WOMEN'S INTERAGENCY HIV STUDY

SECTION 30: CARDIOVASCULAR SUBSTUDY PROTOCOL

A. SPECIFIC AIMS

Specific aims of the WIHS *Cardiovascular Disease (CVD) Substudy* relate to subclinical atherosclerosis, CVD risk factors, and CVD events among HIV-infected and HIV at-risk women.

SUBCLINICAL ATHEROSCLEROSIS

1. To characterize subclinical atherosclerosis markers, including carotid artery intima-media thickness (IMT), carotid stiffness, and carotid atherosclerotic lesions among HIV-infected women and HIV-negative controls. Comparisons with men (both HIV-infected and HIV-negative) will be performed using measurements made under a similar protocol in the Multicenter AIDS Cohort Study (MACS) cohort.
2. To determine the relationship between baseline carotid IMT, stiffness, and atherosclerotic lesions and parameters of HIV disease, including HIV-1 viral RNA level, CD4 cell count, history of clinical AIDS-defining illness, and the inflammatory, immunologic, metabolic, and anthropometric changes associated with HIV infection.
3. To determine the relationship between baseline carotid IMT, stiffness, and atherosclerotic lesions and antiretroviral medication regimens: (1) protease inhibitor-containing HAART therapy (PIT); (2) antiretroviral therapy without PIT; (3) no HAART; (4) specific antiretroviral medications.
4. To assess the associations of baseline carotid IMT, stiffness, and atherosclerotic lesions among HIV-infected women with traditional and novel CVD risk factors, including age, race, smoking, hypertension, diabetes, lipids, inflammation markers, hyperglycemia, hyperinsulinemia, adiponectin, and leptin, and to examine whether these associations differ from those seen among HIV-negative controls.
5. To measure the progression of carotid IMT and stiffness over three years of follow up among HIV-infected and HIV-negative women, and to identify predictors of IMT and stiffness progression including:
    a. HIV parameters (serostatus, HIV-1 RNA level, CD4 cell count, AIDS-defining illness);
    b. inflammatory, immunologic, metabolic, and anthropometric changes associated with HIV infection;
    c. antiretroviral medication use;
    d. sex (through comparisons with the MACS cohort);
    e. other CVD risk factors (e.g., age, race, smoking, hypertension, lipids, glucose, insulin).

CVD RISK FACTORS

6. To characterize the distribution and natural history of traditional CVD risk factors (including hypertension, hypercholesterolemia, hypertriglyceridemia, obesity and diabetes) among HIV-infected women, and to compare risk factors versus HIV-negative controls.

7. To examine the inter-correlation among CVD risk factors and identify variables that predict the changes in risk factors over time among HIV-infected women.

8. Among HIV-infected women with CVD risk factors, to describe the proportion in which risk factors are detected, drug treated, and controlled. Detection, treatment, and control of risk factors will also be compared between HIV-infected women and HIV-negative controls.

CLINICAL CVD EVENTS

9. To analyze CVD events in prospective and nested case-control analyses in relation to subclinical carotid atherosclerosis, HIV/AIDS status, antiretroviral medications and CVD risk factors.

B. OVERVIEW OF WIHS CARDIOVASCULAR SUBSTUDY

The Cardiovascular Substudy will involve additional data collection from all consenting WIHS participants. The three data collection components are: (1) carotid artery ultrasound (US), (2) measurement of a metabolic panel, and (3) collection of fasting CVD blood samples. Refer to Appendix C for the planned overall study timetable.

1. CAROTID ULTRASOUND

   All WIHS women will be invited to receive a baseline carotid ultrasound (US) exam. For a subset of women (~100–150 per site), three follow-up US exams will be performed at two to three year intervals. Standardized interpretations of ultrasound scans will be made under the direction of Dr. Howard Hodis at the USC Ultrasound Reading Center.

2. METABOLIC PANEL

   Using the bloods obtained at the core visit, a metabolic panel will be measured for all WIHS participants as specified in the laboratory protocol (see **MOO, Section 10**). If the subject is fasting at the core visit, a *fasting metabolic panel* will be measured: total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), calculated low-density lipoprotein cholesterol (LDL-C), triglycerides (TRIG), glucose and insulin. If the subject is not fasting at the core visit, a *non-fasting metabolic panel* will be measured: TC, HDL-C, and direct LDL-C.

   CVD Substudy participants who are non-fasting at the core visit at which the metabolic panel is to be measured may (depending on the visit number) be asked to return for a separate fasting visit so that metabolic panel specimens can be collected.

NOTE: In addition to the above assays, direct LDL was measured for all participants (regardless of fasting status) during visits 20 and 21, and HgA1c was measured during visits 13 through 23 and 33+ (odd visits only) for all participants (regardless of fasting status).

In addition, retrospective testing of insulin and lipids at visits 29 and 30 has been completed. Lipid and insulin assays were performed on reposited specimens for women who attended their core visit fasting for visits 29 and 30.

Criteria for visit 29 testing:

a. Fasting at core visit

b. Serum separated within eight hours or less

c. Results not already obtained via Metabolic Substudy (*MS02*)

Criteria for visit 30 testing:

a. Fasting at core visit

b. Serum separated within eight hours or less

c. Results not already obtained via Metabolic Substudy

d. Participant completed form *NC06* as part of NC Battery

3. FASTING CVD BLOOD SAMPLES

NOTE: Fasting CVD blood samples were collected from women participating in the Cardiovascular Substudy during visits 20 through 23 only.

At least once per one-year interval (during visits 20 through 23), women participating in the Cardiovascular Substudy will provide a CVD blood sample, either at a core visit, an ultrasound visit or a separate visit, obtained after an eight-hour fast. Fasting CVD samples will be drawn up to twice a year (twice for women who are fasting at both core visits; once for women who are fasting at only one core visit or who make a separate visit for fasting blood draw).

C. BACKGROUND AND SIGNIFICANCE

1. CORONARY HEART DISEASE (CHD) AND STROKE IN HIV-INFECTED PATIENTS

Multiple published reports have documented the occurrence of premature CHD or other vascular complications in HIV-infected patients (Henry 1998; Behrens 1998; Gallet 1998; Vittecoq 1998). A recent retrospective study of 3,000 HIV-infected patients followed in 10 U.S. cities compared the rate of myocardial infarction (MI) among patients with history of PI use, versus those who had not been exposed to PI-containing therapy. Beginning in 1996 (when PI's first became widely available), an increased risk of MI was found among patients who had taken these agents. While preliminary and retrospective in nature, the HIV Outpatient Study (HOPS) has clearly shown an increased risk of CHD among HIV-infected patients who have taken PI therapy (Holmberg 2001).

Similar conclusions were drawn by Hodder et al. (Hodder 2001) who evaluated all claims codes from the California Medicaid population, which has a large population of HIV-infected individuals; only those patients who were on therapy for their HIV disease were included. Although the database did not contain information on other risk factors for CHD, young HIV-infected men appeared to have an increased incidence of coronary heart disease, as opposed to the non-infected, age-matched controls. While these data are preliminary, it is clear that CHD may be a major emerging problem in HIV-infected individuals. Recent data have also suggested a high rate of stroke among patients with

AIDS (Cole 2004), although the stroke risk associated with HIV infection *per se* versus antiretroviral drug therapies has not been defined.

2. LIPIDS AND CORONARY HEART DISEASE

While LDL (Gordon 1981; Castelli 1986; Schmidt 1985; Hamsten 1986; Newman 1986) and VLDL are atherogenic (Reardon 1985; Steiner 1987), HDL appears to protect against coronary heart disease (Miller 1975; Gordon 1977). Large-scale epidemiologic studies with prolonged periods of follow-up have demonstrated a strong relationship between higher cholesterol levels in young men and an increased risk of coronary heart disease in middle age (Anderson 1987; Klag 1993). The correlation between elevated cholesterol levels and increased risk of death due to CHD has also been demonstrated in women (Jacobs 1992). While most studies have shown that elevation of triglycerides is associated with an increased risk of CHD (Austin 1991; Brunzell 1989), not all studies have confirmed this finding on multivariate analysis, after adjustment for cholesterol, LDL-C and HDL-C levels (Hulley 1980).

3. NON-LIPID CVD RISK FACTORS

Cigarette smoking is a strong risk factor for CHD and stroke among both men and women (LaCroix 1991; Willett 1987), and cessation of smoking can significantly decrease this risk (LaCroix 1991; Willett 1987; Colditz 1988). Sustained hypertension is another risk factor for CHD and stroke (Joint National Committee), as is obesity (Manson 1990). Of interest, visceral obesity, characterized by excess accumulation of adipose tissue in the abdomen, is a particular risk factor for cardiovascular disease, in both men and women (Larsson 1984; Lapidus 1984). While this pattern of obesity is associated with other risk factors, such as glucose intolerance and hypertension, visceral obesity itself is a risk factor for CHD (Kalkhoff 1983). When measured by the ratio of waist-to-hip circumference (Larsson 1984), the preferred ratio for middle aged and elderly women is < 0.8 (Freedman 1990). Physical inactivity is a further risk factor for CHD (Berlin 1990). Additionally, diabetes mellitus, either insulin-dependent or non-insulin-dependent, is a major risk factor for CHD and stroke, with the increased risk even higher for women than for men (Manson 1991; Barrett-Connor 1983).

