining the optimized longitudinal image of the CCA, the transducer is moved away from the OTA view in the anterior direction and 1-2 video clips of CCA IMT images in the anterior view are captured. The transducer is then moved away from the OTA view in the posterior direction and 1-2 video clips of CCA IMT images in the posterior view are captured.

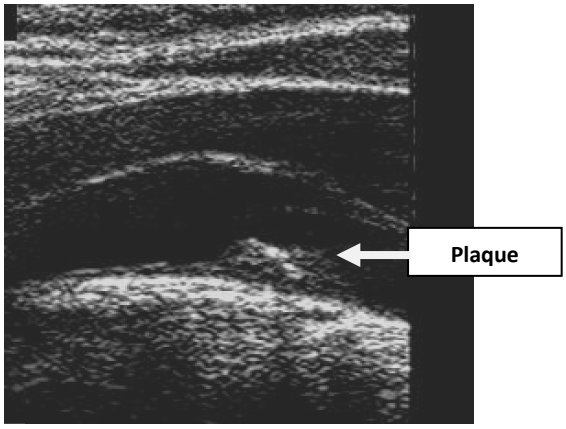
The transducer is then moved cephalad (toward the head) maintaining the jugular vein stacked above the carotid artery to scan the carotid bulb (bifurcation) along the longitudinal plane. The transducer is swept to the anterior direction and then swept to the posterior direction bringing the far wall IMT of the bifurcation into view while maintaining the jugular vein stacked above the bifurcation. Emphasis is placed on optimizing visualization of the IMT and acquiring the thickest IMT image in the bifurcation view. A bifurcation view is defined as an image that contains 100% of the carotid bulb as well as visualization of the proximal internal or external carotid arteries; 1-2 video clips of the bifurcation view are captured.



Carotid artery bifurcation view

Carotid artery plaque imaging

Following acquisition of CIMT, the carotid artery is surveyed for plaques, defined as a focal thickening of the intima >1.5 mm. Beginning with the cross section view of the distal CCA, the transducer is moved cephalad from the distal CCA through the carotid bifurcation to the proximal internal and external carotid arteries then back to the distal CCA while surveying for lesions. The transducer is then slowly turned 90^{o} to acquire a longitudinal plane view of the carotid artery. The transducer is then swept in the anterior direction and then swept in the posterior direction to acquire clear images of plaque(s), if any. If only one plaque is identified, the maximal carotid plaque thickness (MCPT) is obtained and 2 video clips are captured. If multiple plaques are identified, each plaque is labeled (p1, p2, p3, etc.), the MCPT of each plaque is obtained and 1 video clip of each plaque is captured.

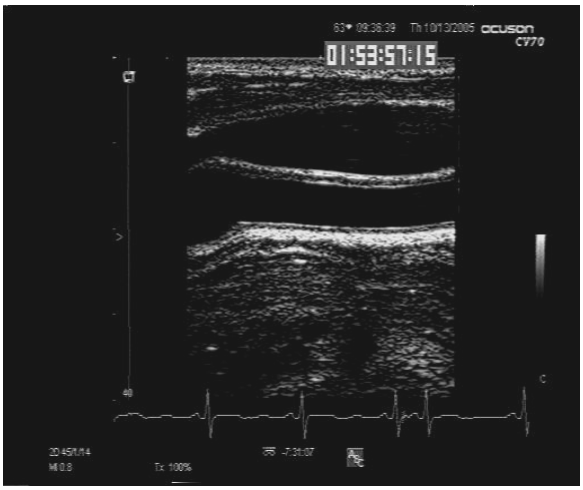


Carotid artery plaque in the bifurcation view

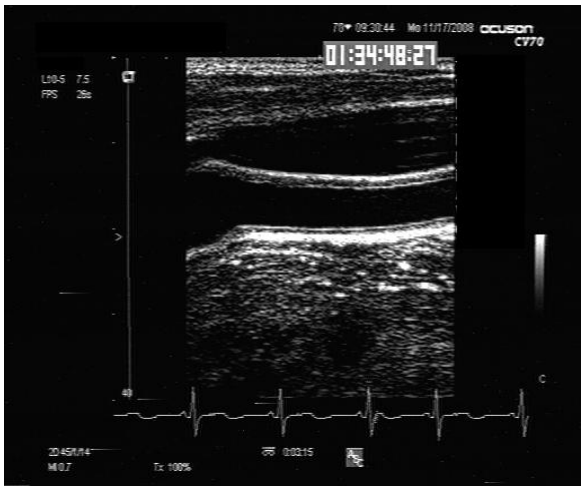
Longitudinal carotid artery imaging

Repeat (follow-up) CIMT image acquisition uses the same ultrasound equipment and transducer during the entire project. Each individual's baseline image of CIMT, arterial diameters and MCPT, if any are used as a guide to match the vascular and surrounding soft tissue structures of the follow-up ultrasound examinations. This is a direct visual aid method for reproducing transducer angulation designed for repeat image acquisition for longitudinal studies (1-5,7,8). In addition, repeat image acquisition

parameters must be the same as those used for baseline image acquisition, including transducer frequency range (MHz), depth, dynamic range, time gain controls (TGC), gain, power, etc., all of which are automatically recorded on the baseline ultrasound image and from which the setup parameters for the ultrasound equipment are derived. This procedure establishes standardization for instrument setup that encompasses the full dynamic range of the ultrasound echo across all examinations within the same participant. The key to repeat image reproducibility is to match all of the technical parameters and the follow-up image to the baseline image as precisely as possible.



CCA baseline image



CCA image 36 month later

The primary trial end point is change in right distal CCA far wall IMT measured (over one centimeter) during end diastole. The transducer angle with the best definition of the blood-intima and media-adventitia echoes at baseline examination will therefore be used in all subsequent studies for quantitative measurement and comparison. The co-primary trial end point is change in carotid artery stiffness determined over serial systolic and diastolic distal CCA diameter measurements.

Phantom images obtained from RMI 404 GS LE, Small Parts Gray Scale-Attenuation coefficients of 0.7 db/cm/MHz are evaluated every 6 months to assure image quality and to standardize calibration pixel size for IMT and diameter measurements. In addition, if ultrasound equipment requires replacement or repair (beyond maintenance), phantom images are evaluated before performing ultrasound examinations.

8.3 Carotid Ultrasound Image Acquisition and Standardization

8.3.1 Equipment and Supplies

1) Mindray DC 7 Digital Diagnostics Ultrasound System

2) Linear array L7-3 (frequency range: 3-7 MHz) transducer

3) Single-lead ECG – contained within the Mindray DC 7 Digital Diagnostics Ultrasound System

4) ECG monitoring electrodes

5) GE – V100 Vital Signs Monitor (automated blood pressure device)

6) Phantom Test Instrument (RMI 404 GS LE, Small Parts Gray Scale-Attenuation coefficients of 0.7

db/cm/MHz)

7) Examination table

8) Ultrasound conducting gel

9) 45º molded head block

10) Personal computer

11) Back up USB flash drives and portable hard drives

8.3.2 General Ultrasound Procedure and Variability

The above overview (Section 8.2) and following details of our ultrasound techniques/methodology provide a high degree of standardization for image acquisition and processing, resulting in significant reduction in measurement variability between scans; inter- and intra-sonographer coefficients of variation (CVs) <3% (1-5,7,8). Even in multicenter studies with multiple acquisition sites, the methodology is highly stable and reproducible with low variability, <3% CV (11-13).

8.3.3 Baseline CIMT Acquisition Procedure

Carotid Artery (right)

1) Turn on the-Mindray DC 7 Digital Diagnostics Ultrasound System

2) Select the study preset - NAPS

3) Check the screen monitor to make sure that the preset parameter settings are as follows:

- Transducer frequency - 7.0 MHz
- Power input - 100 %
- Dynamic range at mid-range - 55
- Line density - M
- FOV - W
- Focus Number - 1
- THI - off
- iBeam - off
- Smooth - 2
- iClear - 4
- Persist - 2
- Gray Map - 5
- Depth - 38 mm
- Zoom mode - normal
- ECG - on
- TGC - should be fixed in mid-range position
-

4) Press **NEW PATIENT** key on the control panel to begin the CIMT examination and enter the following information on the screen monitor:

- Participant identification number (PID)
- Date
- Visit #
- Study name (NAPS)
- RT (scan of right carotid artery) or LT (scan of left carotid artery)
- DEG - 45^{o} (head angle)
- PLN (head plain) - (enter NT, MT or CT during ultrasound examination)
- Optimal transducer angle - (OTA, degree - enter 0, 15, 30, 45, 55, -5, -10, etc., during ultrasound examination)
- OP - operator's name

5) Prepare the participant for scanning

- Bring the participant into the ultrasound examination room.
- Explain CIMT procedure to the participant.
- Properly position participant in the supine position on the examination table. Remove necklaces and other objects that may interfere with the procedure. Participant's head and neck are anatomically aligned; place support pillows under participant's head, neck or shoulders if necessary.
- Head is wedged between square pillow block on right and 45^{o} wedge pillow on left. Left ear touches wedge pillow. Blocks rest upon shoulders.
- Connect ECG leads: white - right wrist; black - left wrist; green - right leg.
- Check ECG tracing on the screen monitor; adjust the ECG gain so that tracing is at the bottom of the screen and fully visible.

6) Apply small amount of conducting gel to the field of scanning (right neck). Begin scanning.

7) Begin scanning in the transverse plane on the proximal right side of the neck. Stack the right jugular vein and CCA. In this position, the cross-sections of both vessels appear near the center of image (do not capture the transverse image).

8) Maintaining the transverse stacked image of the jugular vein and CCA, move the transducer towards the head. Move through the carotid bifurcation then back to the bulb-distal CCA area.

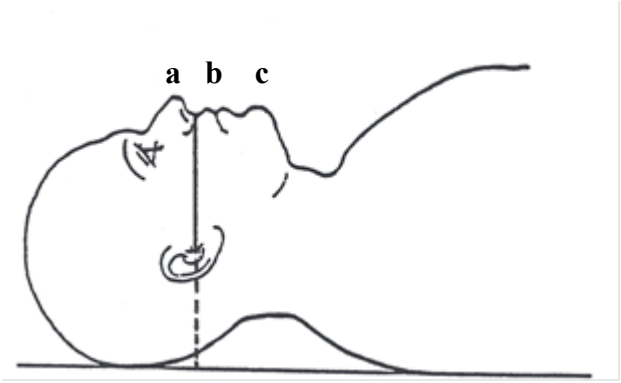
9) Slowly rotate the transducer 90^{o} degrees around the central image line of the stacked jugular vein and CCA, maintaining the jugular vein stacked above the CCA to a longitudinal image. In this position, the carotid artery image will be in a horizontal orientation. Instruct participant to perform a Valsalva maneuver to fully demonstrate the jugular vein and CCA, if necessary (do not capture this image). Keep transducer pressure light and obtain an image that contains the proximal portion of the carotid bulb as well as the mid- to distal- portion of the CCA. Adjust the overall gain to the lowest setting possible to obtain the optimal image (not during Valsalva). Both the near and far wall CCA IMT should be clearly visible.

