

ISSUES IN HIV RESEARCH

**METHODOLOGICAL AND CLINICAL ISSUES IN ANALYSIS OF DATA
FROM HIV CARDIOVASCULAR RESEARCH: VALIDITY OF ULTRASOUND
METHODS, IMPACT OF ANTI-RETROVIRAL THERAPY ON
ATHEROSCLEROSIS, AND IMPUTATION OF MISSING VALUES**

By

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TITLE: Methodological and Clinical Issues in the Analysis of Data from HIV
Cardiovascular Research: Validity of Ultrasound Methods, Impact of Anti-Retroviral
Therapy on Atherosclerosis, and Imputation of Missing Values

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ABSTRACT

Background and Objectives: There are some methodological and clinical challenges in conducting HIV related research. A subset of such challenges include: non-availability of a universally accepted method to quantify subclinical atherosclerosis in HIV patients; ultrasound imaging techniques aimed at quantifying atheroma burden and endothelial dysfunction have been proposed, however there is no universally accepted ultrasound protocol; conflicting inferences on the nature of the relationship between anti-retroviral therapy (ART) and cardiovascular disease (CVD) due to small sample sizes; and missing data from longitudinal studies and ultrasound data. The objective of this thesis is to investigate selected aspects of the afore-mentioned issues, and to provide recommendations for future research.

Methods:

Project 1: We compared the construct validity of carotid artery intima media thickness (IMT) and brachial artery flow mediated vasodilation (FMD); two non-invasive ultrasound techniques used in measuring the extent of sub-clinical atherosclerosis. Baseline and one-year follow-up data were obtained for a sample of 257 subjects aged 35 years or older, recruited into an ongoing study of cardiovascular risk in HIV. An ultrasound technique having statistically significantly strong association with known CVD risk factors was adjudged to have good construct validity. The relationship between baseline IMT or FMD and known CVD risk factors was studied using multiple regression analysis. We modelled the relationship between progression of IMT or FMD and risk factors using fixed-effects models.

Project 2: To more precisely investigate the relationship between ARTs and IMT (as a surrogate for CVD), we pooled cross-sectional baseline, record-level data for 1,032 patients recruited across three cohort studies in Canada, France and USA in a meta-analysis. We investigated the association between exposure to ARTs and CVD using hierarchical linear models.

Project 3: On missing data, we studied the impact of an inclusive strategy for conducting multiple imputation (MI) on the efficiency of regression parameter estimates using Monte-Carlo simulation. In an inclusive strategy, all final analysis variables are included in a multivariate normal model to impute plausible values for missing data. This issue is not well studied for longitudinal HIV data.

Results and Conclusions:

Project 1: Baseline IMT was significantly associated with age ($p < 0.001$), male gender ($p = 0.034$), current smoking status ($p < 0.001$), systolic blood pressure ($p < 0.001$) and total:HDL cholesterol ratio ($p = 0.004$). IMT progression was significantly associated with age ($p < 0.001$), male gender ($p = 0.0051$) and current smoking status ($p = 0.011$). Neither extent nor progression of FMD was significantly associated with any of the examined vascular risk factors. IMT was adjudged to have better construct validity than FMD.

Project 2: Similar to some (but not all) previous studies, ARTs do not appear to lead to CVD independent of traditional risk factors. However, exploratory analysis of two-way interactions suggests statistically significant moderating effects between ARTs and

traditional risk factors. These results warrant further investigation into potential moderating effects between ARTs and known CVD risk factors.

Project 3: In conducting MI, simulation results show that a strategy that includes all final analysis model variables in the imputation model provides the least combined variability and bias for final regression estimates. This is important to note because final regression estimates are used in making clinically relevant inferences in practice.

PREFACE

This thesis is a “sandwich thesis”, which combines three individual projects prepared for publication in peer-reviewed journals. The following are the contributions of A.

Odueyungbo in all the papers included in this dissertation: developing the research ideas and research questions; writing the protocol and analysis plans; conducting all statistical analysis; writing all of the manuscripts; submitting the manuscripts; and responding to reviewers’ comments. The work in this thesis was conducted between Winter 2007 and Fall 2009.

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CHAPTER 1

INTRODUCTION

Human immuno-deficiency virus (HIV) is an organism that compromises the immune system of infected individuals, consequently resulting in life-threatening opportunistic infections [1,2]. The virus can be transferred from an infected person to an uninfected person through the exchange of body fluids such as blood, vaginal fluid, semen or breast milk [3]. HIV infection is a precursor to AIDS (Acquired Immune Deficiency Syndrome) which is a deadly and severe phase in the progression of the disease [4].

According to a 2006 report by the Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO), over 25 million individuals have died from AIDS since its discovery in 1981 [5]. In 2005, approximately 39 million individuals were living with HIV globally, with an estimated four million new infections [5]. In 2008, there were 65,000 cases of HIV/AIDS in Canada, with 2,300 to 4,300 new infections (<http://www.phac-aspc.gc.ca/aids-sida/publication/survreport/estimat08-eng.php>). In Ontario, there were 26,490 cases in 2007, with 1,683 new infections (http://health.gov.on.ca/english/public/program/hivaids/general/charact_epidemic.html).

There is currently no cure for HIV/AIDS. However, the advent of anti-retroviral therapies (ARTs) has been associated with a significant reduction in the number of HIV infections that progress into AIDS, thus leading to improved life expectancy for patients [2,6].