Pre-menopausal women are at significantly decreased cardiovascular risk when compared with men (Castelli 1986). After menopause, however, the incidence of CHD and stroke progressively increases in women, and CVD mortality is ultimately similar among men and post-menopausal women (Thom 1987). Race has been shown to play a role in CHD and stroke, with African Americans at higher risk for reasons that are not fully understood. Lastly, even when all other risk factors are taken into account, a family history of early CHD is yet another independent risk factor for coronary heart disease (Barrett-Connor 1984).

4. LIPID ABNORMALITIES AND OTHER CVD RISK FACTORS IN HIV-INFECTED PATIENTS

Multiple abnormalities in lipid metabolism have been described in HIV-infected patients prior to the availability of HAART (Grunfeld 1992). Elevated triglyceride levels have been demonstrated, and have been found to be associated with more rapid progression of HIV disease (Grunfeld 1991). Decreased cholesterol levels have been noted, with a similar effect on disease progression (Posner 1993). Lower levels of HDL-C and LDL-C have been described (Grunfeld 1992). Aside from aberrations in lipid metabolism, which may be induced by HIV and its milieu of inflammatory cytokines, various antiretroviral

compounds have also been associated with lipid abnormalities. Thus, significant elevations of triglycerides have been demonstrated after use of non-nucleoside reverse transcriptase inhibitors (NNRTI) (Moyle 1999).

More importantly, all currently licensed protease inhibitors (PI) have also been associated with significant elevations of triglycerides and cholesterol (Sullivan 1997; Mulligan 2000). Thus, clinically significant elevations in triglyceride or cholesterol were documented in 74% of 104 PI-treated, and in 28% of 27 PI-naïve HIV-infected patients followed over time by Carr and his group (p=0.0001) (Carr 1999). In 38 HIV-infected patients who received at least one PI, results were compared with those in 17 PI-naïve individuals (Behrens 1999). A total of 82% of the PI group were found to have total cholesterol levels over 200 mg/dl, versus 12% of the PI-naïve group (p=0.0001), while triglycerides over 200 mg/dl were found in 66% of PI-treated and 18% of PI-naïve patients (P=0.001). LDL and VLDL levels were also statistically increased in patients who received PI-containing regimens (Behrens 1999). Similarly, when compared to patients receiving antiretroviral therapy without a PI, Mulligan and colleagues demonstrated patients who received PI-containing ART had a mean increase in cholesterol of 32 mg/dL, including a 27% increase in directly measured LDL cholesterol (Mulligan 2000). These changes occurred after a mean of 3.4 months of therapy.

Ritonavir appears particularly capable of inducing these abnormalities (Sullivan 1997), even when given to HIV-negative individuals (Purnell 2000); lipid abnormalities could be detected within two weeks, consisting of a 24% increase in cholesterol, and a 137% increase in triglycerides (Purnell 2000). Periard and colleagues evaluated plasma lipoprotein levels in 93 HIV-infected adults receiving PI therapy, and compared these results with their pretreatment values, and with those obtained in 28 non-PI-treated HIV-infected patients (Periard 1999). An increase in plasma cholesterol was found in all PI-treated groups, although the abnormalities were most profound in those who received ritonavir, as opposed to nelfinavir or indinavir. Ritonavir was also associated with significant increases in plasma triglyceride levels, a finding not seen with the other PI agents. Plasma HDL-cholesterol levels remained unchanged in the PI-treated subjects (Periard 1999).

Aside from inducing elevations in total cholesterol, LDL-C and triglyceride levels, PI use has also been associated with additional factors which may augment the risk for CHD or stroke. These include development of abdominal visceral fat (Miller 1998), impaired glucose tolerance (Carr 1999; Behrens 1999), and possibly, hypertension.

5. CAROTID INTIMA-MEDIA THICKNESS (IMT) AS A PREDICTOR OF CVD

Patients with established CHD, or those with known atherosclerotic disease of the carotids, are at high risk for development of subsequent MI, stroke, or CHD-related death (Salonen 1991), occurring at a rate which is five to seven-fold higher than expected in the general population (Salonen 1991). Pignoli and co-workers first identified the lumen-intima and media-adventitia echoes in carotid ultrasound images in 1986 (Pignoli 1986), and were able to demonstrate a very high correlation (r=0.90) between the intima-media thickness of the carotids as measured by ultrasound and as measured directly on histologic specimens. Subsequent large-scale epidemiologic studies demonstrated an association between carotid artery IMT and the presence of cardiovascular risk factors in both men (Salonen 1991) and women (Bonithon-Kopp 1991). A relationship between

abnormal carotid IMT and angiographically proven coronary artery disease was also shown (Crouse 1987; Wofford 1991).

Additionally, a relationship between carotid IMT and confirmed history of CHD has also been demonstrated (O'Leary 1992). In these studies, carotid IMT was shown to be a better predictor of CHD than traditional lipid or non-lipid risk factors for cardiovascular disease (O'Leary 1992). These findings led very quickly to the use of B-mode ultrasonography as a direct measure of atherosclerosis (Hodis 1999).

Several studies have now indicated that carotid artery IMT may be related to the risk of clinical coronary events and stroke. The Kuopio Ischaemic Heart Disease Risk Factor Study (KIHD) was a population-based study of Finnish men, who have one of the highest rates of CHD in the world (Salonen 1993). At baseline, carotid IMT was performed on 1,257 subjects who were free of CHD. The maximal common carotid artery IMT at baseline was significantly associated with the risk of myocardial infarction (MI), such that for each 0.1 mm of common carotid IMT, the risk of MI increased by 1.11 ($P<0.001$, 95% CI 1.06 to 1.16).

In the Rotterdam study, B-mode ultrasound images of the common carotid artery were performed in 5,965 patients at baseline, who were then followed for a mean of 2.7 years. A nested case-control approach was used to study the relationship between carotid IMT and incident MI and stroke, based on 99 MIs (31% women) and 95 strokes (60% women). The odds ratio for MI or for stroke increased significantly for each 0.163 mm increment of common carotid IMT among patients without history of prior MI or stroke (Bots 1997).

The Cardiovascular Health Study was a prospective study that evaluated the incidence of cardiovascular disease in 4,476 subjects who underwent B-mode ultrasonography at baseline and had no history of clinical cardiovascular disease (O'Leary 1999). After a median follow up of 6.2 years (maximum 7 years), the relative risk for MI, stroke, and MI with stroke combined was significantly elevated for each 0.2 mm increment of maximal common carotid artery IMT ($p<0.001$).

D. CAROTID ULTRASOUND PROTOCOL

Carotid artery intima-media thickness (IMT) assessed non-invasively by ultrasound (US) has been extensively validated as a surrogate for subclinical atherosclerotic disease. Elevated carotid IMT strongly predicts incident stroke and myocardial infarction. Arterial stiffness is another marker of early atherosclerosis that may be detected via sonography. We will examine whether HIV-related variables (e.g., serostatus, antiretroviral medication use, CD4 count, etc.), standard CVD risk factors (e.g., age, hypertension, smoking, diabetes, etc.), and novel CVD risk factors (e.g., serum inflammation markers, leptin, etc.) are correlated with markers of atherosclerosis, including elevated carotid IMT, carotid artery stiffness, and carotid atherosclerotic lesions, among WIHS participants.

Data to be collected include a baseline carotid US exam, conducted on as many WIHS participants as possible. Follow-up US exams will be obtained for a selected, informative subset. The use of a selected, informative follow-up subset will maximize the study's efficiency for examining the effect of HIV, antiretroviral medications, and other variables of interest on progression of carotid atherosclerosis. In addition, all women in the Cardiovascular Substudy will provide blood samples, which must be provided under eight-hour fasting conditions, according to lab protocol. Standardized interpretations of ultrasound

scans will be performed under the direction of Dr. Howard Hodis at the USC Ultrasound Reading Center.

1. TIMEFRAME

   Baseline US exams will begin during the core visit 20 time window and should be completed as quickly as feasible, in order to minimize measurement drift and maximize the available follow-up time. Baseline US exams will continue through core visit 22. For a subset of approximately 100 to 150 women per site, follow-up US exams will be conducted beginning at core visit 25. Additional follow-up ultrasounds will be conducted on this subset beginning at core visits 29 and 33. The follow-up subset will be chosen according to serostatus, antiretroviral medication use, and other variables. Sites will be notified by WDMAC at a later date which women should be included in the follow-up subset.

2. ENROLLMENT PROCEDURES

   All women should be invited to participate in the Cardiovascular Substudy, except for those at outlying subsites for whom travel to the US lab would not be feasible. Because each site will designate a single ultrasound lab to perform carotid US exams, it may not be feasible to perform US on women enrolled at geographically isolated subsites. However, it is expected that the necessary arrangements will be made for women to travel to the US lab, even if located less conveniently than their usual WIHS clinic. Participants may receive travel reimbursement and a monetary or other incentive for participating in the Cardiovascular Substudy.

   Participation in the Cardiovascular Substudy requires collection of fasting CVD blood samples. If subjects are fasting at the core visit at which Cardiovascular Substudy enrollment occurs, the core phlebotomy will involve collection of fasting CVD bloods. If women are not fasting at the time of Cardiovascular Substudy enrollment, fasting CVD bloods will need to be collected at the carotid US visit, a special (non-core) visit, or at the next core visit. See **Section E2** for additional detail on collection of fasting CVD blood samples.