10) After the 90^{o} transducer rotation, slight transducer angle adjustment may be necessary to obtain the longitudinal optimal transducer angle (OTA) view of the right CCA. Adjust the TGC's and overall gain to obtain the optimal gain. Gain must be as low as possible (optimal gain) and stable throughout CIMT image acquisition. IMT will be brightest when the transducer angle is perpendicular to the vessel wall.

11) Simultaneously optimize the CCA IMT of the near and far walls. Enter from key board the degree of neck extension using one of the following anatomical landmarks as a perpendicular plane to the examination table:

PLN (head plane) - enter any of NT, MT or CT

a) "NT" = tip of nose to external acoustic meatus
b) "MT" = tip of mouth to the external acoustic meatus
c) "CT" = tip of chin to the external acoustic meatus



OTA, degree - enter any of 0,10,15,20,25,30,35,40,45,-5,-10, etc.

a) 0 = transducer's longitudinal view plane is parallel to the plane of the examination table
b) 10 = transducer's longitudinal view plane is 10^{o} above the horizontal of the plane of the examination table
c) -5 = transducer's longitudinal view plane is 5^{o} below the horizontal of the plane of the examination table

12) Repeat steps 7-10 five times to ensure that the OTA image is a high quality CIMT image and repeatable during the scan. Capture at least 5 video clips of the OTA view.

13) Annotate "ANT" on screen monitor. While maintaining the optimized longitudinal image of the CCA, move the transducer away from the OTA in the anterior direction to acquire CCA IMT images and capture 1-2 video clips of the anterior view.

14) Annotate "POST" on screen monitor. Move the transducer away from the OTA in the posterior direction to acquire CCA IMT images and capture 1-2 video clips of the posterior view.

15) Annotate "BIF" on screen monitor. Scan the longitudinal plane view of the bifurcation (carotid artery bulb). Sweep to the anterior direction and then sweep back to the posterior direction. Bring the far wall IMT of the bifurcation into view. Emphasis is placed on optimizing visualization of the IMT and acquiring the thickest IMT image on the bifurcation view. A bifurcation view is defined as an image that contains 100% of the carotid bulb as well as the proximal internal or external carotid arteries. Capture 1-2 video clips of the bifurcation view. Identify the internal and external carotid arteries by Doppler, if necessary. Annotate images to identify structures.

16) Survey the carotid artery for plaques. In the cross section view, move the transducer cephalad (towards the head) from the distal CCA through the bifurcation to the proximal internal and

external carotid arteries then return to the distal DCC. Slowly rotate the transducer 90º to acquire a longitudinal plane view of the carotid artery. Sweep in the anterior direction then sweep in the posterior direction to acquire plaque(s) images, if any. If only one plaque is identified, obtain the maximal carotid plaque thickness (MCPT) and capture 2 video clips. If multiple plaques are identified, label each plaque (as p1,p2,p3…) and obtain the MCPT of each plaque and capture 1 video clip of each plaque. Determine quantitative Doppler at site of all lesions.

17) Annotate "Left" on screen monitor. Scan the left side CCA IMT by turning the participant's head to the right and following steps 6-16.

18) Standard output file format: Both AVI and DICOM video clips. Provide at least 16 clips if possible for each scan. This includes:

5 clips	-	Longitudinal OTA views of the RCCA
1 clip	-	Longitudinal anterior view of the RCCA
1 clip	-	Longitudinal posterior view of the RCCA
2 clips	-	Longitudinal views of the RBIF (IMT)
2 clips	-	Longitudinal views of the MCPT, if any
3 clips	-	Longitudinal OTA views of the LCCA
1 clip	-	Longitudinal anterior view of the LCCA
1 clip	-	Longitudinal posterior view of the LCCA
2 clips	-	Longitudinal views of the RBIF (IMT)
2 clips	-	Longitudinal views of the MCPT, if any

19) Prepare the GE-V100 automated blood pressure device. Measure the participant's upper right arm circumference to select the appropriate blood pressure cuff size. Snugly place the blood pressure cuff on the participant's right upper arm. Note the mark on the inside of the blood pressure cuff that needs to be placed over the brachial artery. Blood pressure cuff should fit snugly but not so tightly as to prevent venous return between determinations. Press POWER ON and then press INFLATE to begin the automated blood pressure determinations every one minute. Record five sequential blood pressure and pulse rate measurements on the participant's data sheet.

20) Completely and accurately complete the CIMT work sheet.

21) Place a back-up USB flash drive or portable hard-drive to the ultrasound system's tray. Click the [iStation] key on the control panel or click the [iStation] button in the review screen and select the source of the scan database, Find the participant's PID and scan date in the review screen and select the examination that has been performed. Click [Send To] or [Back-up Patient Record] in the dialogue box and click [OK] or the [Back-Up] button in the dialogue box to begin writing to the system-supported media (Back-up USB flash drive or portable hard-drive). After the writing process is completed, click the symbol in the lower right corner of the monitor screen to eject the back-up USB flash drive or portable hard-drive.

8.3.4 Repeat (Follow-up) CIMT Acquisition Procedure

Use the same ultrasound equipment and transducer during entire CIMT project. Use participant's baseline CCA, vascular diameter images, carotid artery bifurcation as well as MCPT images, if any as the reference for ultrasound equipment setup parameters and guidance for repeat image acquisition. Repeat image acquisition parameters must be the same as those used for baseline image acquisition including transducer frequency range (MHz), depth, dynamic range, TGC, gain, power, etc., all of which

are automatically recorded on the baseline ultrasound images. The key to image reproducibility is to match the follow-up image to the baseline image as precisely as possible.

Follow the same CIMT acquisition procedure as used for baseline acquisition (See Section 8.3.3, Baseline CIMT Acquisition Procedure) and ensure that the follow up image landmarks look the same as those on the baseline image when performing follow-up image acquisition:

- Top tissue pattern
- Jugular vein pattern
- Bottom tissue pattern
- CCA near-wall IMT pattern
- CCA far-wall IMT pattern
- MCPT pattern, if any

8.4 Automated Computerized Image Analysis of IMT

A computer system developed to assess carotid arterial dimensions from ultrasound images includes a personal computer. Software developed to measure changes in arterial dimensions includes a technique for automated edge detection of the lumen-intima and media-adventitia echo lines for IMT measurements (1-5,7,8). Automated boundary detection provides IMT and luminal measurements with greater precision than manual boundary identification because multiple measurements along the vessel structures replace a single point measurement made with a cursor (7,8). When automated detected edges are observed they do not differ from those chosen by manual analysis although manual variability is considerably greater than that obtained from computer-detected gradient-based edges. A description of the critical parts of the image processing system is given below.

Analysis of each B-mode carotid ultrasound frame involves the steps outlined below.

1) Select the frame to be analyzed from the digital frame loop
2) Enter the frame identification information (participant ID, date, etc.)
3) Manually point to the approximate location of the lumen-intima and media-adventitia echoes for IMT measurements (or the adventitia-media and media-adventitia echoes for arterial diameter measurements) with a mouse
4) Automatically track the lumen-intima and media-adventitia echoes and compute IMT (or automatically track the adventitia-media and media-adventitia echoes and compute arterial diameter)

These steps are further discussed below.

Frame selection: Ultrasound image input to the current ultrasound image processing and analysis workstation system is directly through the digital frame loops generated by the digital ultrasound imager. Digital frame loops are played back through the ultrasound image processing and analysis workstation system and optimum frames are selected through direct visualization of individual digitized frames within the digitized frame loop.

Participant and measurement database: The program database has been structured to allow subjective descriptions of image quality and lesion morphology to be stored along with measurements and other process descriptors. The information stored is shown below. Data relating to image quality reflects on the relative reliability of each measurement and is taken into account when the measurements are analyzed. Participant and image information:

Reader (computer operator)	
Participant identification code	
Visit number	
Examination date	
Frame time code	
Side	(left,right)
Segment code	(CC1, CC2, CC3, BUL, ICA, PA, BA)
Measurement code	(near wall, far wall, lumen diameter)
Tracking method	(automatic, manual)
Image quality	
Lumen-intima lesion?	(yes,no)
Wall characteristics, border	
Wall characteristics, density	
Wall characteristics, structure	
Double line visible?	(yes,no)
Measurement made?	(yes,no)

Ultrasound equipment parameter preset values for the ultrasound examination, such as transducer frequency range (MHz), depth, dynamic range, TGC, gain, power, etc. are automatically recorded on the ultrasound video clips, and are recorded separately to establish identical technical conditions for follow-up examinations. This process standardizes instrument setup that encompasses the full dynamic range of the ultrasound echo across all studies within the same participant.

Automated boundary detection: Automatic detection of the lumen-intima and media-adventitia echo lines is difficult because the intensity of the boundary is highly variable and frequently discontinuous. Our software surmounts these difficulties in a three step edge detection process. Additionally, our computer tracking software searches for edges normal to the curvature of the artery.

The first step is to identify the approximate location of the boundary at four to six points using a mouse. A smooth curve fit to these points serves as a guide to the automated edge tracking algorithm.

In the second step of the edge tracking process, the computer examines image intensity values in the image along a series of paths perpendicular to the smooth curve. For each path, the point in the image where the intensity gradient is largest is conditionally labeled as the boundary of the echo along that path. For each boundary point, both the image coordinates and the gradient value are stored.

The third step in the process compares the gradient value for each conditional edge to the maximum gradient value of all conditional edges of the boundary and eliminates those edges with gradient values less than a preselected fraction of the maximum value. This procedure eliminates weak edges and gaps from the boundary.

IMT and lumen diameter measurement: The boundary tracking described above is applied to the lumen-intima and media-adventitia echoes. Since the longitudinal carotid images are nearly horizontal in the ultrasound pictures, wall thickness is measured along vertical image lines at each point of the wall where both boundary points exist. From these data, maximum and average wall thickness is computed. Lumen diameter is measured in the same way as the wall thickness with the boundary tracking applied to the near and far wall media-adventitia echoes.

Lesion size measurements: The lumen-intima ultrasound echo from raised carotid artery lesions are frequently highly irregular and difficult to track with automated edge finding algorithms. In addition, calcium shadowing can obscure the media-adventitia boundary. To deal with these problems, an option

is available to use manual tracing of lesion boundaries with the mouse. When calcium shadowing completely obscures edges, the opposite common carotid artery is used. If this is also unreadable because of shadowing, and manual tracking is also impossible, the case is discarded. In our experience over tens of thousands of ultrasound examinations, this occurs in <%1 of participants. The type of tracking used (automatic or manual) is indicated in the database when such measurements are stored.

From the resulting analyses by computer of the B-mode images, thickness of the far wall intima-media and arterial diameters are stored electronically. Our software automatically creates and stores averages, maxima and minima vessel dimensions.

8.4.1 IMT Measurement Standardization

Good IMT image processing derives from good image acquisition.

All settings must be consistently and accurately shown along with the ultrasound scan images for each participant. This information is entered as part of the database during image processing.