Clinical and Methodological Issues in HIV Research

Research involving chronic diseases, like HIV, can be quite challenging due to the array of methodologically and clinically relevant issues encountered by investigators. A subset of the challenges may include: lack of a universally acceptable technique for assessing extent of atherosclerosis in HIV infected persons; identifying and quantifying the susceptibility of HIV patients to cardiovascular disease (CVD); and missing data in longitudinal or cross-sectional HIV studies. The objective of this dissertation is to investigate these three issues (discussed below), and provide recommendations or suggestions on how to address some of them in practice.

Issue 1 – Quantifying Atherosclerosis in HIV patients:

Theoretical and empirical research suggests that HIV patients are more susceptible to CVD [7-9] but there is no universally acceptable method to quantify the extent of subclinical atherosclerosis in this patient population. Non-invasive, cost-effective, safe, validated and reliable methods are desirable but the available gold standard (coronary angiography) is invasive, costly and often leads to complications [10]. The absence of a universally acceptable technique makes it difficult for researchers to collaborate or to compare results across studies. Nonetheless, ultrasound measures obtained from arterial wall thickness have been used as surrogates of extent, severity and progression of atherosclerosis in diverse patient populations [11,12]. Examples of such measures include carotid intima media thickness (IMT), brachial artery flow-mediated vasodilation (FMD), plaque area etc [11,12].

Issue 2 - Association between ARTs (anti-retroviral therapies) and CVD:

Along with the positive impacts of ARTs on patients' survival, there are reports of unfavourable outcomes like abnormal lipid profiles, insulin resistance, and lipodystrophy [13-17]. As a result of these adverse effects, there is considerable interest in studying the predisposition of HIV patients to CVD, and the specific role of ARTs in the atherosclerotic process [13,18]. However, results linking ARTs to CVD have been conflicting [16,19-34]. Many of the available studies are characterized by small sample sizes (and limited number of events for binary outcomes like Myocardial Infarction), thereby placing a limit on precision and statistical power for studying research hypotheses. As mentioned in the previous section (Issue 1), the non-uniformity of ultrasound methods for assessing subclinical atherosclerosis also makes it difficult to compare results across studies.

Issue 3 – Missing Data in Longitudinal Studies and Ultrasound Data:

Missing data constitute a major problem in the statistical analysis of prospective clinical studies, especially in HIV research, where patients are often monitored for long durations to ascertain progress against some pre-defined criteria [35]. Incomplete observations can occur as a result of patients' attrition, loss to follow-up, data entry errors, or unreadable scans in ultrasound images [35].

Many statistical methods ignore incomplete observations, but inferences may not be valid (or reliable) when missing values are completely ignored or not properly handled, especially when missingness is related to the outcome of interest [36]. Methods for handling missing data, such as multiple imputation (MI), have been shown to lead to

less biased inferences for a variety of missing data patterns or mechanisms [37-40]. In MI, missing values are predicted from pre-selected variables included in an assumed multivariate normal (imputation) model. A common approach is to include all final analysis model variables (inclusive strategy) in the imputation model [41], but penalties associated with exceptions to this strategy are not well studied for longitudinal HIV data. A technique, such as Monte-Carlo simulation, will be useful in assessing the impact of a deviant MI strategy on the variability and bias of estimated regression parameters.

Outline for the Thesis

This thesis is a sandwich of three papers strategically mapped to each of the Issues (1-3) described above. The three papers are separated into different chapters beginning from Chapter 2.

Chapter 2 discusses a study to compare the construct validity of two commonly used ultrasound techniques for quantifying the extent of atherosclerosis in HIV patients. The methods are: carotid artery intima media thickness (IMT) and brachial artery flow-mediated vasodilation (FMD). While IMT measures anatomic disease in the carotid arteries, FMD is a measure of endothelial dysfunction in the brachial arteries [11,12]. The objectives of this chapter are: to further assist researchers in the quest for ultrasound techniques that are useful for quantifying subclinical atherosclerosis; and to advance one step closer to a universally acceptable ultrasound metric.

In Chapter 3, an individual-patient meta-analysis was conducted to, more precisely, investigate the relationship between ARTs and carotid IMT (as a surrogate for CVD) in a large sample of patients recruited to three cohort studies across North America

and Europe. In a departure from previous studies, we have also explored interactions between ARTs and traditional CVD risk factors in an exploratory analysis of possible moderating effects. Not accounting for interaction effects, when they exist, may lead to incomplete inferences from regression models [42]. Further, an understanding of potentially significant interaction effects, will assist physicians in prescribing appropriate therapies for patients based on empirically determined and theoretically meaningful susceptibility to CVD.

Chapter 4 discusses the problem of missing data in longitudinal HIV studies. We conducted a simulation study to investigate performance - measured using a quantity incorporating bias and variability - associated with estimated regression parameters after MI: (1) when some final analysis models are excluded (restrictive strategy) from the imputation model; and (2) under different percentages of missingness.

Lastly, longitudinal data are often encountered in studies of chronic diseases such as HIV/AIDS, in which patients are tracked, over time, for progress against certain benchmarks [43]. Statistical analyses of longitudinal (or clustered) data ought to account for possible correlations between successive measurements on each subject. Models that fail to account for correlation will likely provide invalid inferences [43], which may result in sub-optimal clinical decisions. We have used appropriate methodology - such as generalized estimating equations and multi-level analysis - in all the papers included in this thesis to obtain statistical models that are closer to reality [35].

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CHAPTER 2

Comparison of brachial and carotid artery ultrasound for assessing extent of subclinical atherosclerosis in HIV: a prospective cohort study

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Abstract

Background: Non-invasive surrogate measures which are valid and responsive to change are needed to study cardiovascular risks in HIV. We compared the construct validity of two noninvasive arterial measures: carotid intima medial thickness (IMT), which measures anatomic disease; and brachial flow-mediated vasodilation (FMD), a measure of endothelial dysfunction.