   For women who refuse enrollment into the Cardiovascular Substudy, complete the *Participation Notification Form* (CVNOTI) form at the core visit and enter into Apollo. Depending upon the reason the participant didn't enroll into the substudy, women can be asked at each core visit through visit 22 if they would be willing to participate in the substudy.

   For women who consent to participate in the Cardiovascular Substudy, a *CVNOTI* will be completed at the time the carotid ultrasound exam is completed (see **Section D12**).

3. INFORMED CONSENT

   Enrollment in the Cardiovascular Substudy will involve a baseline carotid US exam and, for a subset to be identified later, three additional follow-up US exams. Additionally, the first twenty women enrolled at each clinical site that agree to undergo a second scan are scheduled to complete a quality control ultrasound scan within one month of their original scan.

   The carotid US exam is a non-invasive procedure that does not carry risks beyond those associated with routine medical care. In the event that a potentially clinically significant carotid artery lesion is detected, subjects will be advised to obtain appropriate follow up

(see **Section D14**). One or two times per year, additional tubes of fasting blood (up to 23 ml) will be drawn for measurement of CVD-related markers. Suggested language for the informed consent appears in **Appendix A**.

4. SCHEDULING BASELINE US EXAMS

   After a subject accepts the invitation to participate in the Cardiovascular Substudy, schedule the baseline ultrasound exam as soon as feasible. The time of day of the US exam is not important. Women will not be required to fast for US exams. However, there is an opportunity to obtain fasting CVD blood on the same day as the US exam if necessary (i.e., if the subject was not fasting at the core visit at which Cardiovascular Substudy enrollment occurred). The core visits, US exams, and fasting CVD blood draws should be as close together in time as possible (optimally, within two weeks).

5. SCHEDULING FOLLOW-UP US EXAMS

   For a subset of approximately 100 to 150 women per site, follow-up US scans will be conducted as close as possible to the anniversary date of the baseline US. Sites will be notified by WDMAC which women should be included in the follow-up subset.

6. DISENROLLMENTS

   Women will be permitted to withdraw from the Cardiovascular Substudy at any time. Women will also be disenrolled from the substudy if the USC Ultrasound Reading Center determines that the baseline scan is unusable (this will be uncommon). Complete a *Disenrollment Form* (DENR) if disenrollment occurs and document the reason for disenrollment as indicated in the Question-by-Question Specifications.

7. ULTRASOUND LABS

   Established ultrasonography centers will be used as US labs. Each of the six WIHS sites will designate a single lab each to perform all baseline and follow-up US exams.

   The contact person at the USC Ultrasound Reading Center for questions regarding training, equipment, shipping of ultrasound results, and other technical issues is:

   Dr. Yanjie Li
   Project Specialist
   USC Keck School of Medicine
   2250 Alcazar Street, Suite 132
   Los Angeles, CA 90033

   Tel: 323-442-3993
   Fax: 323-442-2345
   Email: yanli@usc.edu

   Contact information for the Director of the USC Ultrasound Reading Center is:

   Dr. Howard Hodis
   USC Keck School of Medicine
   2250 Alcazar Street, Suite 132
   Los Angeles, CA 90033

   Tel: 323-442-1478
   Fax: 323-442-2685
   Email: athero@usc.edu

8. ULTRASOUND PERSONNEL AND TRAINING

Prior to the enrollment of any participants, all sonographers will receive one day of training on-site at the USC Ultrasound Reading Center, with review of practice tapes and follow up by telephone during the following week. Training will be mandatory regardless of the experience of the sonographer. A brief retraining, by telephone, will be conducted before each additional follow-up exam begins. In the event of staff turnover at local US labs, new sonographers will be required to attend training at USC before performing WIHS exams. Sites should alert WDMAC if they are aware of turnover at the US lab.

9. ULTRASOUND EXAMINATION PROCEDURES

Ultrasound exams should take less than 30 minutes to complete. The exam protocol will include the following components, which will capture: (1) standardized measurements of right common carotid artery (CCA) far wall IMT and stiffness (baseline and follow-up), and (2) assessment of atherosclerotic lesions in the right CCA, carotid bulb, and proximal internal carotid (ICA) and external carotid (ECA) at the bifurcation (baseline and follow-up):

- Scan right CCA distal segment for IMT (baseline and follow-up), stiffness (baseline and follow-up) and lesions (baseline only)
- Scan right carotid bulb and proximal internal carotid (ICA) and external carotid (ECA) at the bifurcation for lesions (baseline and follow-up)

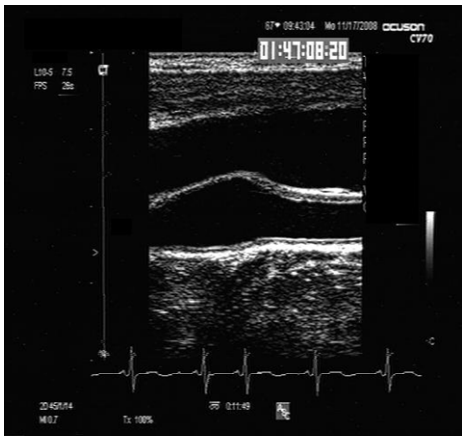
RBIF CIMT Acquisition Procedure

Step for RBIF (Annotate "BIF" on screen)

Carotid Bifurcation IMT Acquisition Procedure

Scan the longitudinal plane view of bifurcation. Sweep to the anterior direction then sweep back to the posterior direction. Try to make the far wall bifurcation IMT visible. Acquire a thickest-IMT image on the bifurcation view. A bifurcation view is an image that has 100% of the carotid bulb as well as the proximal internal or external carotid arteries visible.

Please record on the video tape at least a 1 minute for this step



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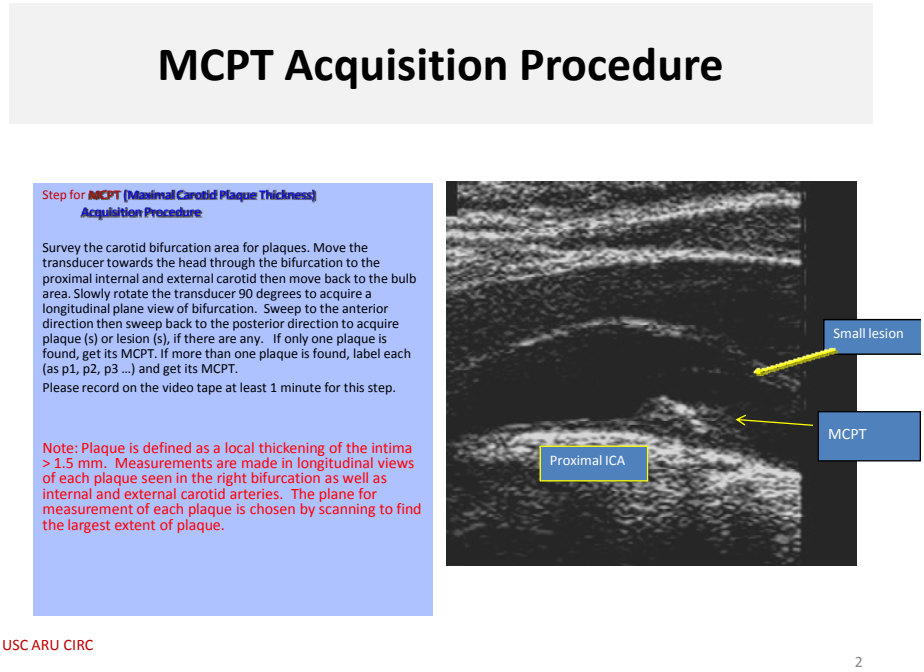


- Three-lead ECG simultaneous with US scans (recorded on same tape)
- Blood pressure (Dinamap): five readings after scan, separated by 60-second intervals

Summary procedures for high resolution B-mode carotid artery ultrasound procedures are as follows. Additional detail will be provided by USC Ultrasound Reading Center at the training sessions.

The following data collection equipment and supplies are required:

a. Ultrasound machine (Machine must have the capacity and software for B-mode and ECG monitoring as part of the system. The ultrasound equipment must be capable of displaying ECG traces.)

b. Linear 7.5 frequency probe or 5-10 MHz multifrequency probe.

c. External Time Code (TG-50 generator/inserter, $379.00). www.HORITA.com, P.O. BOX 3993, Mission Viejo, CA 92690, Phone: 949-489-0240

d. S-VHS video tapes.

e. ECG monitoring electrodes and ECG system connectors.

f. Ultra-gels.

g. Exam table.

h. Two Super VHS data acquisition recorders. (One for recording exams, one for making duplicate tapes for archiving)

i. Special pillow (45 degree head block: Triangle 45) Cat. No.203343 ($10.00), for order information call: 1-800-321-6964

j. Blood-pressure meter (stand/manual or auto) with small to large cuffs. Approximately $1,500 for a factory refurbished model. (Dinamap XL vital signs monitor, Johnson & Johnson Medical Inc., Arlington TX, USA)

B-mode carotid artery images are acquired with a high-resolution ultrasound imager using a linear array 7.5 MHz probe. The ECG (three-lead) and ultrasound images are simultaneously recorded on ½ inch tape with a Panasonic AG-7355 S-VHS videotape recorder or its equivalent.