Equipment information:

a) Transducer frequency range (MHz)
b) Depth (field of view (FOV))
c) Dynamic range
d) TGC
e) Total gain
f) Power
g) Ultrasound instrument

Participant information:

a) Date of scan
b) Visit number
c) Participant ID
d) Study (NAPS)
e) Date of image processing
f) Participant's positioning during ultrasound image acquisition
g) Participant's side scanned (left, right)

Image processing information:

a) Segment analyzed
    - CC1 - First cm from the proximal carotid artery bulb
    - CC2 - Second cm from the proximal carotid artery bulb
    - CC3 - Third cm from the proximal carotid artery bulb
    - BUL - Carotid artery bulb
    - ICA - Internal carotid artery
b) Image processor's initials
c) Frame number analyzed
d) Ultrasound image description
    i) Angle variation of the common carotid artery

ii) Distance of IMT measurement from the proximal carotid artery bulb
iii) Image name for storage

e) Measurement code (near wall, far wall, lumen diameter)
f) Tracking method (automatic, manual)
g) Image quality
h) Lesion notation

Image processing calibration:

a) This may vary from participant to participant (Depth or FOV) and by ultrasound imager
b) Use the phantom test images to calibrate the horizontal and vertical pixel sizes

Frame selection for image processing:

a) Frames selected at QRS wave (R-S interval) and T-wave on each heart cycle (paired image)
b) Frame selected where IMT is brightest and most continuous
c) Must use the same referencing landmarks on each follow-up visit
d) Name and save (at least 15 paired images) images

IMT measurement:

a) A pre-calibrated computer-generated 1 cm electronic ruler is placed above area of interest
b) Using a mouse, points are marked directly above the lumen-intima echo along the 1 cm ruler length. This represents the first edge
c) Using a mouse, points are marked directly below the media-adventitia echo along the 1 cm ruler length. This represents the second edge
d) Steps a through c are repeated for the next consecutive frame (usually the next heart cycle)
e) Based on the baseline measurement, the same measurement location is made on each follow-up scan (absolutely required)
f) The image processed baseline scan is viewed during image processing of all follow-up scans to ensure step e

8.5 Automated Computerized Arterial Diameter Analyses

Procedures outlined in Section 8.4 are followed and applied to measurement of arterial diameter during systole (maximum arterial excursion) and diastole (minimum arterial excursion). Arterial diameter is defined from adventitia-media (near wall) to media-adventitia (far wall). Arterial diameter is measured along the same 1 cm segment identified by the computer-generated electronic ruler used for the measurement of IMT.

8.6 Vascular Stiffness

Measurements of right CCA lumen diameters at systole (D_S) and diastole (D_d) are determined at the same site where CIMT is measured (8,14-16). Standardized brachial artery blood pressure measurements are determined using an automated oscillometric method (GE – V100 Vital Signs Monitor) at the end of each ultrasound examination (average of 5 supine measurements) in order to measure systolic (P_s) and diastolic (P_d) pressures and pulse pressure ($PP=P_s-P_d$) (8,14-16). The CV for D_s=1.28-2.33%, D_d=1.18-2.23%, P_s=6.17-8.75% and P_d=6.96-8.77% (8,15). Two indices of arterial stiffness and arterial compliance are calculated as follows:

$$\text{Arterial distensibility} = [2(D_s-D_d)/D_d]/PP$$
$$\text{Arterial compliance} = (D^2_s-D^2_d)/PP$$

8.7 Quality Control

These steps provide a measure of consistency of image analysis.

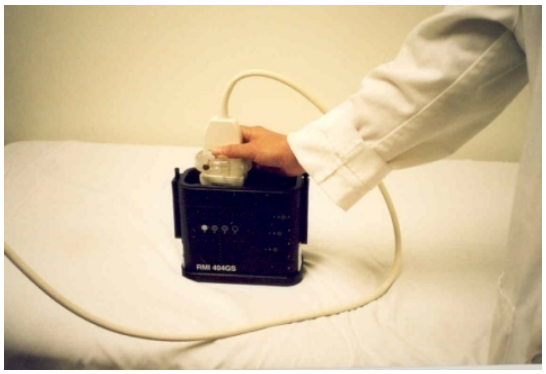
1) The Mindray DC 7 Digital Diagnostics Ultrasound System - To monitor operation of Mindray DC 7 Digital Diagnostics Ultrasound System, a precision small parts grey scale phantom (RMI 404 GS LE, Small Parts Gray Scale-Attenuation coefficients of 0.7 db/cm/MHz) simulating the lumen-intima and media-adventitia boundaries is scanned every 6 months. The distance between vertical and horizontal targets is measured with our IMT assessment software. Since the true size of the phantom distances do not change and the power and gain of the scanner is kept constant, any change in the measured distances indicates a performance change in the ultrasound system that will be corrected. Results of these measurements are plotted and monitored for drift.

2) Phantom images obtained from the RMI 404 GS LE, Small Parts Gray Scale-Attenuation coefficients of 0.7 db/cm/MHz scanning test are evaluated before the project initiates. Subsequently, phantom scanning tests are required every 6 months to assure image quality, image resolution and measurement accuracy (see above) as well as to standardize calibration pixel size for IMT and diameter measurements. In addition, if ultrasound equipment requires replacement or repair (beyond usual maintenance), a phantom scanning test is required.

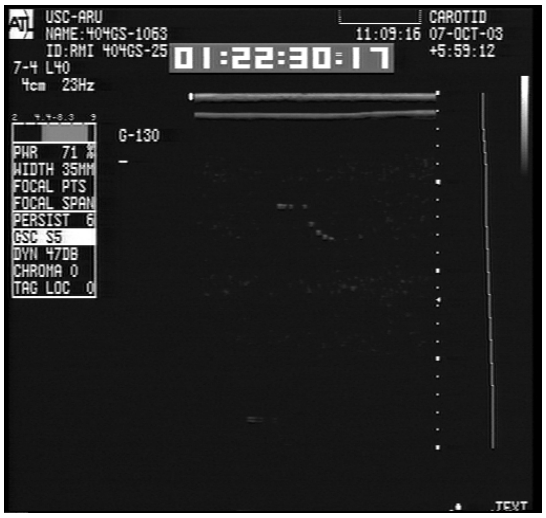
3) Phantom scan procedures:

A) Fill the water dam on the top of the phantom equipment. Ensure that water temperature is between 70-76^{o}F.

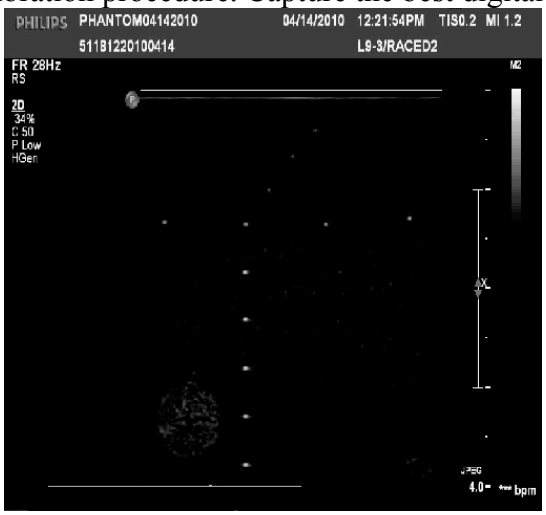
B) Set ultrasound equipment image depth to 38 mm. Use the same transducer that is used for the CIMT project. Set the probe's frequency range to the same range as is set for the CIMT project. For example, if in the CIMT project the linear probe is set to 7.0 MHz, then in the phantom scanning test the transducer is set to 7.0 MHz. Likewise, if in the CIMT project the linear transducer is set to 7.5 MHz, then in the phantom scanning test the transducer is set to 7.5 MHz.



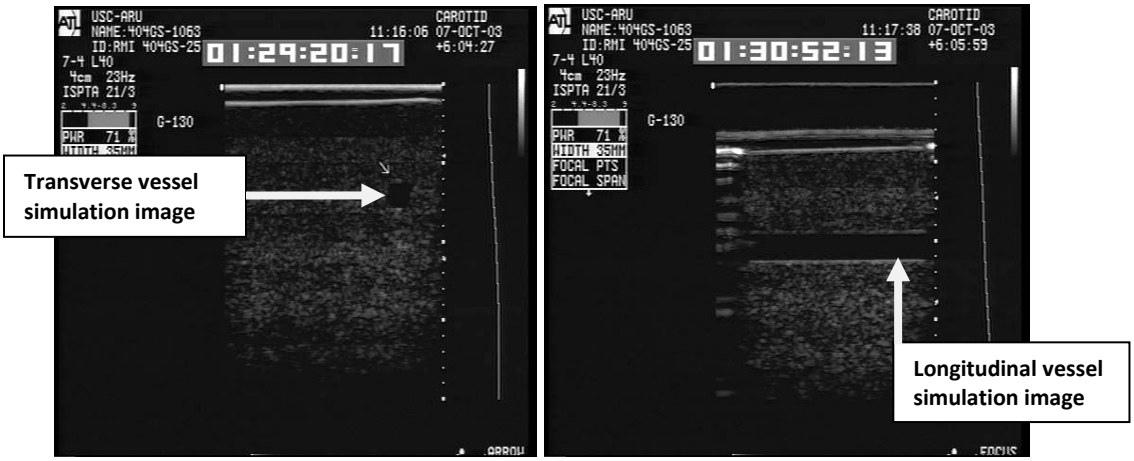
C) Acquire an image that has 4 horizontal pins and 4 curved-down pins. Set gain as low as possible. Obtain the best axial and lateral resolution for the target group. Capture a digital video clip.



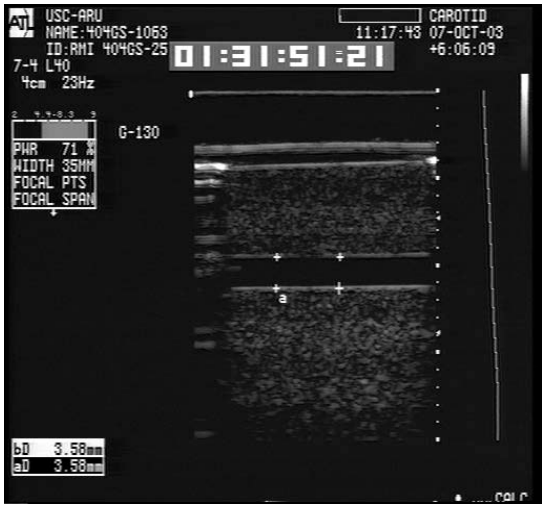
D) Acquire an image that has 4 horizontal pins and 6 vertical pins. Adjust transducer pressure to obtain all pins in the normal positions of the horizontal and vertical lines. Move the focal zone slowly down the imaging field. Set gain as low as possible. The image background must be as dark as possible to display sharper horizontal and vertical pins. Bright background and blurred pins are not acceptable for the calibration procedure. Capture the best digital video image clip two times.