Methods: A sample of 257 subjects aged 35 years or older, attending clinics in five Canadian centres, were prospectively recruited into a study of cardiovascular risk among HIV subjects. The relationship between baseline IMT or FMD and traditional vascular risk factors was studied using regression analysis. We analyzed the relationship between progression of IMT or FMD and risk factors using fixed-effects models. We adjusted for use of statin medication and CD4 count in both models.

Results: Baseline IMT was significantly associated with age ($p < 0.001$), male gender ($p = 0.034$), current smoking status ($p < 0.001$), systolic blood pressure ($p < 0.001$) and total:HDL cholesterol ratio ($p = 0.004$), but not statin use ($p = 0.904$) and CD4 count ($p = 0.929$). IMT progression was significantly associated with age ($p < 0.001$), male gender ($p = 0.0051$) and current smoking status ($p = 0.011$), but not statin use ($p = 0.289$) and CD4 count ($p = 0.927$). FMD progression was significantly associated with current statin use ($p = 0.019$), but not CD4 count ($p = 0.84$). Neither extent nor progression of FMD was significantly associated with any of the examined vascular risk factors.

Conclusions: IMT correlates better than FMD with established cardiovascular risk factors in this cohort of HIV patients. Standardization of protocols for FMD and IMT will facilitate the comparison of results across studies.

Background

HIV patients may have a higher risk of developing cardiovascular diseases than the general population [1-3]. This higher risk *may* be attributed to HIV infection or to individual drugs (or drug classes) used in treating the infection [1,4]. In particular, studies have shown that protease inhibitors [4] and nucleoside reverse transcriptase inhibitors such as abacavir and didanosine are associated with increased risk of myocardial infarction in HIV patients [5].

Cardiovascular disease is often characterized by development of atherosclerosis, in which plaque is accumulated on the inside of arterial walls [6]. The reference standard for assessing extent of atherosclerosis is coronary angiography, which is costly, invasive and has occasional complications such as vascular injury [7]. Inexpensive, reproducible, validated, non-invasive measurement of sub-clinical atherosclerosis involves the use of ultrasound (US) methods for imaging the carotid and brachial arteries [8-10]. Summary measures obtained from arterial wall thickness have been used as surrogates of extent, severity and progression of atherosclerosis in numerous studies of cardiovascular health involving diverse patient populations [10]. Examples of such measures include carotid intimal medial thickness (IMT), brachial artery flow-mediated vasodilation (FMD) and plaque area [10,11].

Carotid IMT is a measure of anatomic disease, used to identify and determine the extent of early arterial wall changes or structural vascular abnormalities [10,12-14]. Increased carotid IMT is a strong predictor of acute coronary events [10,14,15], and is

significantly associated with established cardiovascular risk factors among various study populations [1,9,10,13,14,16-18].

Brachial FMD is a non-invasive and validated measure of endothelial function [19,20]. The endothelium helps to maintain vascular health by releasing both *paracrine* and *autocrine* factors such as nitric oxide (also called *endothelium-derived relaxing factor*). Nitric oxide (NO) promotes smooth muscle relaxation, inhibition of platelet aggregation and adhesion, vasodilation and increased blood flow [21,22]. Thus, endothelial generation of NO is protective against atherogenesis [22]. A reduction in endothelial release of NO indicates endothelial dysfunction and is regarded as an early evidence of atherosclerosis [21-25]. Individuals with coronary artery disease (CVD) may exhibit impaired brachial FMD responses in the brachial arteries [11,20,26].

Impaired brachial FMD has been shown to be significantly associated with cardiovascular risk factors in some [11,24,27], but not all, studies [13,28]. Also, there are conflicting results regarding the association between brachial FMD and cardiovascular events in various patient populations [20,29].

Non-invasive surrogate measures which are valid and responsive to change are needed to study cardiovascular risks associated with HIV or HIV treatment regimens. There are limited data on the relationship between extent/progression of carotid IMT or brachial FMD and traditional vascular risk factors in HIV patients. Further, the relationship between carotid IMT and brachial FMD has not been well studied in HIV patients. In this study, we compare the validity and responsiveness to change of two ultrasound measures: 12-segment carotid artery IMT and brachial artery FMD in

Canadian HIV vascular study participants. We also investigate the relationship between these two measures.

Methods

Study design and study population:

HIV patients aged 35 years or older, attending university-affiliated clinics in five Canadian centers (Hamilton, Toronto, Calgary, Quebec City and Vancouver) are being recruited into an ongoing five-year, prospective, multi-center cohort study to evaluate the association between atherosclerotic progression, anti-retroviral drug regimen, immune reconstitution and standard cardiovascular risk factors. Subjects are recruited regardless of cardiovascular risk factors or past cardiac history. The study was approved by research ethics boards of each study site, and informed consent was obtained from all participants.

All participants provide a medical history and undergo yearly high-resolution ultrasound using a standardized protocol and centralized reading. As of March 2008, 257 subjects had baseline measurements for carotid IMT and brachial FMD, with 168 patients having one-year follow-up assessments. Measurement of carotid IMT is ongoing, but brachial FMD was discontinued after one-year follow-up due to cost considerations. For this ancillary study, two datasets were created namely: **(1) cross-sectional data** consisting of 257 patients with baseline carotid IMT and brachial FMD; and **(2) progression data** consisting of 168 patients with baseline and follow-up measurements for carotid IMT and brachial FMD (Figure 1).