Lying face up, subjects are positioned so the neck is extended to present the optimal angle for ultrasound examination. Using B-mode, the right distal common carotid artery (CCA) is imaged in cross-section and the scan head moved laterally until the jugular vein and the CCA are stacked with the former above the latter. In this position, the central image line passes along the common diameter of both vessels. The scan head is then rotated around the central image line 90 degrees maintaining the jugular vein stacked above the CCA while obtaining a longitudinal view of both vessels. In this longitudinal view, the distal CCA far wall is horizontal. The proximal portion of the carotid bulb is included in all images. Minimum gain necessary for clear visualization of structures is used, and once the transducer is positioned, a minimum of 10 seconds of videotape is recorded. Emphasis of ultrasound imaging is on the distal centimeter of the CCA since the least variability in measurement has been reported for this area. When imaging of the distal CCA is complete, the transducer is left on the surface of the skin and slowly moved cephalad to bring into view the flow divider and first segments of the proximal internal and external carotid arteries. This image will also contain the distal bulb. At the end of the ultrasound examination, five blood pressure measurements at the brachial artery will be acquired at 60-second intervals with an automated blood pressure device (automated cuff inflation preferred) (Dinamap XL vital signs monitor, Johnson & Johnson Medical Inc., Arlington TX, USA).

Copies of videotapes and the *Ultrasound Tracking Form* (CV01) forms will be sent to the USC Ultrasound Reading Center for interpretation. The original *CV01* should be entered into Apollo and filed locally. In addition, one copy of all VHS tapes must be kept either at the WIHS clinic or ultrasound lab.

For analysis of CCA far wall IMT, the Prosound computer system (copyright, University of Southern California, Los Angeles CA, USA) will be used, which utilizes automated boundary detection to locate the lumen-intima and media-adventitia echo boundaries at sub-pixel resolution. The IMT measurement consists of an average of 80–100 independent measurements made along a 1-cm distance in the far wall of the distal right CCA. Using this methodology, the coefficient of variation for average IMT is 2.5% within operators (Zheng 2003).

10. FOLLOW-UP ULTRASOUND EXAMINATION PROCEDURES

For each participant, the same depth of field, gain, monitor intensity setting, and other instrumentation settings used at the baseline exam must be used at each follow-up exam. Follow-up scans must use the same ultrasound imager, transducer, and other hardware used at the baseline exam. If possible, follow-up scans should also be performed by the same sonographer and at the same time of day. At the time of a follow-up examination, the ultrasound lab will be provided with a copy of the corresponding baseline scan to permit more accurate and reproducible measurements of disease progression.

11. QUALITY CONTROL

Quality control (QC) will accomplished through: (a) collection of duplicate carotid ultrasound exams, (b) evaluation of phantom images, (c) inspection of scans by the Reading Center, (d) ongoing communication regarding US lab personnel/equipment changes, and (e) collection of blinded repeat scans during the first follow-up period.

a. QC duplicate carotid exams

Each site will collect duplicate baseline carotid ultrasound examinations for a QC subsample of 20 subjects at baseline and 10 subjects at each follow-up. The duplicate exam should be conducted at a separate visit within one month of the initial baseline or follow-up examination. The identical imaging protocol will be used. The women receiving QC duplicates should be a broadly representative subsample of women in the carotid study. There are no specific requirements regarding the composition of the QC subsample, and the first 20/10 women at each site who agree to have repeat visits will constitute the QC subsample.

b. Phantom images

Phantom images will be evaluated on an annual basis to assure image quality and standardize calibration pixel size for various types of ultrasound equipment. In addition, if ultrasound equipment requires replacement or repair (beyond usual maintenance), phantom images must be evaluated before performing WIHS ultrasound exams. Each ultrasound lab must perform a phantom scan once per year and send the phantom test tape to the USC Ultrasound Reading Center for evaluation and calibration. Each ultrasound lab should perform and have analyzed one phantom scan prior to beginning ultrasound measurements on participants.

We will purchase a single phantom device to be circulated among the WIHS sites. The specific handling instructions for shipping the phantom will be described in the training sessions. The phantom device to be used is:

RMI 404 GS, Small Parts Gray Scale-Attenuation coefficients of 0.7 db/cm/MHz.

Order information call: 1-800-GAMMEX 1 (426-6391) or 1-608-831-1188.

Sites should notify WDMAC upon shipment of the phantom to any other WIHS site.

c. Reading Center image evaluation

The USC Ultrasound Reading Center will alert the local site in the event that US scans are unreadable or incomplete. Sites should schedule a repeat US exam if feasible. Participants will be disenrolled if a good-quality baseline scan is not completed (see **Section D6**).

d. Changes in personnel/equipment

Sites are asked to notify WDMAC in the event of personnel turnover, equipment breakdown or other changes at ultrasound labs.

e. CIMT Substudy QC Blinded Repeat Scan Protocol

At Bronx and Brooklyn sites, enroll the first five women who agree to return for an additional (QC) carotid ultrasound scan.

When participant returns for her QC scan, assign to her a “sham ID” and write her WIHSID in the “assignment of sham ID’s” table distributed by WDMAC next to the

assigned sham ID. Complete a *CV01* form for the QC scan, entering the sham ID into the WIHSID field and circling "1" (for baseline) in Question A3, carotid ultrasound type. Complete the remainder of the form in the usual manner. Send a copy of the completed *CV01* form (with the sham ID) to the USC reading center with the ultrasounds tape.

After the *CV01* form has been copied, write the participant's actual WIHSID in the top margin of the form copy you intend to store on site. To enter the *CV01* form into Apollo, go to the "QC CV01" data entry screen and select the participant's actual WIHSID from the drop-down list. The actual WIHSID will be automatically entered on the form as usual. In addition, there will be a separate field entitled "sham ID" into which the assigned sham ID should be entered. Enter the rest of the form data in the usual manner.

12. TRACKING

After completion of the US exam, complete and enter a *CVNOTI* to document Cardiovascular Substudy enrollment and the date of the completed US exam. Serostatus and antiretroviral medication use (based on the most recent available *F22MED* data) will also be documented on *CVNOTI.* In addition, *CVNOTI* will be used to document whether Cardiovascular Substudy participants have provided fasting blood samples; this will help to track which women will need to make separate (non-core) visits for fasting blood draws (see **Section E2**).

Complete and enter a *Carotid Ultrasound Tracking Form* (CV01) with each completed ultrasound exam.

The sites should also maintain a log of completed exams to help with follow-up of shipments to the USC Ultrasound Reading Center (containing: date exam completed, WIHSID, date mailed to Reading Center, etc.).

13. SENDING ULTRASOUND STUDIES TO READING CENTER

Tapes of ultrasound studies should be mailed via courier to the USC Ultrasound Reading Center. In addition, a copy of the *CV01* for each exam will be sent to the USC Reading Center (includes equipment used, settings, blood pressure readings, etc.). A copy of all tapes and forms should be retained at the ultrasound lab or WIHS clinic. Studies should be sent to the USC Reading Center at one-week intervals. The mailing address for the Reading Center appears in **Section D7** above.

Label S-VHS tapes as depicted:

Face label: Avery 5199-F, 3.0625" x 1.8375"

WIHSID Visit# US exam date

Spine label: Avery 5199-S, 5.8" x 0.69"

WIHS Tape #_____ Site code:_____ Site Name: _____

14. CLINICAL ALERTS

Study examinations may reveal previously undetected disease of possible clinical significance. Sonographers will receive training at the USC Ultrasound Reading Center regarding the appropriate follow-up procedures for possible disease. If the sonographer detects any potentially significant carotid artery lesion (specifically, an apparent 50% stenosis), it must be documented on form *CV01* and the local Project Director or Principal Investigator must be notified as soon as possible. Ultrasound scans will be sent by overnight mail to the USC Ultrasound Reading Center for expedited review. If the finding of a possible carotid lesion is confirmed by visual inspection at the USC Reading Center, a report stating the abnormality will be faxed to the local site.

Letters to participants and their physicians will be generated by the local WIHS site PI. Sample letters appear in **Appendix B**. No clinical alerts will be generated based on central readings performed by the USC Ultrasound Reading Center, aside from the expedited review of potential disease detected by sonographers as described in the preceding paragraph. These results are for research purposes only.

15. DATA TRANSMISSION TO WDMAC

Completed *CVNOTI* and *CV01* forms will be entered into Apollo by site study staff, and data will be incorporated into the central edit process and distributed with other clean Apollo data.

Ultrasound data will be transmitted from the USC Reading Center (Wendy Mack) to WDMAC (Gayle Springer) on a regular basis. These data will include results for IMT (two frames, wall thickness of right CCA), diameters (two frames each for minimum and maximum) and lesions. Ultrasound data will be incorporated into the frozen database at the end of each visit cycle, as with other centrally-generated data.