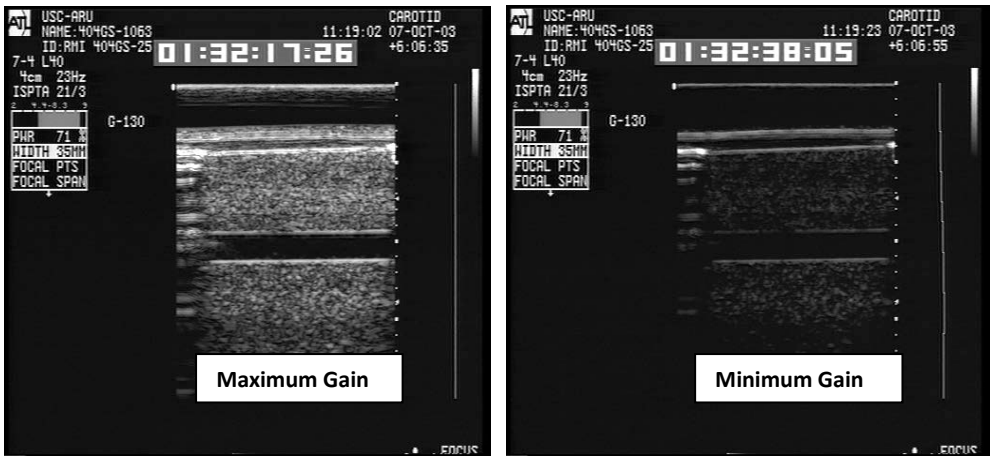
E) Acquire a transverse view of the vessel simulation image by adjusting the imaging gain. Turn the transducer 90º to get a vessel simulation image with optimal gain in the longitudinal view. Capture a digital video clip.



F) Freeze image and measure vessel simulation diameter that is approximately 3.5 mm long. Capture the best digital image.



G) Unfreeze the image to obtain the original vessel simulation image with optimal gain. Then change time gain to maximum. Capture the best digital video image. Change time gain to minimum. Capture the best digital video image.



H) Complete the phantom work sheet.

I) Provide 6 video clips for each phantom test:

1 video clip	-	Image that has 4 horizontal pins and 4 curved-down pins
2 video clips	-	Image that has 4 horizontal pins and 6 vertical pins
1 video clip	-	Longitudinal vessel simulation image with optimal gain
1 video clip	-	Longitudinal vessel simulation image with maximum gain
1 video clip	-	Longitudinal vessel image with minimum gain

8.8 Data Security

To assure security of data, processed images are stored electronically and maintained in locked facilities.

8.9 Data Transfer

Participant records are exported to Excel-readable files. Data are down-loaded into an Excel file and delivered to the DCC.

8.10 References

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9.0
DATA COLLECTION AND MANAGEMENT

9.1 Study Personnel and Contact Information

9.1.1 Investigators

1) Howard N. Hodis, M.D. (323) 442-1478, athero@usc.edu

2) Vijay Kalra, Ph.D. (323) 442-1526 or (626) 388-5924, vkalra@usc.edu

3) Howard A. Liebman, M.D. (323) 865-3950, liebman_h@med.usc.edu

4) Wendy J. Mack, Ph.D. (323) 442-1820, wmack@usc.edu

5) Herbert J. Meiselman, Sc.D. (323) 442-1268 or (323) 442-1267, hmeiselman@gmail.com or meiselman@usc.edu

9.1.2 Data Coordinating (DCC) Personnel

1) DCC Director: Wendy Mack, Ph.D. (323) 442-1820, wmack@usc.edu

2) Data Programmer/Analyst: Laurie Dustin, M.S. (323) 442-1566, labree@usc.edu

3) Data Programmer/Analyst: Naoko Kono, M.S. (323) 442-1802, kono@usc.edu

4) Data Monitor: Olga Morales (323) 442-1568, molga@usc.edu

5) Data Systems Engineer: Adrian Herbert (323) 442-2248, aherbert@usc.edu

9.1.3 Clinic Personnel

1) Study Coordinator: Liny Zurbrugg, R.N. (323) 442-2425

2) Patient Coordinator: TBA (323) 442-1754

3) Study Nurse: TBA (323) 442-2257

4) Phlebotomist: TBA (323) 442-1488

5) Phlebotomist: Esther Bhimani (323) 442-2014

6) Project Coordinator: Janie Teran (323) 442-1478; FAX (323) 442-2685, athero@usc.edu

7) Clinic FAX: (323) 442-2345

9.1.4 Ultrasound Personnel

1) Yanjie Li, M.D. (323) 442-3993, yanli@usc.edu

2) Mingzhu Yan, M.D., Ph.D. (323) 442-1303, mingzhuy@usc.edu

3) FAX: (323) 442-2345

9.1.5 Participating Laboratories

1) Quest Corporation

Contact Person: Ellie Hetman (818) 737-8347

To measure chemistry panel, insulin, complete blood count (CBC) with platelet count, prothrombin time (PT), activated partial thromboplastin time (aPTT) and fibrinogen (see Section 7).

2) USC Hematology Laboratory

Laboratory Director: Howard A. Liebman, M.D. (323) 865-3950, liebman_h@med.usc.edu

Contact Person: Leanne Rochanda, (323) 865-0965, rochanda@usc.edu

To measure vWF antigen, tissue plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1), D-dimers, platelet aggregation (ADP, collagen, epinephrine), ristocetin cofactor, factor VIII, plasma nattokinase levels and anti-nattokinase antibodies (see Section 7).

3) USC Rheology Laboratory

Laboratory Director: Herbert J. Meiselman, Sc.D. (323) 442-1268 or (323) 442-1267, hmeiselman@gmail.com or meiselman@usc.edu

Contact Person: Rose Wenby, (323) 442-1268 or (323) 442-1267, rwenby@usc.edu

To measure erythrocyte sedimentation rate (ESR), red blood cell aggregation (plasma), red blood cell aggregability, plasma viscosity and whole blood viscosity (see Section 7).

4) USC Biochemistry Laboratory

Laboratory Director: Vijay Kalra, Ph.D. (323) 442-1526, vkalra@usc.edu

Contact Person: Caryn Gonsalves, Ph.D. (323) 442-1528 or (626) 353-8232

To measure MCP-1, IL-8, TNFα, IL-1β, IL-10, E-selectin, P-selectin, endothelial injury in-vitro assay: VCAM-1, ICAM-1 and monocyte activation (FACS): CD11b, CD11c, VLA-4 (see Section 7).

5) USC Atherosclerosis Research Unit (ARU) Core Lipid Laboratory

Laboratory Director: Juliana Hwang, Pharm.D. (323) 442-1160 or (323) 442-2297, julianah@usc.edu

Contact Person: Gail Izumi (323) 442-1160 or (323) 442-2297, izumi@usc.edu

To measure lipid, insulin and hemoglobin A1c levels as well as prepare samples for -80ºC storage (see Section 7).

9.2 Laboratory Data

Because the study is double-blinded, all laboratory data except Quest safety data (chemistry, CBC with platelets, PT, aPTT and fibrinogen) will be entered by laboratory personnel directly into the trial data system; clinic personnel will be blinded to these laboratory data. Pre-randomization screening values for LDL-C will be made available to the study clinic personnel. On-trial LDL-C levels will also be routinely reported to the clinic.

9.2.1 Sample Labeling and Tracking

Bar-code labeling will be used for collection, processing, storage and disposition of all laboratory samples by the following procedure:

1) The samples to be collected at each clinic visit will be listed in pre-made requisition forms, generated through the trial data system; the requisition form will contain pre-printed participant ID, visit number (linking visit number to samples to be collected), number and type of blood tubes to be used for that particular clinic visit and a bar code.

2) Sample collection tube labels will be generated at the study clinic using the trial data system. On the initial collection tubes, labels will include participant ID, visit number and the type of tube and specimen collected, thus assuring that the proper sample is collected in the appropriate collection tube for each clinic visit.

3) Receipt of samples by staff at the Core Lipid Laboratory will be compared and verified against the bar-coded laboratory requisition form that shows which samples should be received according to clinic visit. Any missing samples will be immediately brought to the attention of the clinic staff.

4) The Core Lipid Laboratory staff will use the trial data system to generate bar-coded labels (cryolabels for stored samples) for each tube. The bar code will contain data on participant ID, visit number, type of sample collected and a laboratory ID number.

5) In addition to the data included in the bar code, the sample data table in the trial database will include date of sample collection, the directed analyte(s) for future measurements of stored samples, storage location (for stored samples), as well as information on ultimate disposition and receipt of the sample (date sent, date received, to what laboratory, comments), thereby serving as the laboratory log as to the receipt and disposition of all samples.

6) A sample flow-sheet that shows the number and type of samples to be stored at -80ºC will be used by Core Lipid Laboratory staff to compare and verify against the pre-made requisition forms for each clinic visit.

7) Personnel from the following laboratories will enter their respective laboratory measurements directly into the sample data table: Core Lipid Laboratory, Hematology Laboratory, Rheology Laboratory, Biochemistry Laboratory.

8) The DCC will run routine checks of the laboratory data tables to determine whether and which data may be missing. If data are missing because of shipment loss or sample loss at the laboratory level, back-up storage samples will be re-shipped to the appropriate laboratory so that the analyte can be measured.

9.2.2 Lipid, C-Reactive Protein (CRP) and Hemoglobin A1c Data

Samples will be analyzed by the ARU Core Lipid Laboratory to obtain lipid, CRP and hemoglobin A1c data. The contact person at this laboratory is:

Gail Izumi, (323) 442-1160 or (323) 442-2297, izumi@usc.edu

9.2.2.1 Data Transmission

All data will be entered directly into the trial data system by personnel at the Core Lipid Laboratory. A random sample of hard copies of data will be requested by the DCC for double entry and estimation and monitoring of data entry error rates.

9.2.2.2 Study Data

The following data will be obtained:

A) Lipids (precipitation method):

1) total plasma cholesterol
2) total plasma triglycerides
3) plasma HDL-C
4) calculated VLDL-C
5) calculated LDL-C

LDL-C will be calculated by the Friedewald equation:

LDL = Total Cholesterol - HDL-cholesterol - (Triglycerides/5)

This equation is applicable only when triglycerides are less than 500 mg/dl. When triglycerides are greater than this value, the calculated LDL-C will be set to missing in the lipid database. Calculated LDL-C and VLDL-C (Triglycerides/5) will be computed by the DCC after data entry of laboratory lipids.

B) Metabolic

6) C-reactive protein
7) hemoglobin A1c

9.2.2.3 Data Collection Schedule

1) Lipid data by the precipitation method will be assessed at the screening visit (SV) and v06 and every 6 months on-trial (study visits v12, v18, v24, v30, v36).

2) CRP will be assessed at the screening visit (SV) and v06 and every 6 months on-trial (study visits

v12, v18, v24, v30, v36).

3) Hemoglobin A1c will be obtained at SV, v03 and v06 and every 6 months on-trial (study visits v12, v18, v24, v30 and v36).

9.2.2.4 Clinic Responsibilities

1) Phlebotomist to draw, label and send samples to the Core Lipid Laboratory.

2) Phlebotomist to use trial data system to generate pre-printed requisition forms and tube labels.

3) Phlebotomist to indicate on trial data system date and samples sent to Core Lipid Laboratory.

9.2.2.5 ARU Core Lipid Laboratory Responsibilities

1) Receipt and storage of samples from phlebotomist.

2) Compare and verify samples received against the bar-coded laboratory requisition form that shows which samples should be received according to clinic visit. Immediately notify clinic staff of any missing samples.