Clinical characteristics:

Data on demographic and certain clinical characteristics of subjects were collected at each centre using questionnaires administered by research staff, or by chart review. Blood pressure was measured twice using a mercury sphygmomanometer, and results averaged. Lipids (total and HDL cholesterol and triglycerides) were measured after overnight fast. LDL-cholesterol concentration was calculated by the Friedewald formula. CD4-T-lymphocyte counts were obtained by FACS analysis performed by the Hamilton Regional Laboratory Medicine Program, and plasma HIV viral load were measured by Chiron bDNA assay at the Central Public Health Laboratory in Toronto, Ontario.

Ultrasound methods:

Ultrasound imaging and readings are conducted by trained personnel using high resolution B-mode ultrasonography, standardized protocol and centralized reading. The ultrasound laboratory in each study site uses imaging systems equipped with 7.5 to 10 MHz linear phase-arrayed vascular transducers. The same imaging system is used for all ultrasound imaging within each center. Ultrasound measurements are recorded on S-VHS tapes, which are later digitized and analyzed offline at the Core Carotid Ultrasound Laboratory (Hamilton, Ontario) by a certified reader blinded to patients' clinical information.

Patients were advised to fast and abstain from caffeine/vasoactive medications 12 hours prior to measurement, and were advised to avoid cigarette smoking (second-hand

inclusive) at least four hours prior to imaging. Imaging for carotid IMT was done before brachial FMD on the same day.

(A) 12-segment carotid intimal medial thickness (IMT)

Carotid IMT identifies and quantitates early arterial wall changes or structural vascular abnormalities [10,12,13]. A rigorously-standardized, reliable, validated method of ‘12-segment carotid IMT’ developed by Lonn et al [8,30] was used to assess the global extent of atherosclerosis in patients. Images of six well-defined segments (near and far wall of the common carotid, the bifurcation and the internal carotid) were obtained in each of the left and right carotid arteries using high resolution B-mode ultrasonography.

Ultrasound measurements were recorded on S-VHS tapes, which were later digitized and analyzed using the Image-Pro V4.5.1 software (Glen Burnie, Maryland). For each segment a minimum of three frames were measured. The maximum of all measurements from each segment were summed-up and divided by 12 to obtain the “12-segment mean-maximal carotid IMT” [8]. Twelve-segment mean-maximal carotid IMT is higher in individuals with CVD [8,30].

(B) Brachial flow-mediated vasodilation (FMD)

Brachial FMD was measured using an extensively validated and reliable method [13,31-33]. End-diastolic ultrasound images of the brachial artery diameter (longitudinally and slightly above the *antebrachial fossa* or upper arm) were obtained at rest and during vasodilator response induced by passive hyperemia (endothelium-dependent dilation).

Each patient rested in a quiet room for 10 minutes, after which sequential images of the brachial artery were obtained within a 45 second interval. Subsequently, a blood

pressure cuff was inflated around the right lower arm to at least 200 mm Hg, resulting in occlusion of blood flow to the upper arm. The cuff was released after five minutes, resulting in a marked increase in blood flow due to resistance vessel dilation. The increase in blood flow stimulates the release of NO which mediates the dilation of conduit vessels. Peak brachial artery dilation occurs approximately one minute after cuff release [26]. Another set of sequential images was obtained during peak dilation.

The ultrasound image frames obtained were recorded on S-VHS tapes, from which brachial artery diameters were calculated using Dynamic Endothelial Assessment (DEA) software (Montreal, Quebec). Average diameter of brachial artery (before and after dilation) was obtained from nine sequential images taken at rest and 12 taken during peak artery dilation. Percent flow mediated dilation was expressed as

$$\text{FMD\%} = \left[\frac{(\text{average diameter at peak dilation} - \text{average diameter at rest})}{\text{average diameter at rest}} \right] * 100$$

Conduit vessel dilation is attenuated (smaller %FMD) in individuals with CVD [26].

Twelve-segment carotid IMT and brachial FMD have been standardized and validated in previous studies at the Core Carotid Ultrasound Laboratory (Hamilton, Ontario), with intraclass correlation > 90% and coefficient of variation < 5% for repeat examinations [13,30].

Statistical analysis:

Continuous variables are expressed as mean (standard deviation), while categorical variables are expressed as count (percent) unless otherwise stated.

We hypothesized that “brachial FMD and carotid IMT should correlate well with traditional vascular risk factors for them to be considered good measures of extent, severity or progression of atherosclerosis”. This formed the basis for assessment of construct validity. Multiple linear regression models were used to examine the association between baseline carotid IMT or brachial FMD and the well-validated traditional “Framingham” cardiovascular risk factors of age, male gender, current smoking status, systolic blood pressure (SBP) and total:HDL cholesterol ratio using the cross-sectional data. Goodness-of-fit was evaluated by plotting the residuals from models to assess the normality assumption. The distribution of residuals should approximate the normal distribution for good model fit. We also used the co-efficient of determination (R^2) to quantify the proportion of variation in the dependent variable explained by the independent variables included in the multiple regression models [34].