16. CIMT DIGITAL IMAGE ACQUISITION PROTOCOL

a. Equipment and supplies

i. Ultrasound machine:

- Ultrasound machine must have the capacity for simultaneous B-mode imaging and ECG monitoring (ECG input)
- Linear 7.0-12 MHz frequency transducer
- QuickSet (preset) feature: The preset feature enables image capture with an optimized configuration of imaging parameter settings for a specific transducer and exam. Presents for the CIMT study can be created.
- Digital dynamic image acquisition, storage, review and copy to CD or DVD
    - Internal hard drive for image and data storage
    - Storage of real-time dynamic clips (AVI format or DICOM video file format (uncompressed video is preferred)

- Standard output file format: AVI or DICOM uncompressed video file – export of AVI format is preferable
- ECG traces must be stored in the AVI file or DICOM file so that the traces can be viewed along with the B-mode images

ii. Supplies: DVDs or CDs

b. CIMT digital acquisition procedure

Digital CIMT Acquisition Procedure is very similar to the analog CIMT Acquisition Procedure except during the CIMT study, the sonographer should capture at least seven to nine AVI images if possible for each subject. This includes five longitudinal views of the RCAA, two longitudinal views of the RBIF images and at least two longitudinal views of the Carotid Plaque(s) or Lesion(s) images, if there are any.

c. Archiving and exporting CIMT

i. Digital video file requirements

- Standard output file format: AVI or DICOM video file format. First choice is AVI. If AVI is not available, DICOM video is acceptable. However, DICOM video is preferred to be uncompressed video or less compressed video.
- Each AVI or DICOM video file must be at least five seconds in duration.
- Provide at least seven to nine AVI images if possible for each subject. This includes five longitudinal views of the RCCA, two longitudinal views of the RBIF (IMT) and at least two longitudinal views of the Carotid Plaque or Lesion(s) images, if possible.

ii. Archive all CIMT study files on a CD/DVD. Send to USC ARU.

- Video file formatting must be consistent throughout the study. If AVI file format is chosen, then use AVI format throughout the entire study. If DICOM video file format is chosen, then use DICOM video file format throughout the entire study. Avoid compression (preferred) or minimize compression.
- DICOM still images are not acceptable.
- ECG traces must be stored in the AVI file or DICOM file so that the traces can be viewed simultaneously with the B-mode images.

d. Labeling and shipping

i. Number each CD or DVD sequentially. In addition, label each CD or DVD cover with the following information:

- CIMT-CD# (001, 002, 003…)
- Protocol name: WIHS
- Site# (777777, 888888…)

ii. Duplicate the CIMT CD or DVD and the label information and save on site.

iii. Photocopy the Intima-Media Thickness imaging work sheets for each subject. All of the information on the CIMT work sheets should match the information on the CD or DVD image labels.

iv. Ship the package overnight on a weekly basis if possible. Add a CIMT log for each CD or DVD. This log should have all relevant information about each scan.

v. All information on CD or DVD labels and CIMT work sheets will be reviewed for accuracy and verified if necessary with the on-site sonographer.

vi. The USC ARU mailing address:

Yanjie Li
Atherosclerosis Research Unit
University of Southern California Keck School of Medicine
2250 Alcazar Street, CSC 132
Los Angeles, CA 90033
(Tel) 323-442-3993

E. BLOOD SAMPLES AND ASSAYS

The Cardiovascular Substudy will involve additional blood samples and laboratory assays:

1. METABOLIC ASSAYS

For all WIHS participants, a *metabolic panel* will be measured. The fasting metabolic panel consists of: total cholesterol, HDL-C, calculated LDL-C, triglycerides, glucose, insulin and Hemoglobin A1c. For non-fasting core blood samples, a modified version of the metabolic panel omits assays that are uninformative in non-fasting conditions.

The components of the metabolic profile, as well as the subgroup of women on whom it has been performed, has changed over time in WIHS:

- Visits 13 through 19*:* Lipid, insulin, glucose and HgA1c assays were performed at every visit for all women fasting at their core visit. These specimens were initially reposited, and then batch shipped for central testing.
- Visits 20 through 23*:* Lipid and HgA1c assays were performed at every visit for all women, regardless of fasting status. Insulin and glucose assays were performed for all women fasting at their core visit. These specimens were batch tested monthly. If not fasting at their core visit, Cardiovascular Substudy participants were asked to return for a fasting blood draw. Specimens (for lipids, insulin, glucose and HgA1c) collected at these visits were reposited; some were later sent for central testing.
- Visits 24 through 27*:* Lipid assays were performed at odd-numbered visits for all women, regardless of fasting status. Insulin and glucose assays were performed at odd-numbered visits for all women fasting at their core visit. (If a participant missed an odd-numbered visit, specimens were to be collected at the next even visit, plus the next odd visit to get the participant back on an odd-visit schedule.) These specimens were batch tested monthly.
- Visits 29 and 30*:* Lipid and insulin assays were performed on reposited specimens for women who attended their core visit fasting. Additional criteria for visit 29 testing included: serum separated within eight hours or less, results not already obtained via Metabolic Substudy. Additional criteria for visit 30 testing included: serum separated within eight hours or less, results not already obtained via Metabolic Substudy, participant completed form *NC06* as part of the NC Battery.

- Visits 31 through 35: Hemoglobin A1c (HgA1c) assays will be performed at odd-numbered visits for all women, regardless of fasting status. Insulin and glucose assays will be performed at odd-numbered visits for all women fasting at their core visit. Complete lipid assays will be performed at odd-numbered visits for all women fasting at their core visit. These specimens will be batch tested monthly. If a woman is not fasting at her core visit, site discretion is allowed in determining whether or not to perform partial lipid assays on a monthly schedule. If lipid testing is not performed, sites should send those specimens to SeraCare for long-term storage.
- Visits 36 through 46: Hemoglobin A1c (HgA1c) assays will be performed at even-numbered visits for all women, regardless of fasting status. Insulin and glucose assays will be performed at even-numbered visits for all women fasting at their core visit. Complete lipid assays will be performed at even-numbered visits for all women fasting at their core visit. These specimens will be batch tested monthly. If a woman is not fasting at her core visit, site discretion is allowed in determining whether or not to perform partial lipid assays on a monthly schedule. If lipid testing is not performed, sites should send those specimens to SeraCare for long-term storage.

Assay	Fasting metabolic panel	Non-fasting metabolic panel
Total cholesterol	Yes	Yes
HDL-C	Yes	Yes
LDL-C	Yes (calculated; direct if TG>400)	Yes (direct)
Triglycerides (TRIG)	Yes	No
Insulin	Yes	No
HgA1c	Yes	Yes
Glucose	Yes	No

Detailed instructions appear below (see **Section E4**) for specimen processing and creation of aliquots for metabolic assays. Collection tubes for metabolic assays include the following:

- SST tube: metabolic *(10ml tube, 8.5ml draw)* for cholesterol/TRIG/insulin
- EDTA (lavender) tube: whole blood *(3ml tube, 3ml draw)* for HgA1c
- NaF/K oxalate (gray-top) tube: *(3ml tube, 3ml draw)* for glucose

2. FASTING CVD BLOOD SAMPLES

NOTE: Collection of fasting CVD blood samples was discontinued at visit 24.

One or two times per year (during visits 20 through 23), additional volumes of fasting blood will be drawn for measuring lipoproteins (via NMR) and other novel CVD markers among women participating in the Cardiovascular Substudy.

Fasting CVD blood specimens should be incorporated into the core phlebotomy if Cardiovascular Substudy participants are fasting at core visits. Otherwise, phlebotomy can be performed at the carotid US visit, or at a separate (non-core) visit specifically for the collection of fasting blood, if necessary, to meet the requirement of at least one

annual fasting draw (i.e., if core visits do not yield fasting bloods). Optimally, separate fasting blood draw visits should be within two weeks of core visits. Fasting CVD samples will be drawn up to twice a year (twice for women who are fasting at both core visits; once for women who are fasting at only one visit or who make a separate visit for fasting blood draw).

Detailed instructions appear below (see **Section E4**) for specimen processing and creation of aliquots for fasting CVD bloods. Collection tubes include the following:

- SST tube: CVD *(10ml tube, 8.5ml draw)* for various novel CVD markers
- EDTA (lavender) tube: plasma *(3ml tube, 3ml draw)* for NMR analysis of lipoproteins

All processed specimens will be placed in 1 ml aliquots and batch-shipped to the WIHS Central Repository. At a later date, assays will be performed using these specimens at a central laboratory for selected, informative subjects such as case-control pairs. A variety of assays are of interest as correlates of HIV-related variables and predictors of carotid atherosclerosis and CVD events, including:

- lipid-related assays *(lipoprotein particle size, HDL subclass, VLDL, IDL measured via NMR spectroscopy methods)*
- adipocytokines *(leptin, adiponectin)*
- inflammation markers *(high-sensitivity C-reactive protein, soluble intercellular adhesion molecule-1, matrix metalloproteinase-2 and -9, E-selectin)*
- markers of glucose metabolism *(proinsulin, C-peptide)*

3. DEFINITION OF FASTING

Fasting will be defined as no food or beverage intake except water for eight hours prior to specimen collection. Fasting bloods may be drawn at any time of day. Fasting status at time of blood draw will be recorded on WIHS forms *F29* (for core blood draw) and *CV29* (for cardiovascular draw, if performed).

4. PHLEBOTOMY AND ALIQUOTTING

The table below summarizes the tubes to be drawn for the metabolic panel, lipoproteins (by NMR methods) and other novel CVD assays. Detailed instructions for each tube appear on the pages that follow.