3) Use the trial data system to generate bar-coded labels (cryolabels for stored samples) for each tube.

4) Timely and accurate processing of laboratory data.

5) Prepare data sheets with laboratory results and enter laboratory data of lipids, CRP and hemoglobin A1c into the trial database.

6) Perform ongoing laboratory quality control and standardization procedures.

7) Provide DCC with random samples of hard copies of laboratory data on DCC request.

9.2.2.6 DCC Responsibilities

1) Review newly entered laboratory data for completeness and apparent accuracy.

2) Perform double data entry on random 10% of laboratory data. Compare double-entered files (entered at laboratory versus DCC). Calculate and report error rates in weekly data reports. Report unacceptably high error rates to Steering Committee.

3) Monitor timeliness of entry of data into trial data system.

4) Routinely inform laboratory of data not yet entered.

5) Compare participant's data with past data for possible within-subject outliers; generate queries to laboratory and monitor responses.

6) Monitor possible drift in laboratory values over time.

7) Inform laboratory director and laboratory personnel of outliers and data drift in weekly reports.

8) Edit database as appropriate in response to data queries.

9.2.3 Hematology Laboratory Data

Samples will be analyzed by the Hematology Laboratory to obtain coagulation factors (vWF antigen, tPA, PAI-1, D-dimers), platelet aggregation (ADP, collagen, epinephrine), ristocetin cofactor, factor VIII, plasma nattokinase levels and anti-nattokinase antibodies. The contact person at this laboratory is:

Leanne Rochanda, (323) 865-0965, rochanda@usc.edu

9.2.3.1 Data Transmission

All data will be entered directly into the trial data system by personnel at the Hematology Laboratory. A random sample of hard copies of data will be requested by the DCC for double entry and estimation and monitoring of data entry error rates.

9.2.3.2 Study Data

The following data are obtained:

A) Coagulation factors:

1) vWF antigen
2) tPA
3) PAI-1
4) D-dimers
5) factor VIII

B) Platelet aggregation:

6) ADP
7) collagen
8) epinephrine
9) ristocetin cofactor

C) Plasma product levels:

10) nattokinase levels
11) anti-nattokinase antibody

9.2.3.3 Data Collection Schedule

1) Coagulation factors and ristocetin cofactor will be assessed at baseline (v00), week 1, v01, v06 and every 12 months on-trial (study visits v18 and v30).

2) Platelet aggregation will be assessed at baseline (v00), v01 and v06.

3) Nattokinase levels will be determined at baseline (v00) and at every subsequent on-trial clinic visit; every month for the first 6 months and then every 3 months for the remainder of the trial (study visits week 1, v01, v02, v03, v04, v05, v06, v09, v12, v15, v18, v21, v24, v27, v30, v33,

36).

4) Anti-nattokinase antibodies will be determined at baseline (v00), v01, v06 and every 12 months on trial (study visits v18 and v30).

9.2.3.4 Clinic Responsibilities

1) Phlebotomist to draw, label and send samples to Core Lipid Laboratory.

2) Phlebotomist to use trial data system to generate pre-printed requisition form and tube labels.

3) Phlebotomist to indicate on trial data system date and samples sent to Core Lipid Laboratory.

9.2.3.5 ARU Core Lipid Laboratory Responsibilities

1) Receipt of samples from phlebotomist.

2) Compare and verify samples received against the bar-coded laboratory requisition form that shows which samples should be received according to clinic visit. Immediately notify clinic staff of any missing samples.

3) Use the trial data system to generate bar-coded labels (cryolabels for stored samples) for each tube.

4) Deliver samples to Hematology Laboratory and enter delivery into trial data system.

9.2.3.6 Hematology Laboratory Responsibilities

1) Receive samples from Core Lipid Laboratory and enter receipt into trial data system.

2) Timely and accurate processing of laboratory data.

3) Prepare data sheets with laboratory results and enter into the trial database.

4) Perform ongoing laboratory quality control and standardization procedures.

5) Provide DCC with random samples of hard copies of laboratory data on DCC request.

9.2.3.7 DCC Responsibilities

1) Review newly entered laboratory data for completeness and apparent accuracy.

2) Perform double data entry on random 10% of laboratory data. Compare double-entered files (entered at laboratory versus DCC). Calculate and report error rates in weekly data reports. Report unacceptably high error rates to Steering Committee.

3) Monitor timeliness of entry of data into trial data system.

4) Routinely inform laboratory of data not yet entered.

5) Compare participant's data with past data for possible within-subject outliers; generate queries to

laboratory and monitor responses.

6) Monitor possible drift in laboratory values over time.

7) Inform laboratory director and laboratory personnel of outliers and data drift in weekly reports.

8) Edit database as appropriate in response to data queries.

9.2.4 Rheology Laboratory Data

Samples will be analyzed by the Rheology Laboratory to obtain rheology measures (ESR, red blood cell aggregation (plasma), red blood cell aggregability (polymer), plasma viscosity, whole blood viscosity). The contact person at this laboratory is:

Rose Wenby, (323) 442-1268 or (323) 442-1267, rwenby@usc.edu

9.2.4.1 Data Transmission

All data will be entered directly into the trial data system by personnel at the Rheology Laboratory. A random sample of hard copies of data will be requested by the DCC for double entry and estimation and monitoring of data entry error rates.

9.2.4.2 Study Data

The following data will be obtained:

A) Rheology measures:

1) ESR
2) red blood cell aggregation (plasma)
3) red blood cell aggregability (polymer)
4) plasma viscosity
5) whole blood viscosity

9.2.4.3 Data Collection Schedule

1) Rheology measures are assessed at baseline (v00), week 1, v01, v06 and every 12 months on-trial (study visits v18 and v30).

9.2.4.4 Clinic Responsibilities

1) Phlebotomist to draw, label and send samples to Core Lipid Laboratory.

2) Phlebotomist to use trial data system to generate pre-printed requisition form and tube labels.

3) Phlebotomist to indicate on trial data system date and samples sent to Core Lipid Laboratory.

9.2.4.5 ARU Core Laboratory Responsibilities

1) Receipt of samples from phlebotomist.

2) Compare and verify samples received against the bar-coded laboratory requisition form that shows which samples should be received according to clinic visit. Immediately notify clinic staff of any missing samples.

3) Use the trial data system to generate bar-coded labels (cryolabels for stored samples) for each tube.

4) Deliver samples to Rheology Laboratory and enter delivery into trial data system.

9.2.4.6 Rheology Laboratory Responsibilities

1) Receive samples from Core Lipid Laboratory and enter receipt into trial data system.

2) Timely and accurate processing of laboratory data.

3) Prepare data sheets with laboratory results and enter into the trial database.

4) Perform ongoing laboratory quality control and standardization procedures.

5) Provide DCC with random samples of hard copies of laboratory data on DCC request.

9.2.4.7 DCC Responsibilities

1) Review newly entered laboratory data for completeness and apparent accuracy.

2) Perform double data entry on random 10% of laboratory data. Compare double-entered files (entered at laboratory versus DCC). Calculate and report error rates in weekly data reports. Report unacceptably high error rates to Steering Committee.

3) Monitor timeliness of entry of data into trial data system.

4) Routinely inform laboratory of data not yet entered.

5) Compare participant's data with past data for possible within-subject outliers; generate queries to laboratory and monitor responses.

6) Monitor possible drift in laboratory values over time.

7) Inform laboratory director and laboratory personnel of outliers and data drift in weekly reports.

8) Edit database as appropriate in response to data queries.

9.2.5 Biochemistry Laboratory Data

Samples will be analyzed by the Biochemistry Laboratory to obtain plasma inflammatory markers (MCP-1, IL-8, TNFα, IL-1β, IL-10, E-selectin, P-selectin), endothelial injury in-vitro assay inflammatory markers (VCAM-1, ICAM-1) and monocyte activation (FACS) markers (CD11b, CD11c, VLA-4). The contact person at this laboratory is:

Caryn Gonsalves, Ph.D. (323) 442-1528 or (626) 353-8232

9.2.5.1 Data Transmission

All data will be entered directly into the trial data system by personnel at the Biochemistry Laboratory. A random sample of hard copies of data will be requested by the DCC for double entry and estimation and monitoring of data entry error rates.

9.2.5.2 Study Data

The following data will be obtained:

A) Plasma inflammatory markers:

1) MCP-1
2) IL-8
3) TNFα
4) IL-1β
5) IL-10
6) E-selectin
7) P-selectin

B) Endothelial injury in-vitro assay inflammatory markers:

8) VCAM-1
9) ICAM-1

C) Monocyte activation (FACS) markers:

10) CD11b
11) CD11c
12) VLA-4

9.2.5.3 Data Collection Schedule

1) Plasma inflammatory markers are assessed at screening visit (SV), v02, v09 and every 12 months on-trial (study visits v21 and v33).

2) Endothelial injury in-vitro assay inflammatory markers are assessed at baseline (v00), v02, v09 and every 12 months on-trial (study visits v21 and v33).

3) Monocyte activation (FACS) markers are assessed at baseline (v00), v02, v09 and every 12 months on-trial (study visits v21 and v33).

9.2.5.4 Clinic Responsibilities

1) Phlebotomist to draw, label and send samples to Core Lipid Laboratory.

2) Phlebotomist to use trial data system to generate pre-printed requisition forms and tube labels.

3) Phlebotomist to indicate on trial data system date and samples sent to Core Lipid Laboratory.

9.2.5.5 ARU Core Lipid Laboratory Responsibilities

1) Receipt of samples from phlebotomist.

2) Compare and verify samples received against the bar-coded laboratory requisition form that shows which samples should be received according to clinic visit. Immediately notify clinic staff of any missing samples.

3) Use the trial data system to generate bar-coded labels (cryolabels for stored samples) for each tube.

4) Deliver samples to Biochemistry Laboratory and enter delivery into trial data system.

9.2.5.6 Biochemistry Laboratory Responsibilities

1) Receive samples from Core Lipid Laboratory and enter receipt into trial data system.

2) Timely and accurate processing of laboratory data.

3) Prepare data sheets with laboratory results and enter into the trial database.

4) Perform ongoing laboratory quality control and standardization procedures.

5) Provide DCC with random samples of hard copies of laboratory data on DCC request.

9.2.5.7 DCC Responsibilities

1) Review newly entered laboratory data for completeness and apparent accuracy.

2) Perform double data entry on random 10% of laboratory data. Compare double-entered files (entered at laboratory versus DCC). Calculate and report error rates in weekly data report. Report unacceptably high error rates to Steering Committee.

3) Monitor timeliness of entry of data into trial data system.

4) Routinely inform laboratory of data not yet entered.

5) Compare participant's data with past data for possible within-subject outliers; generate queries to laboratory and monitor responses.

6) Monitor possible drift in laboratory values over time.

7) Inform laboratory director and laboratory personnel of outliers and data drift in weekly report.

8) Edit database as appropriate in response to data queries.

9.2.6 Quest Laboratory

Chemistry panel, insulin, CBC, platelet count, PT, aPTT and fibrinogen data are analyzed by Quest. Because these laboratory results constitute clinical safety data are not crucial to blinding of study personnel, these data are transmitted directly from Quest to the study clinic. At the time of receipt, a

photocopy of the laboratory results is made and sent to the DCC.