Fixed effects models were used to study the relationship between progression of carotid IMT or brachial FMD and known cardiovascular risk factors using the progression data. Fixed effects models are useful for longitudinal data in which changes in time-varying covariates such as age, total:HDL cholesterol and SBP may affect the repeated outcome of interest [35]. There is no reason to assume that these quantities are constant over time. Further, the correlation between baseline and follow-up response is incorporated into model specification by assuming a plausible correlation structure. We assumed a “continuous time” version of the auto-regressive (AR(1)) correlation structure (available only for mixed/fixed effects models in SAS[®] software), to adjust for irregularities in follow-up times [36]. The reason is that many scheduled follow-up visits

were not feasible due to circumstances beyond the control of investigators, thus resulting in differential follow-up times for patients. A *time* variable was created by designating the first visit for each patient as ($t_1 = 1$) and follow-up visits as

$$t_2 = \left\{ t_1 + \left(\frac{\text{Date of second visit} - \text{Date of first visit}}{365} \right) \right\}$$

The time component is closer to reality by making it a continuous, rather than a discrete, variable. Model fit was assessed using the “Null Model Likelihood Ratio Test” [37]. The “Null Model Likelihood Ratio Test” is a likelihood ratio test of whether the model with a *specified* covariance structure fits better than a model where repeated responses are assumed *independent*. An independent covariance structure is often implausible for repeated measures data. A p-value < 0.05 for the likelihood ratio test shows that the fitted model is better than an *independent* covariance structure model [37]. Model adequacy was also evaluated using Akaike’s Information Criterion (AIC) to compare between “continuous time” and “fixed time” AR(1) structures. A smaller AIC indicates better fit [37].

We evaluated the nature of the relationship between baseline carotid IMT and brachial FMD using Pearson correlation co-efficient.

Patients were classified as very low, low, medium/high risk if individual Framingham risk scores were < 5%, 5-9% and $\geq 10\%$ respectively [38]. The medium and high risk categories were combined due to limited numbers of subjects in these categories. Framingham risk scores quantify the 10-year risk of developing “hard” coronary heart disease including myocardial infarction and coronary death [38].

Framingham risk score is a strong predictor of coronary heart disease [38]. One-way analysis of variance (ANOVA) models were used to cross-sectionally examine differences in brachial FMD or carotid IMT by Framingham risk group classification.

We adjusted for current use of statin medication and CD4 count in each regression model. All statistical tests were conducted at 5% significance level. Graphs and analysis results were obtained using SPSS Version 15.0 (SPSS Inc., Chicago, Illinois, USA) and SAS Version 9.1 (SAS Institute Inc., Cary, NC, USA).

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Baseline and follow-up characteristics:

Cross-sectional data - There were 257 patients in the baseline extent data with 232(90.3%) males and 25(9.7%) females. Carotid IMT ranged from 0.47mm to 2.24 mm, with mean(SD) of 0.81(0.23) mm. Brachial FMD ranged from -7.36% to 29.96%, with mean(SD) of 4.95(4.50)%. We found a weak inverse relationship between carotid IMT and brachial FMD at baseline ($r = -0.126$; $p = 0.043$; see Figure 2). Other patient characteristics are listed in Table 1.

Stratifying by Framingham risk group, *dose-response* relationships were found between risk group classification and carotid IMT or brachial FMD (Table 2). Carotid IMT differed significantly between risk groups from ANOVA analysis ($p < 0.001$).

Brachial FMD did not differ significantly across the risk groups from ANOVA results ($p = 0.227$).

Of the 257 patients assessed at baseline, information on anti-retroviral therapy was available for 253 individuals. There were 85 (34%) patients who were currently on Abacavir, 106 (42%) were on Zidovudine, 61 (24%) on Stavudine, 21 (8%) on Didanosine, 98 (39%) on Efavirenz, 21 (8%) on Nelfinavir and 21 (8%) on Nevirapine. However, we did not test the effects of HIV medications on Carotid IMT/brachial FMD as that was not part of our main goal, which was to validate these measures against traditional risk factors.

Progression data - There were 168 patients in the progression dataset with 151(89.9%) males and 17(10.1%) females. Median (interquartile range) follow-up time was 1.02 (0.43) years. At baseline, carotid IMT varied from 0.47 mm to 1.57 mm with mean(SD) of 0.82(0.22) mm, while brachial FMD varied from -6.81% to 29.96% with mean(SD) of 5.10(4.58)%. At one-year follow-up, the measures ranged from 0.50 mm to 1.57 mm with mean(SD) of 0.84(0.23) mm and -13.61% to 25.52% with mean(SD) of 4.40(4.96)% respectively. On average, carotid IMT progressed at 0.02(standard error (SE) = 0.01) mm/year while brachial FMD decreased at 0.84(SE = 0.79)%/year. Summary statistics for other variables are listed in Table 3. Summary data for patients excluded from the progression analyses are summarized in Table 4. Patient distribution appears to be comparable in both included and excluded data, except for viral load and current statin use.

Examining the data cross-sectionally at baseline and follow-up, there was a *dose-response* relationship between carotid IMT and risk group classification (Table 5).

Carotid IMT differed significantly by risk group classification at baseline and follow-up ($p < 0.001$ respectively in each case). There was neither a *dose-response* relationship nor significant difference in brachial FMD across risk groups at baseline and follow-up ($p = 0.540$ and 0.312 respectively).

Validity of baseline extent measures (cross-sectional data):

Goodness-of-fit tests were satisfied. The distribution of residuals did not deviate systematically from the normal distribution. Validity of measurement method was assessed by how well each method correlated with classical cardiovascular risk factors at baseline. From multiple regression models: older patients ($p < 0.001$), male patients ($p = 0.034$), current smokers ($p < 0.001$), patients with higher SBP ($p < 0.001$), or higher total:HDL cholesterol ($p = 0.004$) were statistically significantly associated with higher carotid IMT (Table 6). The cardiovascular risk factors explained approximately 45% of the variation in carotid IMT ($R^2 = 0.45$). Neither current statin use nor CD4 count were statistically significantly associated with IMT ($p = 0.904$ and 0.929 respectively).

In contradistinction, none of these risk factors was significantly associated with brachial FMD (Table 6). The cardiovascular risk factors explained only 3% of the variation in brachial FMD ($R^2 = 0.031$). Current use of statins explained negligible amount of variation in both IMT and FMD regression models. It should however be noted that the percentage of patients on statin was very small to make strong inferences regarding the effect of the drug.