Tube	Analytes	Core visit, if fasting	Core visit, if non-fasting	Separate (non-core) fasting blood visit * °
SST tube – Metabolic *(10ml tube, 8.5ml draw)*	Cholesterol, TRIG, insulin	√ (Quest)	√ (Quest) ♦	√ * ° (SeraCare)
EDTA (lavender) tube: whole blood *(3ml tube, 3ml draw)*	HgA1c	√ (Quest)	√ (Quest)	
NaF/K oxalate (gray-top) tube *(3ml tube, 3ml draw)*	Glucose	√ (Quest)		√ * ° (SeraCare)
SST tube – CVD *(10ml tube, 8.5ml draw)*	Novel CVD assays	√ * (SeraCare)		√ * ° (SeraCare)
EDTA (lavender) tube: plasma *(3ml tube, 3ml draw)*	Lipoproteins by NMR	√ * (SeraCare)		√ * ° (SeraCare)

♦ Site discretion is allowed in determining whether to perform the non-fasting lipid panel for visit 33.

* For women in Cardiovascular Substudy only.

° Separate (non-core) visits may be necessary to meet the lab protocol requirement among CVD Substudy participants.

NOTE: Specimens in the above table shaded in gray are no longer collected as part of the Cardiovascular Substudy.

SST Tube (for metabolic panel)
(10 ml tube, draws 8.5 ml blood)
Draw for all participants at specified core visits, regardless of fasting status.

Collect from all participants at all specified visits (i.e., even-numbered visits beginning with visit 36), regardless of fasting status.

Processing instructions

1. Gently invert plastic SST tube eight times immediately after specimen collection. (This activates clotting.)
2 Let blood clot 30 minutes in vertical position.
3. Within one hour of blood collection, spin SST for 10 minutes in a swinging bucket centrifuge or for 15 minutes in a fixed angle centrifuge at 1100 to 1300 X g. Centrifuge at room temperature or refrigerated temperature.
4. Maintain separated serum at refrigeration temperature prior to freezing.
5. Freeze serum in aliquots within eight hours of collection at -70ºC. Please be careful to label with the correct specimen codes (indicating whether or not the specimens are fasting).
6. Leftover specimens may be kept locally until Question Diagnostics has confirmed testing. Then, leftover specimens should be sent to SeraCare for long-term storage.

Yields 4-5 ml serum.

Specimens for metabolic assays need to be centrifuged within one hour of collection, and frozen in 1 ml aliquots at -70ºC within eight hours of collection. Metabolic assays will be performed within one month after collection at Quest Diagnostics (Baltimore, MD).

SST: Metabolic	Analytes	Aliquots	Send for analysis
At core visit, fasting	a. TC, HDL-C, TRIG, calculated LDL-C, insulin b. Long-term storage	1 ml x 2 1 ml x 2-3	to Quest: within 1 mo central save/batch ship to SeraCare
At core visit, non-fasting	a. TC, HDL-C, direct LDL-C b. Long-term storage	1 ml x 2 1 ml x 3-4	site discretion regarding real-time testing central save/batch ship to SeraCare
At separate (non-core) fasting blood visit	a. Long-term storage	1 ml x 4-5	central save/batch ship to SeraCare

EDTA (lavender) tube: whole blood (for HgA1c)
(3 ml blood, draws 3ml blood)
Draw for all participants at core visit regardless of fasting status.

Processing instructions

1. Gently invert EDTA-anticoagulated whole blood tube eight times immediately after blood collection.
2. Store EDTA-anticoagulated whole blood tube at room or refrigeration temperatures.
3. Within 30 hours of blood collection prepare aliquots of whole blood. (Gently invert whole blood tube eight to ten times immediately prior to aliquotting.)
4. Freeze whole blood aliquots at -70^{o}C within 30 hours of blood collection.
5. Leftover specimens may be kept locally until Question Diagnostics has confirmed testing. Then, leftover specimens should be discarded.

Yields 3 ml whole blood.

EDTA tube: whole blood	Analytes	Aliquots	Send for analysis
At core visit, fasting	a. HgA1c b. Short-term storage	1 ml 1 ml	to Quest: within 1 mo locally, until result confirmed; then discard
At core visit, non-fasting	a. HgA1c b. Short-term storage	1 ml 1 ml	to Quest: within 1 mo locally, until result confirmed; then discard
At separate (non-core) fasting blood visit	Do not draw this tube		

NaF/K oxalate (gray-top) tube (for glucose)
(3 ml tube, draws 3ml blood)
Draw for all participants at specified core visit (if fasting).

Collect from fasting participants only at all specified visits (i.e., even-numbered visits beginning with visit 36).

Processing instructions

1. Gently invert NaF/K oxalate anticoagulated whole blood tube eight times immediately after blood collection.
2. Maintain NaF/K oxalate anticoagulated whole blood tube at room or refrigeration temperature prior to centrifugation.
3. Within 30 hours of blood collection, spin blood tube for 10 minutes in a swinging bucket centrifuge or for 15 minutes in a fixed angle centrifuge at 800-1,000 X g to obtain plasma. Centrifuge at room temperature or refrigerated temperature.
4. Aliquot separated plasma immediately after centrifugation.
5. Freeze plasma aliquots at –70ºC within 30 hours of blood collection.
6. Leftover specimens may be kept locally until Question Diagnostics has confirmed testing. Then, leftover specimens should be discarded.

Yields 1.5 ml plasma.

NaF/K oxalate (gray-top)	Analytes	Aliquots	Send for analysis
At core visit, fasting	a. Glucose b. Short-term storage	1 ml 1 ml	to Quest: within 1 mo locally, until result confirmed; then discard
At core visit, non-fasting	Do not draw this tube		
At separate (non-core) fasting blood visit	a. Long-term storage	1 ml x 2	central save/batch ship to SeraCare

SST Tube (for novel CVD assays)
(10 ml tube, draws 8.5 ml blood)
Draw for CV Substudy women only, at core visit (if fasting).
NOTE: Collection of this tube was discontinued at visit 24.

Processing instructions

1. Gently invert plastic SST tube eight times immediately after specimen collection. (This activates clotting.)
2. Let blood clot 30 minutes in vertical position.
3. Within one hour of blood collection, spin SST for 10 minutes in a swinging bucket centrifuge or for 15 minutes in a fixed angle centrifuge at 1100 to 1300 X g. Centrifuge at room temperature or refrigerated temperature.
4. Maintain separated serum at refrigeration temperature prior to freezing.
5. Freeze serum in aliquots within eight hours of collection at -70ºC.

Yields 4-5 ml serum.

CVD blood samples need to be centrifuged within one hour of collection, and frozen in 0.5ml aliquots at -70ºC within eight hours of collection. Fasting CVD blood specimens will be assayed centrally at a later date for novel CVD-related markers including lipoproteins (via NMR).

SST: CVD	Analytes	Aliquots	Send for analysis
At core visit, fasting	a. Long-term storage (novel CVD assays)	0.5 ml x 8-10	central save/batch ship to SeraCare
At core visit, non-fasting	Do not draw this tube		
At separate (non-core) fasting blood visit	a. Long-term storage (novel CVD assays)	0.5 ml x 8-10	central save/batch ship to SeraCare

EDTA (lavender) tube: plasma (for NMR lipoproteins)
(3 ml tube, draws 3 ml blood)
Draw for CV Substudy women only, at core visit (if fasting).
NOTE: Collection of this tube was discontinued at visit 24.

Processing instructions

1. Gently invert EDTA-anticoagulated tube eight times immediately after blood collection.
2. Maintain EDTA-anticoagulated tube at room or refrigeration temperatures prior to centrifugation.
3. Within 30 minutes of blood collection, spin EDTA tube for 10 minutes in a swinging bucket centrifuge or for 15 minutes in a fixed angle centrifuge at 1100 to 1300 X g. Centrifuge at room temperature or refrigerated temperature.
4. Maintain separated plasma at refrigeration temperature prior to freezing.
5. Freeze plasma in aliquots within eight hours of collection at -70ºC.

Yields 1.5 ml plasma

EDTA tube: plasma	Analytes	Aliquots	Send for analysis
At core visit, fasting	a. Long-term storage (NMR lipoproteins)	0.5 ml x 3	central save/batch ship to Central Repository
At core visit, non-fasting	Do not draw this tube		
At separate (non-core) fasting blood visit	a. Long-term storage (NMR lipoproteins)	0.5 ml x 3	central save/batch ship to Central Repository

5. USE OF S-CODES AND LABELING

Metabolic panel assays will be run by Quest Diagnostics on a monthly basis. Fasting specimens will be sent directly from the clinical sites to Quest Diagnostics for testing. If a woman is not fasting at her core visit, site discretion is allowed in determining whether or not to perform lipid assays on a monthly schedule. If lipid testing is not performed, sites should send those specimens to SeraCare for long-term storage.

The tests ordered will differ slightly for participants who have fasted for eight or more hours versus those participants who have not. Therefore, it is imperative that clinic staff accurately assesses the participant's fasting status, record it on WIHS forms *F29* and *CV29* as appropriate, and relay that information to the local processing laboratory. After aliquoting, the vials must be labeled using the correct s-codes for fasting versus non-

fasting specimens. This information will determine the type of tests ordered and the cost of the panel. (For entire s-code table, see **WIHS Manual of Operations, Section 10, Appendix A, or Section 31, Appendix A**.)