The contact person in this laboratory is:

Ellie Hetman (818) 737-8347

9.2.6.1 Data Transmission

All data are sent from Quest to the study clinic. These data are reviewed by the research nurse/phlebotomist and abnormal values reported to Dr. Hodis.

9.2.6.2 Study Data

The following data will be obtained:

A) Chemistry:

1) albumin
2) albumin/globulin ratio
3) alkaline phosphatase
4) ALT (SGPT)
5) AST (SGOT)
6) bicarbonate
7) BUN
8) BUN/creatinine ratio
9) calcium
10) chloride
11) creatinine
12) GGTP
13) glucose
14) iron
15) LDH
16) phosphorous
17) potassium
18) sodium
19) total bilirubin
20) total protein
21) uric acid

B) Metabolic

22) insulin

B) Complete blood count:

23) WBC
24) RBC
25) hemoglobin
26) hematocrit
27) MCV
28) MCH
29) MCHC
30) RDW
31) platelet count

C) Clotting times:

32) prothrombin time
33) activated partial thromboplastin time
34) fibrinogen

9.2.6.3 Data Collection Schedule

1) Chemistry panel is determined at screening visit (SV), v01 and every month for 6 months (study visits v02, v03, v04, v05, v06) and then every 6 months on-trial (study visits v12, v18, v24, v30 and v36).

2) Fasting plasma insulin is determined at screening visit (SV), v03, v06 and then every 6 months on-trial (study visits v12, v18, v24, v30 and v36).

3) CBC and platelet count data are assessed at screening visit (SV), at v01 and every month for 6 months (study visits v02, v03, v04, v05, v06) and then every 6 months on-trial (study visits v12, v18, v24, v30 and v36).

4) PT and aPTT are determined screening visit (SV), v01, v03 and v06 and every 6 months on-trial (study visits v12, v18, v24, v30, v36).

5) Fibrinogen is determined at baseline (v00), week 1, v01 and v06 and every 6 months on trial (study visits v12, v18, v24, v30 and v36).

9.2.6.4 Clinic Responsibilities

1) Phlebotomist to draw, label and send samples to Core Lipid Laboratory.

2) Phlebotomist to use trial data system to generate pre-printed requisition forms and tube labels.

3) Phlebotomist to indicate on trial data system date and samples sent to Core Lipid Laboratory.

4) Receive data from Quest laboratory; enter receipt date into trial data system.

5) Use DCC weekly reports to monitor timeliness of receipt of data.

6) Routinely inform Quest laboratory of data not yet received.

7) Send hard copy of data to the DCC data monitor.

8) Review data and inform Dr. Hodis of abnormal values.

9) Enter abnormal values onto Adverse Event log and complete Adverse Event form.

9.2.6.5 ARU Core Lipid Laboratory Responsibilities

1) Receipt of samples from phlebotomist.

2) Compare and verify samples received against the bar-coded laboratory requisition form that shows which samples should be received according to clinic visit. Immediately notify clinic staff of any missing samples.

3) Use the trial data system to generate bar-coded labels (cryolabels for stored samples) for each tube.

4) Place samples in Quest pick up box for messenger to deliver to Quest Laboratory.

5) Enter delivery into trial data system.

9.2.6.6 Quest Laboratory Responsibilities

1) Timely and accurate processing of laboratory data.

2) Send laboratory results to ARU Research Clinic.

9.2.6.7 DCC Responsibilities

1) Receive data from clinic and review for completeness and apparent accuracy.

2) Perform double data entry on random 10% of laboratory data. Compare double-entered files (entered at laboratory versus DCC). Calculate and report error rates in weekly data report. Report unacceptably high error rates to Steering Committee.

3) Monitor timeliness of entry of data into trial data system.

4) Routinely inform clinic of data not yet entered.

5) Compare participant's data with past data for possible within-subject outliers; generate queries to laboratory and monitor responses.

6) Monitor possible drift in laboratory values over time.

7) Inform laboratory director and laboratory personnel of outliers and data drift in weekly reports.

8) Edit database as appropriate in response to data queries.

9) Monitor safety data and prepare routine reports on adverse laboratory values; confirm that abnormal laboratory values have been reported as Adverse Events.

9.2.7 Sample Storage

Sample storage will be conducted by the ARU Core Lipid Laboratory. The contact person at this laboratory is:

Gail Izumi, (323) 442-1160 or (323) 442-2297, izumi@usc.edu

9.2.7.1 Sample Tracking

All sample storage information (i.e., that samples were collected and storage location) is tracked in the trial data system using bar coding of tubes.

9.2.7.2 Study Data

Serum and plasma (EDTA, citrate) samples are obtained for measurements to be determined at a future date:

1) Fasting homocysteine and B-vitamins (vitamins B_6 and B_{12} and folate)

2) Apolipoproteins
    a) Apo AI
    b) Apo B
    c) Apo E
    d) Apo CIII (total plasma)
    e) Apo CIII-HP
    f) Apo CIII-HS
    g) Apo CIII-HS/Apo CIII-HP ratio

3) Lipoprotein particles:
    a) LP-B
    b) LP-B_c
    c) LP-B:C + LP-B:C:E
    d) LP-AII:B:C:D:E (LP-AII:B complex)
    e) LP-AI
    f) LP-AI:AII

4) Lp(a)

5) Nitric oxide

6) Coagulation factors
    a) Activated protein C (APC)

7) Hormones
    a) Estradiol
    b) Estrone
    c) Estrone-sulfate
    d) Testosterone
    e) Progesterone
    f) Sex hormone binding globulin
    g) Prolactin

8) Bone markers
    a) Bone-specific alkaline phosphatase
    b) Osteocalcin
    c) Osteoprotegrin
    d) RANKL

9) Buffy coat

10) Apolipoprotein E genotype

9.2.7.3 Data Collection Schedule

Blood samples for storage will be collected at baseline (v00) and at every subsequent clinic visit on-trial (study visits week 1, v01, v02, v03, v04, v05, v06, v09, v12, v15, v18, v21, v24, v27, v30, v33 and v36).

9.2.7.4 Clinic Responsibilities

1) Phlebotomist to draw, label and send serum and plasma samples to Core Lipid Laboratory.
2) Phlebotomist to use trial data system to generate pre-printed requisition forms and tube labels.

3) Phlebotomist to indicate on trial data system date and samples sent to Core Lipid Laboratory.

9.2.7.5 ARU Core Lipid Laboratory Responsibilities

1) Receipt of samples from phlebotomist.

2) Compare and verify samples received against the bar-coded laboratory requisition form that shows which samples should be received according to clinic visit. Immediately notify clinic staff of any missing samples.

3) Use the trial data system to generate bar-coded labels (cryolabels for stored samples) for each tube. Storage samples contain labels with participant initials, participant identification number, date of sample collection, visit number, laboratory ID number, study name (NAPS) and the vial contents (EDTA Plasma, Citrated Plasma, Serum and Buffy Coat) and what future determination is to be performed with the sample.

4) Timely aliquoting and properly storing plasma, serum and buffy coat samples.

5) Store samples at -80^{o}C in Core Lipid Laboratory.

6) Enter all samples received for storage into trial data system.

7) Create and maintain storage maps for all stored samples.

9.3 Primary Study End Point Data: Carotid Ultrasound Data

The following ultrasound data will be obtained:

1) Primary ultrasound end point measurement will be obtained by:

   B-mode ultrasonograms in the posterior view for intima-media thickness (CIMT) measurements of the distal centimeter of the right common carotid artery far wall.

2) Co-primary ultrasound end point measures will be obtained by:

   B-mode ultrasonograms in the posterior view for average diameter measurements during systole and diastole of the distal centimeter of the right common carotid artery lumen. The luminal dimensions will be used to calculate the following stiffness measurements:

   a) Arterial distensibility = $[2(D_s\text{-}D_d)/D_d]/PP$

   b) Arterial compliance = $(D^2_s\text{-}D^2_d)/PP$

   where, D_s = average diameter in systole; D_d = average diameter in diastole; PP = pulse pressure = systolic BP – diastolic BP

9.3.1 Data Transmission

Ultrasound measurements will be obtained by the study ultrasonographers. Data will be routinely sent to the DCC electronically. SV ultrasounds will be immediately processed for the primary end point measurement; SV CIMT data will be sent to the DCC to perform the stratified randomization. Remaining ultrasounds will be processed in batch as the participant completes the trial.

9.3.2 Study Data

1) For each study ultrasound image, the following measures will be obtained:

    a) Carotid artery IMT, averaged over the distal centimeter of the right common carotid artery far wall (primary trial end point).

    b) Carotid artery luminal diameter, averaged over the distal centimeter of the right common carotid artery lumen during systole and diastole. Carotid artery diameters will be used in conjunction with pulse pressure to calculate the two measures of arterial stiffness (co-primary trial end points – arterial distensibility and arterial compliance).

9.3.3 Data Collection Schedule

Study ultrasounds will be obtained at two pre-randomization visits, SV and v00 and every six months on-trial (study visits v06, v12, v18, v24, v30 and v36).

9.3.4 Responsibilities of Ultrasound Laboratory

1) Follow protocol for ultrasound acquisition and obtain carotid ultrasounds on each participant at scheduled study visits.

2) Follow protocol for analysis of ultrasound images and obtain study measures.

3) Regular backup of study data.

4) Immediately process SV ultrasounds for right carotid artery IMT measurement and transmit data to DCC.

5) Use data reports from DCC identifying participants who have completed the trial. Process completed participant ultrasound data and electronically transmit study data to the DCC.

6) Work with the DCC personnel in rectifying data outliers and errors.

9.3.5 DCC Responsibilities

1) Routinely send reports of completed participants to ultrasound laboratory.

2) Receive data electronically from study ultrasonographers.

3) Review new ultrasound data for completeness. This will include identification of any missing ultrasound records as well as identification of missing/inaccurate data within an ultrasound record.

4) Review new ultrasound data for accuracy. This will include identification of errors in study visit numbers, dates and possible outliers in ultrasound data.

5) Compare participant's data with previous ultrasound data to monitor possible errors in obtaining ultrasound images or in analysis of these images.

6) Work with study ultrasonographers in rectifying missing/inaccurate data.

7) Once new ultrasound data are deemed clean, update master ultrasound database.

9.4 Cognition

9.4.1 Data Transmission

Cognitive measurements will be obtained by the cognitive tester. The cognitive tester will complete the cognitive test scores, complete the summary score form and enter the summary scores into the trial database.

9.4.2 Study Data

Cognitive function will be determined with a neuropsychological battery designed to evaluate 7 cognitive domains including: attention, concentration, working memory, executive function; visuospatial/visuoconstructive skills; naming/semantic memory; and verbal and nonverbal episodic memory. Neuropsychological tests and corresponding cognitive skills are the following:

1) Symbol Digit Modalities Test
2) Trail Making Test, Part B
3) Shipley Institute of Living Scale, Abstraction scale
4) Letter-Number Sequencing
5) Block Design
6) Judgment of Line Orientation
7) Animal Naming
8) Boston Naming Test
9) California Verbal Learning Test
10) East Boston Memory Test
11) Faces I and II
12) Benton Visual Retention Test
13) Stroop Color and Word Test
14) Wechsler Test of Adult Reading (verbal intelligence quotient)

9.4.3 Data Collection Schedule

Cognitive assessments will be performed at v00 (baseline) and at study visits v18 and v36.