Responsiveness to change (progression data):

The “continuous time” AR(1) structure was assumed for carotid IMT while the “fixed time” structure was assumed for brachial FMD using results from the AICs. Both models provided better fits than the independent correlation structure model from the “Null Model Likelihood Ratio” tests.

From fixed-effects models, positive change in carotid IMT was statistically significantly associated with older age ($p < 0.001$), male gender ($p = 0.005$), and current smoking status ($p = 0.011$). Increase in SBP or total:HDL cholesterol was not statistically significantly associated with progression of carotid IMT (Table 7).

In comparison to non-statin users, patients on current (baseline) statin medication had significantly better FMD response after one-year follow-up (mean difference = 3.11, 95% CI: 0.53 to 5.69). None of the traditional cardiovascular risk factors was significantly associated with progression of brachial FMD (Table 7).

Discussion

Non-invasive, validated and reproducible arterial imaging techniques such as brachial FMD and carotid IMT are often used to measure the extent, severity or progression of subclinical atherosclerosis in vascular health studies [13,20]. Brachial FMD is a measure of endothelial dysfunction [13,20] whereas carotid IMT measures structural vascular integrity [13]. Studies have shown that anti-atherogenic interventions such as statins, angiotensin-converting enzyme (ACE) inhibitors and other blood-pressure lowering agents help to improve brachial FMD [13,32,39,40], and retard carotid IMT

progression [12,13,30,31], thus highlighting the importance of both measures in the atherosclerotic process.

In our study of HIV patients, neither extent nor progression of brachial FMD was significantly associated with any of the examined classical vascular risk factors. The cardiovascular risk factors explained only 3% of the variation in brachial FMD. Use of statin medication led to statistically significant improvement in brachial FMD, thus replicating results from other studies [39]. Extent of carotid IMT was significantly associated with age, male gender, current smoking status, SBP and total:HDL cholesterol, whereas progression of carotid IMT was significantly associated with age, male gender and current smoking status. The cardiovascular risk factors explained approximately 45% of the variation in carotid IMT.

Our results on carotid IMT are similar to results obtained in other vascular studies in both non-HIV [13,15,41,42] and HIV subject populations [1,43]. In a cross-sectional study involving 119 indigenous Australians at risk of cardiovascular disease, carotid IMT was significantly associated with traditional cardiovascular risk factors, while brachial FMD was associated with none of the examined risk factors [28]. A case-control study by Lekakis et al [3] found a significant association between extent of IMT and blood pressure, cholesterol and glucose levels, duration of HIV disease and use of protease inhibitors. In contrast, brachial FMD was only associated with triglyceride measurements [3]. Yan et al [13] in a cross-sectional analysis of data from a large cohort of middle-aged healthy men found significant associations between carotid IMT increase and age, SBP, body mass index, total and LDL cholesterol and fasting plasma glucose. Among all

risk factors examined, increasing SBP was the only one associated with impaired brachial FMD [13]. In a small study (total sample size = 37) involving a relatively homogenous sample of adult HIV patients on anti-retroviral therapy, Stein et al [44] found an association between impaired brachial FMD and VLDL (very low density), IDL (intermediate density), HDL and total cholesterol levels [44]. Brachial FMD has been shown to correlate with vascular risk factors in non-HIV subjects [24,27] and use of protease inhibitors in HIV subjects [44].

We also found a weak inverse relationship between carotid IMT and brachial FMD with borderline significance ($r = -0.126$, $p = 0.043$). A much larger study (sample size of 1,578) by Yan et al [11] found no significant correlation ($r = -0.006$, $p = 0.82$) between IMT and FMD in healthy middle-aged men without cardiovascular disease [11]. Irace et al [45] found a moderate linear association between FMD and IMT in treatment naïve subjects at risk of CVD ($r = -0.217$, $p = 0.058$). In a large study involving 2,109 healthy adults aged 24 to 39 years in Finland, Juonala et al [46] found a statistically significant inverse relationship ($p < 0.001$) between IMT and FMD, thus adding to a series of conflicting results on the “true” nature of the relationship between these two important measures. Several relatively smaller studies have found significant inverse relationship between IMT and FMD suggesting that these two measures assess the same “aspects and stages of early atherosclerosis” [47-52]. The results from smaller studies are suspect due to sample size limitation. Findings from Yan et al [13] suggest that brachial FMD and carotid IMT are likely “unique” and unrelated surrogates that assess varying aspects and stages vascular disease [13]. In contrast, Juonala et al [46] suggest a strong

inverse relationship between FMD and IMT, which would be expected if both measures are assessing the same construct. However, we note that while Yan et al [13] employed an IMT method that includes both far and near walls of all segments in the right and left carotid arteries (similar to our study), Juonala et al [46] employed a method that includes only the far wall of the left carotid artery. Perhaps this may serve to explain the contrasting results.

Various explanations have been proposed for conflicting results regarding brachial FMD in the literature. These include heterogeneity in patient populations being studied, different measurement protocols or inadequate sample sizes [11,13,14]. In our study, brachial FMD was measured using an extensively validated and reliable method [13,31-33]. Rundek et al [11] suggest a possibly *direct* relationship between endothelial dysfunction and atherosclerosis, independent of traditional vascular risk factors. Thus beyond traditional vascular factors, endothelial dysfunction may independently provide additional prognostic information on atherosclerosis through other *risk factors* not currently assessed [11,13,20]. Nevertheless, the validity of brachial FMD as a measure of cardiovascular risk in HIV remains largely unproven. There is need for large, long-term observational studies (with standardized FMD protocols) to critically evaluate the specific role of brachial FMD in atherosclerosis relating to HIV patients. The results presented in our paper were based on baseline and one-year follow-up results.