Specimen Type	Alpha Code	S-Code	Expected Tube Quantities
Serum (tiger-top or gold SST, non-fasting)	NTS	003	1000
Whole blood (lavender, non-fasting)	NWB	008	1000
Plasma (lavender, fasting)	FEP	009	500
Whole blood (lavender, fasting)	FWB	041	1000
Serum (tiger-top or gold SST, fasting)	FTS	042	1000
Plasma (gray-top, fasting)	FGP	043	1000

NOTE: As of visit 24, specimens in the above table shaded in gray will no longer be collected as part of the Cardiovascular Substudy.

6. SPECIMEN SHIPMENTS

Metabolic assays will be performed centrally at Quest Diagnostics (Baltimore, MD). Specimens should be sent to Quest for analysis no less frequently than once per month. Leftover specimen volumes will be placed into 1 ml aliquots and batch-shipped to the WIHS Central Repository for future use in both CVD-related and non-CVD-related research projects.

Contact information for Quest Diagnostics is as follows:

William A. Meyer III, PhD
Virology Department
Quest Diagnostics
1901 Sulphur Spring Road
Baltimore, MD 21227

24-Hour Phone # 410-247-9100
Virology Department (ext 1713)
FAX # 410-536-1474

Denise Bopst is the contact person at Quest.

a. Shipment Manifest

A manifest template in Microsoft Excel was provided to sites at the beginning of visit 20. The following fields must be in the manifest:

1) Unique vial identifier
2) WIHSID
3) WIHS Visit
4) Date of Collection
5) WIHS Specimen Code
6) Volume
7) Quest Diagnostics Incorporated test number (fasting panel is 81892; non-fasting panel is 81876)
8) Protocol Number (WIHS)
9) Box
10) Row
11) Column

The following fields can be provided in the manifest but are not required:

1) LDMS primary, additive, and derivative codes
2) Custom, site-specific identifier

b. Shipment Notification

Sites must notify Quest at least 24 hours prior to shipment with a completed fax. (See **WIHS Manual of Operations, Section 10, Appendix C.**)

c. Shipping Requirements

Sites are responsible for shipping materials and the cost of shipping. All vials for a participant at a visit should be grouped in adjacent locations within a box. It is preferable that a set of participant's vials remains in one box. Depending on the fasting status of the participant, 16-27 complete sets should fit in one box. A set of a participant's vials should not be sent in multiple shipments. Boxes within a shipment should have a unique identifier and ideally all boxes sent to Quest should have a unique identifier across all shipments. Prior to shipment, sites should verify that barcodes are readable and that vials match the manifest.

d. Shipping Schedule

All specimens collected for central metabolic testing should be shipped to Quest once a month. Shipments can arrive between Tuesdays and Fridays only. Sites are expected to adhere to the agreed-upon shipping schedule and notify Quest if they will not ship in their designated week.

Week of the month	Site that will ship to Quest
First	Brooklyn
Second	San Francisco
Third	Chicago
Fourth	Los Angeles/Hawaii

e. Shipping Errors and Confirmation of Receipt

Quest will not be able to immediately confirm receipt of shipment if there are no problems with the shipment. Sender contacts listed on the notification fax will be contacted in the event of a problem with the shipment, manifest, or vials. Received shipments will be summarized monthly by Quest.

7. DATA TRANSMISSION TO WDMAC AND SITES

Completed *F29* and *CV29* forms will be entered into Apollo by site study staff, and data will be incorporated into the central edit process and distributed with clean Apollo data.

Assay results for all tests performed in real time by Quest will be distributed on a monthly basis to both the clinical sites and WDMAC (Gayle Springer). Cardiovascular Substudy laboratory data will be incorporated into the frozen database at the end of each visit cycle, as with other centrally-generated data.

F. CARDIOVASCULAR EVENTS

1. OVERVIEW

   For the Cardiovascular Substudy, we will perform ongoing identification of cardio- and cerebro-vascular disease (CVD) events occurring among all WIHS subjects. CVD events will be analyzed in prospective and nested case-control analyses in relation to subclinical carotid atherosclerosis, HIV/AIDS status, antiretroviral medications, and CVD risk factors. This is a long-term study aim, since the number of CVD events may too small in the short term for adequately-powered analyses.

2. IDENTIFICATION OF POTENTIAL CVD EVENTS

   The means for identifying potential CVD events will be the same as those currently in place, including self-report at core visit interviews. (See **Manual of Operations, Section 12**, for more detailed information on outcomes ascertainment in the WIHS.)

   CVD events for which self-report data will be collected include:

   - Coronary revascularization procedure *to look for* or *to open* blocked vessels in the heart performed on an outpatient or inpatient basis (e.g., cardiac catheterization, angioplasty or coronary artery bypass graft): *F22HX*, Question C44a; disease code: 337.
   - Myocardial infarction (heart attack): *F22HX*, Question C42c; disease code: 331.
   - Stroke: *F22HX*, Question C42d; disease code: 333.
   - Hospitalization for congestive heart failure: *F22HX*, Question C42bi; disease code: 332.
   - Hospitalization for angina (chest pain related to heart disease): *F22HX*, Question C42ai; disease code: 334.
   - Transient ischemic attack (TIA or mini-stroke): *F22HX*, Question C42e; disease code: 335.

   Note that some events (e.g., cardiac catheterization, percutaneous revascularization) are frequently performed on an outpatient basis. Other events such as MI, stroke, angina and CHF will be limited to hospitalizations.

   Note: Medical record abstraction of CVD events was discontinued as of visit 26. Sites should still complete an ATC if a participant reports one of the above events.

APPENDIX A

Suggested Informed Consent Language – Carotid Ultrasound

You will receive an ultrasound examination that will take pictures of the arteries in your neck using sound waves. We will also measure your heart rhythm and blood pressure. These examination procedures are considered safe. You will not be exposed to any X-rays. Ultrasound is widely used in the evaluation of pregnancy and in other clinical applications. Your exposure to ultrasound in this examination will be no greater than a typical clinical examination.

If the examination uncovers any medical problems in the arteries of your neck that require evaluation, you will be so advised. We will also provide that information to a physician or clinic that you choose. In the case that clinical follow up care is required, the WIHS study will not be responsible for paying for this. It is important to note that the examination you receive here does not substitute for a medical examination your doctor might give you. The ultrasound examination you receive here is different from a medical ultrasound examination and does not provide the same information to a physician. It is possible that you may have disease in other arteries in your neck or other parts of your body that are not part of this examination. We will not report results that are of research value only.

APPENDIX B

Sample reports of carotid ultrasound findings

(Centers will adapt these samples for their own use)

Sample Letter to Participant

Dear (participant):

The following abnormal finding has come to our attention at the (name of clinical center) where you are a participant in the WIHS Study.

By visual inspection, 50% stenosis was noted in your (right common carotid artery, right internal carotid artery, etc.). This stenosis indicates that you have some narrowing of this artery in your neck due to atherosclerosis or hardening of the arteries.

*Note: The ultrasound procedure was performed as part of a research protocol to image the carotid arterial wall. This incidental finding is based solely on visual inspection.

We suggest you seek medical attention regarding this matter. Please feel free to contact me at (phone number) if you have any questions.

Sincerely,

Sample Letter to Participant's Physician

Dear Dr. :

The following abnormal finding has come to our attention at the (name of clinical center) where your patient, (name), is a participant in the WIHS Study.