9.4.4 Responsibilities of Cognitive Tester

1) Follow protocol for cognitive testing and obtain cognition data on each participant at scheduled study visits.

2) Follow protocol for analysis and summarization of cognitive data and obtain study measures.

3) Enter cognitive summary data into trial data system as it is completed.

4) Work with the DCC personnel in rectifying data errors.

9.4.5 DCC Responsibilities

1) Monitor timeliness of data entry by study cognitive tester.

2) Review newly entered cognition data for completeness. This will include identification of any missing cognition data as well as identification of missing/inaccurate data within a cognitive assessment.

3) Review new cognitive data for accuracy. This will include identification of errors in study visit numbers, dates and possible outliers in cognitive data.

4) Work with study cognitive tester in rectifying missing/inaccurate data.

5) Once new cognition data are deemed clean, update master cognition database.

9.5 DCC Tasks

9.5.1 Data Entry

Prior to data entry, all forms will be inventoried and undergo a 2-step review (study coordinator and data monitor) for completeness and apparent accuracy; incomplete/inaccurate forms are returned to the study coordinator for rectification. Key features of the data entry system at the time of data entry will include: data entry screens that mimic the data forms, must-enter fields for key variables and range checks. A random sample of study data will be double entered by 2 individuals and program-compared to monitor data entry error rates and to identify need for remediation of data entry processes. Data from external laboratories will be entered directly into the trial data system by personnel at the external laboratories. Hard copies of the laboratory data will also be routinely obtained by the DCC to verify the accuracy of data entry.

9.5.2 Study Reports

9.5.2.1 Recruitment and Study Progress

For each meeting of study personnel as well as the External Data Safety Monitoring Board (EDSMB), a report of recruitment and participant progress throughout the study will be generated. The recruitment portion of the report will include numbers of participants prescreened by telephone and reasons for ineligibility, numbers of participants screened in the ARU research clinic and reasons for ineligibility and numbers of participants randomized. The study progress portion of the report will include numbers of participants who have completed various phases of the study up to the date of the report, numbers of study dropouts and reasons for dropouts.

9.5.2.2 Ideal Clinic Visit Schedule

As each participant is randomized, a schedule of "ideal" visit dates and visit windows will be generated by the trial data system. An ideal visit date is defined to be the exact date on which the clinic appointment should occur, given the participant's randomization date and the study visit schedule. The

visit windows will be computed as the ideal visit date, plus or minus fourteen (14) days.

Each participant's ideal visit schedule will be generated at randomization. The ideal schedule will be available to clinic personnel through the trial data system, with an option to print the schedule as needed. The ideal visit schedule will be used to monitor missed visits and compliance to visit schedule (in/out of window visits).

9.5.2.3 Data Quality

The data quality report will be generated on a monthly basis and for the EDSMB, and will include data on the numbers of errors in completion of study forms. Inaccuracies as well as incompleteness in data forms will be considered errors and will be reported separately. Errors will be computed by study form and will be presented since the last report (i.e., in the past month) and on a cumulative basis.

The data quality report will also include reports of data queries, including number of queries responded to, timeliness of response and number of outstanding data queries.

This report will also include summarization of missed visits and visits outside of the ideal visit window.

9.5.2.4 Participant Safety and Adverse Events

For the EDSMB, the DCC will generate a safety report enumerating the following events, both on a cumulative basis (from the start of the study to present) and new events in the past year. For the EDSMB, adverse events will be presented by blinded study group.

1) Laboratory chemistry values out of range (enumerating abnormally low and high values separately). This will be separately reported for first time versus repeated abnormalities.

2) Triglycerides >500 mg/dL. First time versus repeated elevations will be separately reported.

3) Out of range coagulation and hemostatic factors, PT, aPTT, fibrinogen and platelet count (enumerating abnormally low and high values separately). This will be separately reported for first time versus repeated abnormalities.

4) Abnormal bleeding events ascertained from the Bleeding Assessment Questionnaire **[FORMS 6a, 6b]**.

5) Abnormal urine dipstick.

6) Major cardiovascular events: fatal/nonfatal MI, sudden coronary death, hospitalization for unstable angina, and revascularization procedures (CABG and PTCA).

7) Major cerebrovascular events: TIA, RIND, CVA.

8) Peripheral vascular events including surgical reconstruction and bypass.

9) Deep vein thrombosis

10) Pulmonary thromboembolism

11) Cancers

12) Deaths

13) Other adverse events; serious and non-serious

9.5.2.5 Screening and On-Trial Lipid Levels

As each participant completes all screening visits, clinic personnel will use the trial data system to generate a report of lipid levels. On-trial LDL-C levels will also be generated as needed from the trial data system by clinic personnel.

9.5.2.6 Participant Compliance

For each participant, compliance to the study products will be monitored and summarized in a monthly report. Non-compliance will be reported to the principal investigator. Compliance will be assessed by the following:

1) Compliance to active nattokinase and placebo nattokinase will be assessed by capsule count. Compliance will be defined as $\geq$80% of study product consumed. Capsule count will be the primary determinant of compliance.

2) Overall study compliance will be determined by number of missed study visits.

9.5.3 Data Management

9.5.3.1 Study Databases

The following data tables will comprise the study data:

1) Telephone screening

2) Demographic information

3) Cardiovascular disease history

4) Bleeding assessment questionnaire

5) Active/passive smoking history

6) Alcohol and other drug history

7) Reproductive history (female)

8) Medical history

9) Rose questionnaire

10) Claudication questionnaire

11) Cardiovascular disease risk factors

12) Non-study medications

13) Nutraceuticals and supplements

14) Vital signs (blood pressure, pulse rate); weight and height

15) Final eligibility checklist

16) Dietary food frequency

17) Nutritional supplements

18) Soy Food Questionnaire

19) Physical activity

20) CES-D Scale

21) Cognitive assessment data

22) Waist:hip ratio

23) ECG

24) Study product compliance

25) Termination/Dropout

26) Carotid ultrasound data

27) Closeout

28) Adverse events including: on-trial cardiovascular, cerebrovascular, peripheral vascular events; on-trial non-vascular events; on-trial deaths.

29) Quest data: chemistry panel, insulin, complete blood count, platelet count, prothrombin time, activated partial thromboplastin time, fibrinogen

30) Hematology laboratory data: coagulation factors (vWF antigen, tPA, PA-1, D-dimers, platelet aggregation (ADT, Collagen, Epinephrine), ristocetin cofactor, factor VIII

31) Nattokinase plasma levels, anti-nattokinase antibodies

32) Rheology laboratory data: erythrocyte sdimentation rate, red blood cell aggregation (plasma), red blood cell aggregability (polymer), plasma viscosity, whole blood viscosity.

33) Biochemistry laboratory data: plasma inflammatory markers (MCP-1, IL-8, TNFα, IL-1β, IL-10, E-selectin, P-selectin), endothelial injury in-vitro assay inflammatory markers (VCAM-1, ICAM-1) and monocyte activation (FACS) markers (CD11b, CD11c, VLA-4)

34) ARU Core Lipid Laboratory data: lipids, C-reactive protein, hemoglobin A1c

35) Sample and buffy coat storage inventory

36) ApoE genotype

9.5.3.2 Data Dictionary

The DCC will maintain a data dictionary that contains the following:

1) Copies of all study forms. This will include a historical accounting of all study forms. Thus, all versions of a study form that are used in the study will be kept with the date of use of each version indicated on that form.

2) A notated copy of each study form that lists the trial database on which these data are entered and the variable names used for each variable on the form.

3) Coding lists that will detail the variable name (and associated forms) for which the coding is used.

4) A complete listing of all study variables that includes the following information:

    a) Variable name (database system name)

    b) Variable description

    c) Form from which the variable is derived

    d) Data table in which the variable is entered

    e) Notation of relevant variable codes (or reference to the appropriate coding list)

9.5.3.3 Quality Control

Quality control of the data will include the following:

1) Clinical databases:

    a) Clinic coordinator to review all study forms for completeness and accuracy prior to sending to DCC.

    b) Study programmers and data clerk to review all data forms for completeness and accuracy prior to data entry.

    c) Run error check routines on newly entered data. These programs will identify logical errors in the data, missing data, out-of-range or implausible values, and data outliers (both within a given visit and in comparison to a subject's earlier visits).

d) Based on b and c above, generate data queries in the trial data system to the appropriate study personnel.

e) Monitor resolution and completeness of responses to data queries.

f) Edit databases.

2) Laboratory and end point data:

a) Monitor measures for any apparent longitudinal data drift.

b) Monitor timeliness of receipt of laboratory data and end point data and identify outstanding laboratory samples.

c) Perform error check programs and data queries as in section 1 above.

d) Edit databases.

3) Generate routine quality control reports that will include reporting (by study month) of the numbers of errors in completion of study forms, by form, as well as timeliness and completeness of response to data queries.

4) Monitor study for protocol violations.

5) Double entry of random key study data for monitoring of key stroke error rate.

6) Biannual audit of random data sample for comparison of hard copies to complementary databases.

7) Inform Data Safety Monitoring Board and principal investigator of ongoing/repeated irregularities in any study data.

9.5.3.4 Backup of Databases

All study databases will be backed up to secure servers on a daily basis. Each data file will also be copied to an external hard drive on a weekly basis and stored off-campus. External hard drive copies of the data base will capture all versions of the data base for the previous 4 weeks.

9.6 Statistical Analysis of Study Data

9.6.1 Preliminary Analyses

Standard procedures, including frequency distributions and histograms, summary statistics (means, medians, variances, ranges), and EDA procedures (box plots, stem and leaf displays), will be used to describe the distributions of data, determine normalizing transformations (if necessary), and identify data outliers. These analyses will be conducted for all study outcome measures as well as study covariates. If continuous outcome variables are not normally distributed, normalizing transformations will be considered so that parametric analytic methods may be used; non-parametric methods may be used as an alternative.

9.6.2 Baseline Comparability

All participants will be compared between treatment arms with respect to demographic (e.g., age, race, gender), cardiac and other medical history factors, cardiovascular risk factors (including blood pressure, smoking status), dietary factors, baseline CIMT, vascular stiffness, cognition, plasma insulin, lipids/CRP, hemoglobin A1c, coagulation factors, rheology parameters and inflammatory variables. Analytical methods will include two-sample t-tests (or a non-parametric analog) for continuous variables and chi-square procedures for categorical variables. The significance level for all analyses will be set at a two-sided α=0.05.

If multiple baseline laboratory measures are completed (e.g., at SV and at v00), the baseline value will be computed as the average of the screening measure and the v00 visit determined prior to intervention. All treatment group comparisons on baseline variables will be performed for the entire randomized study sample, for the group of participants with repeat ultrasounds, and for the group of participants without repeat ultrasounds.