From our study, IMT progressed at an annual rate of 0.02 mm/year. Hsue et al [1] estimated the annual progression of IMT as 0.074 mm/year in an ancillary cohort study involving 121 HIV-infected adults [1]. The distinction between progression estimates

from different studies may result from demographic or clinical differences in the HIV populations studied. Further, more precise progression estimates can be obtained from studies with longer follow-up such as the ongoing “Canadian HIV vascular study”. The Canadian HIV vascular study also aims to investigate the relationship between atherosclerotic progression, anti-retroviral drug regimen and immune reconstitution.

There were significant cross-sectional *dose-response* relationships between baseline (or follow-up) carotid IMT and Framingham risk group classification. Framingham risk classification was a strong predictor of extent of carotid IMT, thus highlighting the prognostic value of risk group classification.

The use of fixed effects models to analyze progression data is one of the strengths of our study. Fixed effects models allow for the inclusion of time-varying covariates such as age, SBP and total:HDL cholesterol. Changes in these covariates are likely to affect progression of either brachial FMD or carotid IMT, thus including this information in model specification is vital to obtaining a closer representation of reality. Secondly, the use of the “continuous-time autoregressive correlation structure” option in SAS software allowed for patients to have differential follow-up times, which more closely depicts circumstances surrounding our study. Also, information on the correlation between baseline and follow-up outcome measures was included as part of model specification.

Conclusions

Carotid IMT is a useful surrogate marker of extent and progression of cardiovascular risk in HIV patients 35 years of age and older, correlating better than

FMD with established cardiovascular risk factors. Extent of carotid IMT correlates well with current risk stratification of patients using Framingham risk scores. Use of carotid IMT in ongoing and future observational studies and randomized trials may help to better define the atherosclerotic risk associated with HIV infection and with specific HIV treatments.

Comparison of results across studies is often quite difficult due to differing measurement protocols employed by different investigators. Standardization of protocols for FMD and IMT will aid the comparison of results across studies.

Competing interests

MS has investigator-initiated grant support from Gilead Sciences and Pfizer. LT consults with GlaxoSmithKline Inc. (GSK) on statistical and other methodological issues. No other potential conflicts to report.

Authors' contributions

AO wrote data analysis plan, conducted data analysis and wrote the first draft of manuscript with inputs from MS and LT. MS is principal investigator on HIV vascular cohort study. MS, LT, FS, KG, JG, TA, DE, SS, JB, EL and AO made substantial contributions to manuscript content through subsequent drafts. MS, FS, KG, JG, TA, DE, SS and EL participated in data collection at the various centers. All authors read and approved the final manuscript.

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Figure 1. Flowchart of patients