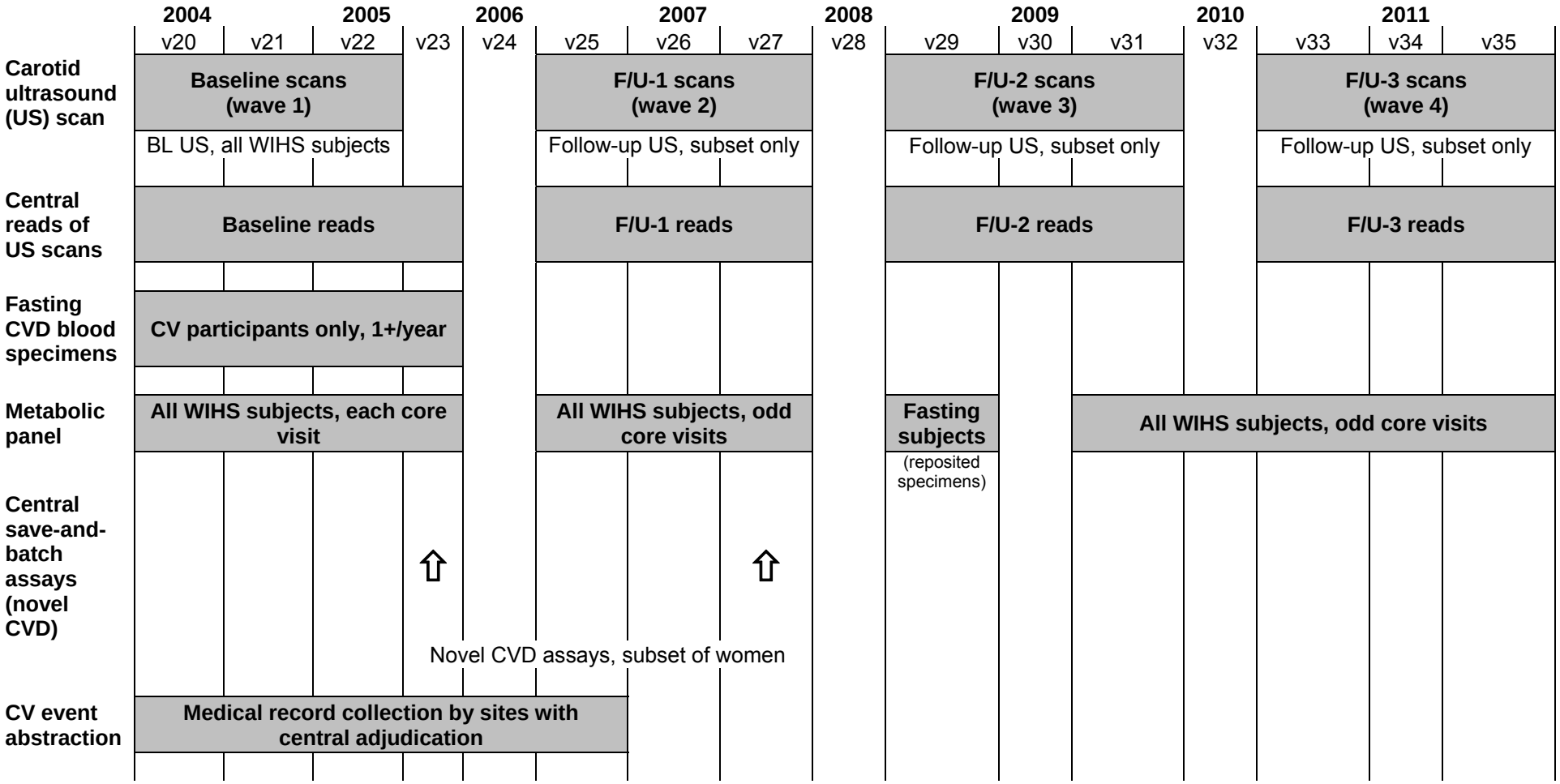
By visual inspection, 50% stenosis was noted in her (right common carotid artery, right internal carotid artery, etc.).

*Note: The ultrasound procedure was performed as part of a research protocol to image the carotid arterial wall. This incidental finding is based solely on visual inspection.

We have advised (name) of the above finding and suggested that she contact you regarding this matter. Please feel free to contact me at (phone number) if you have questions.

Sincerely,

APPENDIX C
Approximate Timetable and Scope of WIHS CVD Substudy



APPENDIX D

Sample size and power considerations

I. Sample size, baseline carotid ultrasound

A. All subjects (assume ~85% of WIHS cohort participates in ultrasound protocol)
- 1600 HIV+
- 650 HIV-

B. AIDS-defining illness (assume 35% of HIV+ have AIDS)
- 560 HIV+ with AIDS
- 1040 HIV+ without AIDS

C. HAART use vs no ART (assume 10% of HIV+ use no ART, 75% use HAART)
- 1200 HIV+ treated with HAART
- 160 HIV+ not treated with ART

D. Among HAART users, PI vs no PI (assume 2/3 of HAART includes PI)
- 800 HIV+ with PI-containing HAART
- 400 HIV+ with PI-sparing HAART

E. Limit to 40 years and older (matches eligibility MACS criteria)
- 800 HIV+
- 195 HIV-

II. Power, cross-sectional analysis of carotid lesions

Hsue, et al 2004 reported an approximate doubling of lesions in any of 12 carotid segments among n=148 HIV+ patients compared with n=63 controls (45% vs 24%). Depairon, et al 2001 found femoral and carotid artery plaques in 93 of 168 (55.4%) HIV-infected subjects compared with 26 of 68 (38.2%) HIV-negative subjects. We assumed a relatively low prevalence of lesions due to the use of a unilateral, limited procedure for imaging lesions.

Control Group	%Lesions	Exposed Group	Exposed %Lesions for Power=.80
HIV-	1%	HIV+	3%
HIV-, >40y	5%	HIV+, >40y	12%
HIV, no AIDS	5%	AIDS	9%
No ART	5%	HAART	9%
HAART, no PI	5%	PI-HAART	11%

Figure Ia. Power for cross sectional analysis of carotid lesions

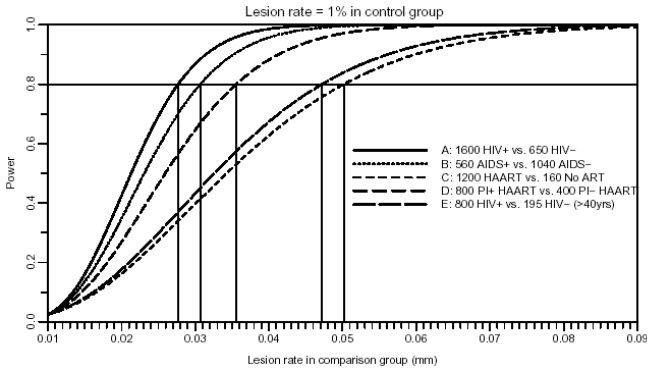
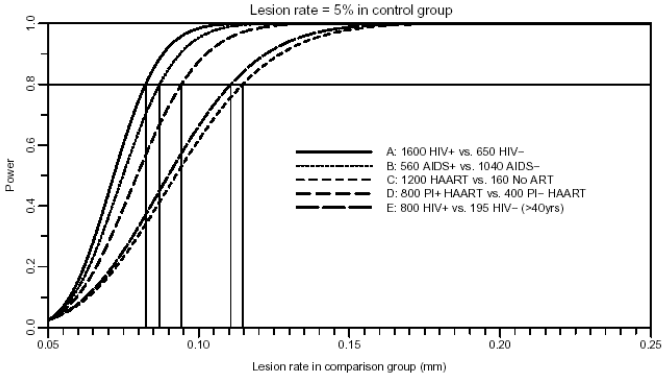


Figure Ib. Power for cross sectional analysis of carotid lesions



III. Power, cross-sectional analysis of right common carotid IMT

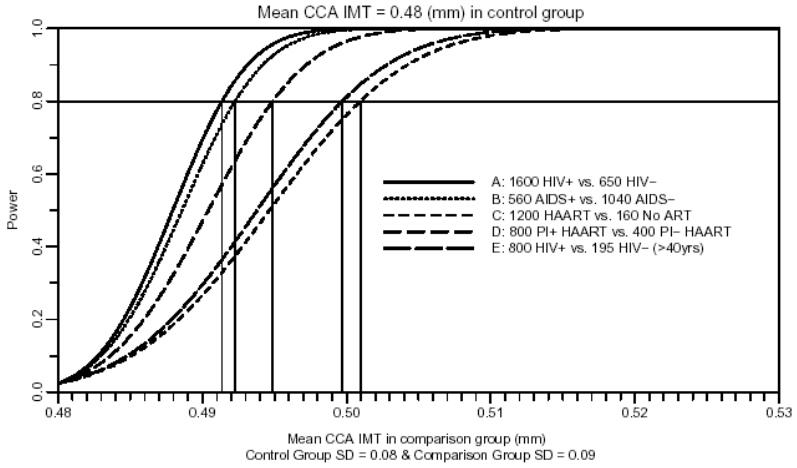
Sample size for IMT progression analyses will be n=760.

For control groups (ie, no HIV, AIDS, PI use), assumed rCCA IMT = 0.48 +/- 0.08 mm based on HIV- control group in Chironi et al, 2003 (n=36). Chironi et al reported rCCA IMT among HIV+ subjects = 0.53 +/- 0.09 mm (n=36).

Control Group	Exposed Group	Exposed rCCA-IMT for Power=.80
HIV-	HIV+	0.491 mm
HIV-, >40y	HIV+, >40y	0.501 mm
HIV, no AIDS	AIDS	0.492 mm
No ART	HAART	0.495 mm
HAART, no PI	PI-HAART	0.500 mm

Control rCCA-IMT = 0.480 mm

Figure II. Power for cross sectional analysis of right common carotid IMT



IV. Power, progression of right common carotid IMT

For control groups (ie, no HIV, AIDS, PI use), assumed control CCA IMT progression reported in metaanalysis by Bots, et al, 2003 = 0.0147 +/- 0.053 mm per year (or 0.0441 mm per 3 years). Hsue, et al 2004 reported CCA progression among HIV+ subjects = 0.074 +/- 0.13 mm per year (or 0.222 mm per 3 years) (n=121), vs no progression in HIV- controls (n=27).

Overall sample size for follow-up ultrasound is 760. Detectable effects are as follows:

Control Group	Exposed Group for Power=.80	Exposed rCCA-IMT Progression
HIV-	HIV+	0.09 mm/3yr
HIV-, >40y	HIV+, >40y	0.14 mm/3yr
HIV, no AIDS	AIDS	0.09 mm/3yr
No ART	HAART	0.11 mm/3yr
HAART, no PI	PI-HAART	0.14 mm/3yr

Control rCCA-IMT Progression = 0.04 mm/3yr

Figure III. Power for progression of right common carotid IMT