9.6.3 Comparison of On-trial Changes in Laboratory and Lifestyle Variables

Treatment groups will be compared with respect to on-trial levels and on-trial changes in plasma insulin, lipids/CRP, hemoglobin A1c, coagulation factors, rheology parameters and inflammatory variables. Study groups will also be compared for on-trial levels and on-trial changes of other factors that might confound the analysis of primary end point differences. These will include analysis of dietary factors (e.g., total calories, and total calorie-adjusted calories from total, saturated, polyunsaturated, and monounsaturated fats, calories from protein, calories from carbohydrates, and dietary genistein, daidzein, and glycitein), physical activity, cardiovascular risk factors (including blood pressure, smoking status), and use of relevant nonstudy medications (including antihypertensive and lipid-lowering medications). For descriptive purposes, on-trial laboratory, dietary, clinical, and physical activity variables will be computed as the average of all on-trial determinations, weighted by the length of time between determinations. On-trial smoking will be compared as percentage of smokers versus percentage abstainers; average on-trial smoking levels (cigarettes per day) will also be computed for smokers as the average of all on-trial determinations as above. For all laboratory and clinical measures, a change variable will be computed at each on-trial measurement time as: on-trial value minus baseline value. Changes in laboratory and clinical variables from baseline will be compared within each treatment arm using generalized estimating equations to account for the repeated measurements over the study period; the model-specified correlation structure of the repeated measures will be determined by examination of the observed correlation structure. The test that the model intercept differs from 0 will comprise the overall test for significant change from baseline; incorporation of covariates for time periods of the study will test for differential change from baseline over the study period. Comparison of on-trial levels and changes in laboratory and clinical variables across treatment groups will also use generalized estimating equations, with an added covariate for randomized treatment group; interaction terms of treatment group-by-study time period will test whether treatment group differences vary over the study period. Compliance with the study product will be compared across treatment groups, also using generalized estimating equations as described. All analyses will be performed for the entire study sample and for the group of participants whose overall study product compliance is >80%. The significance level for all analyses will be set at a two-sided α=0.05.

9.6.4 Evaluation of Potential Biases and Study Validity Introduced by Differential Dropout

Possible biases introduced by differential study dropout will be evaluated by comparing:

1) Baseline variables (Section 9.6.2) in participants with and without repeat ultrasounds, and in participants with and without repeat cognitive testing. Analyses will be performed by two-sample t-tests, Wilcoxon rank sum, or chi-square procedures as appropriate.

2) On-trial variables (Section 9.6.3) evaluated within treatment group in participants with and without repeat ultrasounds, and in participants with and without repeat cognitive testing. Analyses will be performed by analysis of variance (factors for repeat ultrasound/cognitive test (yes/no) and treatment group) and Mantel-Haenszel chi-square (with stratification on treatment group) procedures.

3) Participants without repeat ultrasounds (or cognitive testing) by treatment group on baseline and on-trial variables (Sections 9.6.2 and 9.6.3). Analyses will be performed by two-sample t-test, Wilcoxon rank sum or chi-square procedures as appropriate.

9.6.5 Analysis of Study Outcomes

All analyses of study outcomes will be conducted on an intent-to-treat basis. All participants with baseline and any follow-up end point data, regardless of their dropout and compliance status, will be analyzed according to their randomized treatment group. Statistical assumptions of the planned analyses will be verified. Alternative analyses will be performed if the assumptions of the planned analyses are not justified. Key assumptions that will be verified are: 1) normality of primary end point; 2) homogeneity of variances between treatment groups; and, 3) deviations from linearity in the regression of end point measurements (CIMT, arterial stiffness, cognition) against time on-study.

9.6.5.1 Primary Efficacy Analysis: Carotid Artery IMT Analyses

The primary end point will be the per-subject rate of change in the right distal CCA far wall IMT. The rate of change in the right distal CCA far wall CIMT will be computed for each participant by fitting a regression line of CIMT on years since baseline ultrasound. The estimated slope of the regression line will be used as that subject's CIMT rate of change. Analyses of trial end points will be performed on the intent-to-treat sample, which will be defined as all randomized participants who had a baseline and at least 1 follow-up CIMT measurement taken after randomization.

The statistical approach outlined above will be accomplished using mixed effects models for longitudinal data, with the randomization stratification variable (dichotomized baseline CIMT) used as a covariate. Each CIMT measure will be included as a dependent variable. The regression coefficient associated with the model covariate of time on-study will estimate the average group progression rate of CIMT (in mm/year). Random effects terms for the regression intercept and slope (change over time) will allow for individual participant deviations from their group average in baseline CIMT and CIMT rate of change, respectively. The statistical test for differences in the CIMT rate by treatment group will be accomplished by adding a treatment group by time on-study interaction term. The significance of the regression coefficient associated with this interaction term is the test that the CIMT rates differ by treatment group. We will also evaluate baseline plasma levels of insulin, lipids/CRP, hemoglobin A1c, coagulation factors, rheology parameters and inflammatory variables, dietary nutrients, smoking, blood pressure, physical activity and use of non-study medications as potential confounders. If any of these variables are found to be confounders of treatment-rate of CIMT relationship, they will be included as

covariates in the analysis.

9.6.5.2 Co-Primary Efficacy Analysis: Carotid Artery Stiffness Analyses

Statistical methods to evaluate the arterial stiffness outcomes will mirror those specified for the CIMT analyses above. A normalizing transformation will be applied to the arterial stiffness measures prior to analysis if required.

9.6.5.3 Secondary Efficacy Analysis: Cognition Analyses

Three composite cognitive measures will be used to test for randomized treatment group differences in cognition; each composite will be considered as co-secondary endpoints. (1) The global composite will use scores from all neuropsychological tests. (2) The verbal memory composite will use the California Verbal Learning Test and East Boston Memory Test immediate and delayed recall scores. (3) The executive function composite (determined from principal components of prior trial data) will use scores from the Symbol Digit Modalities Test, the Trail Making Test, the Shipley Abstraction scale, Letter-Number Sequencing, and category fluency. The composites will be calculated as the average of component standardized scores weighted by the inverse interest correlation matrix. Absolute values of change from baseline on each cognitive endpoint will be calculated at both post-randomization cognitive follow-ups (18 and 36 months). General estimating equation models will be used to analyze the repeated measures of change in the cognitive endpoints. The primary independent variables will be randomized treatment group; covariates will include the CIMT randomization stratification variable as well as the baseline value of the cognitive endpoint. Differences in cognitive change will be tested by treatment group. Further analyses will test for interactions with test time (18 versus 36 months) to evaluate whether treatment effects (treatment by time interaction) vary by follow-up time.

9.6.5.4 Analysis of Clinical Events, Adverse Experiences, and Safety Measures

The following clinical events will be tabulated and compared between treatment groups using chi-square or Fisher's exact methods:

1) Major cardiovascular events: fatal/nonfatal MI, sudden coronary death, hospitalization for unstable angina, and revascularization procedures (CABG and PTCA).

2) Major cerebrovascular events: TIA, RIND, CVA.

3) Deep vein thrombosis.

4) Pulmonary thromboembolism.

5) Incident cancers.

6) All cause mortality and cause-specific mortality, including cardiovascular, cerebrovascular, peripheral vascular, cancer, other medical, and non-medical and non-coronary mortality.

7) The treatment groups will also be compared for standard clinical and laboratory safety tests.

In the analysis of laboratory safety variables, treatment groups will be compared with regard to proportions of participants with clinically meaningful changes (either increase or decreases from normal range). Analyses of these data will be done using a chi-square test or Mantel-Haenszel test that allows for stratification, as appropriate.

9.6.6 Interim Analysis

At each meeting of the EDSMB, summary tables of safety data (Section 9.5.2.4) without any formal statistical analyses will be reviewed. The EDSMB will be masked to treatment assignment (i.e., the treatments will be labeled as A and B). If in the best judgment of the EDSMB, trends or patterns are observed in the safety data, the EDSMB may request a formal statistical analysis of the data. The Lan-DeMets rule to determine the current (and all future) alpha levels will be used. Early termination is predicated on attaining statistical significance on major safety issues to be defined by the EDSMB.

Interim analyses will not be performed on the primary (CIMT, arterial stiffness) and secondary (cognition) trial outcomes.

9.6.7 Ancillary Statistical Analyses

9.6.7.1 Within-Group Analyses

The effects of on-trial end point variables other than treatment modality on the per-subject rate of change in CIMT, arterial stiffness and cognition will be separately assessed. The parameters to be tested will include laboratory data (plasma insulin, lipids/CRP, hemoglobin A1c, coagulation factors, rheology parameters, inflammatory variables and nattokinase levels), clinical variables (blood pressure, body mass, waist:hip ratio, etc.), and lifestyle variables (dietary intake, physical activity, smoking, etc.). Baseline and on-trial values of these data will be collected in order to address questions of clinical significance within each treatment group such as: 1) which baseline characteristics have differential effectiveness on the rate of change in CIMT; and, 2) what is the role of changes in these variables on the rate of change in CIMT. These analyses will utilize mixed effects models and generalizing estimating equations with CIMT (arterial stiffness, cognition) as the dependent variable. Analyses will be conducted in the combined sample with a covariate included to control for treatment group. Interaction terms with treatment group will be introduced to test whether relationships between independent variables and the trial end point are of equal magnitude between the 2 study groups. Experiment-wise error rates in these analyses will be controlled. All participants contributing at least one follow-up ultrasound (or cognitive test) will be included in these analyses.

9.6.7.2 Modifiers of Treatment Efficacy

The extent to which treatment effects are modified by other subject-specific factors will be examined. Potential treatment effect modifiers include baseline CIMT, smoking status (on-trial smokers versus non-smokers), BMI, age and gender. The statistical significance of differences in the treatment effect by subgroup will be tested by inclusion of an interaction term (treatment by subgroup) in the efficacy models detailed above. For descriptive comparisons, treatment effect sizes will be computed for each level of the possible modifier.

9.6.7.3 Mediators of Treatment Efficacy

We will test the hypothesis that nattokinase treatment-related reductions in atherosclerosis and cognitive measures will be mediated through hemostatic, fibrinolytic and hemorheological factors as well as attenuation of inflammation, monocyte activation, vascular endothelium injury and activation of

vascular endothelium by circulating monocytes. We will use structural equation models to test for mediation. Observed outcome variables will be changes in CIMT (annualized change rate), stiffness (annualized change rate) or cognitive composite scores. For each of the potential mediator that shows a significant treatment effect, we will test the direct effects of randomized treatment and the indirect effects of treatment (i.e., treatment acting through the potential mediators). Bootstrapping will be used to obtain 95% confidence intervals on the direct and indirect effects; confidence intervals that exclude the value of 0 will be considered as evidence for statistical significance.

9.6.7.4 Correlations in Atherosclerosis and Cognitive Outcome Measures

We will use mixed effects models to test the hypothesis that the reduction in subclinical atherosclerosis progression and cognitive decline with nattokinase will be correlated. The dependent variable will be each value of the CIMT (or arterial stiffness) measure at each study time point. The primary independent variables will be the 18-month and 36-month change in cognitive composites. Interaction terms of time on study with the cognitive composite change scores will test whether the rate of change in CIMT (or arterial stiffness) is associated with the change in cognition.