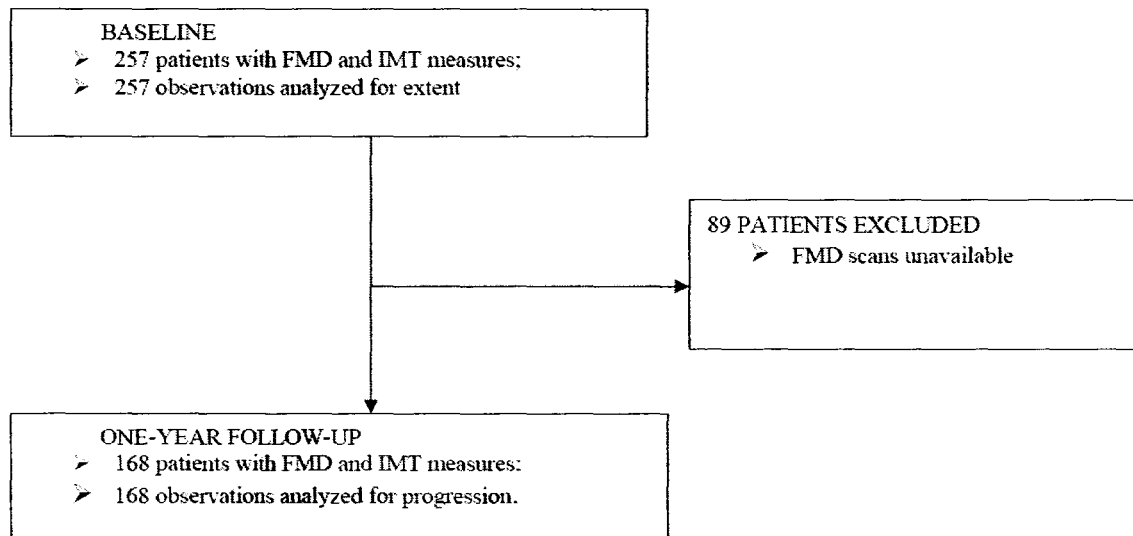


Figure 2. Carotid IMT versus brachial FMD at baseline

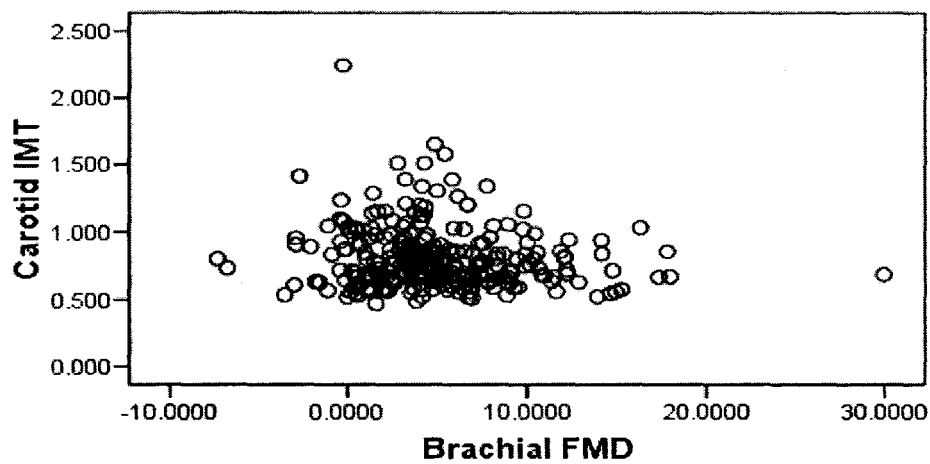


Table 1. Baseline characteristics for extent data

Variable	Estimate
Male [*]	232 (90.3)
Age (years) [#]	46.48 (7.86)
Carotid Artery Intima Media Thickness (IMT, mm) [#]	0.81 (0.23)
Flow Mediated Vasodilation (FMD, %) [#]	4.95 (4.50)
Total:HDL Cholesterol [#]	5.28 (1.33)
Systolic Blood Pressure (mm Hg) [#]	120.5 (15.6)
Current Smoking Status [*]	1 96 (37.5)
Current STATIN use [*]	1 18 (7.0)
CD4 Count [#]	479.9 (270.6)
Log ₁₀ Viral Load [#]	2.2 (1.2)

*NB) I=current smoker/user; * = count(%); # = mean(standard deviation)*

Table 2. Baseline characteristics for extent data by Framingham risk group

Risk group	Number of subjects	IMT (mm)	FMD (%)
Very low (< 5%)	88	0.68 (0.13)	5.58 (5.45)
Low (5 to 9%)	64	0.78 (0.16)	4.86 (3.59)
Medium/High (10% and above)	105	0.93 (0.27)	4.47 (4.08)

NB) Entries for IMT and FMD are reported as mean(standard deviation); IMT increases significantly with increasing Framingham risk ($p < 0.001$)

Table 3. Baseline and follow-up characteristics for progression data

Variable		Baseline	Follow-up
Male*		151 (89.9)	
AGE (years) [#]		47.19 (8.29)	48.25 (8.34)
IMT (mm) [#]		0.82 (0.22)	0.84 (0.23)
FMD (%) [#]		5.10 (4.58)	4.40 (4.96)
SBP (mm Hg) [#]		120.4 (15.7)	121.1 (13.7)
Total: HDL Cholesterol [#]		5.40 (1.39)	5.18 (1.17)
Current smoking status*	1	60 (35.7)	
Current STATIN use*	1	9 (5.4)	
CD4 Count [#]		495.0 (267.6)	571.3 (883.2)
Log ₁₀ Viral Load [#]		2.0 (1.1)	2.1 (1.2)

*NB) 1=current smoker/user; * = count(%); # = mean(standard deviation)*

Table 4. Baseline characteristics of excluded cases (n = 89)

Variable	Baseline
Male [*]	81(91)
AGE (years) [#]	45.16 (6.80)
IMT (mm) [#]	0.79 (0.26)
FMD (%) [#]	4.67 (4.36)
SBP (mm Hg) [#]	120.8 (15.6)
Total: HDL Cholesterol [#]	5.04 (1.18)
Current smoking status [*]	1 36 (40.9)
Current STATIN use [*]	1 9 (10.1)
CD4 Count [#]	451.14 (275.51)
Log ₁₀ Viral Load [#]	2.4 (1.3)

*NB) 1=current smoker/user; * = count(%); # = mean(standard deviation)*

Table 5. Baseline and follow-up characteristics for progression data by Framingham risk group

Risk group	Number of subjects	IMT 1 (Baseline)	IMT 2 (Follow-up)	FMD 1 (Baseline)	FMD 2 (Follow-up)
Very low (< 5%)	54	0.70 (0.14)	0.72 (0.15)	5.67 (5.88)	4.35 (4.36)
Low (5 to 9%)	46	0.78 (0.17)	0.78 (0.17)	4.83 (3.54)	5.29 (5.13)
Medium/High (10% and above)	68	0.94 (0.24)	0.97 (0.25)	4.83 (4.02)	3.84 (5.27)

NB) Entries for IMT and FMD are reported as mean(standard deviation); Reported to two decimal places.

Table 6. Estimates from multiple regression models for baseline Carotid IMT and Brachial FMD (%)

PARAMETER	CAROTID IMT			BRACHIAL FMD		
	Est.*	95% CI	p-value	Est.*	95% CI	p-value
Age (years)	0.016	(0.014, 0.019)	<0.001	-0.021	(-0.093, 0.051)	0.569
Male	0.081	(0.006, 0.155)	0.034	-1.738	(-3.601, 0.124)	0.067
Current smoking status	0.096	(0.050, 0.143)	<0.001	0.294	(-0.874, 1.462)	0.620
SBP (mm Hg)	0.003	(0.002, 0.005)	<0.001	-0.021	(-0.058, 0.016)	0.262
Total:HDL Cholesterol	0.026	(0.008, 0.043)	0.004	0.001	(-0.435, 0.438)	0.995
Current STATIN use	-0.006	(-0.096, 0.085)	0.904	1.578	(-0.683, 3.839)	0.171
CD4 Count	-0.000004	(-0.00009, 0.00008)	0.929	-0.001	(-0.003, 0.001)	0.512

NB) *Est. - Estimate.

Table 7. Estimates from fixed effects models for progression of Carotid IMT and Brachial FMD (%)

PARAMETER	CAROTID IMT			BRACHIAL FMD		
	Est.*	95% CI	p-value	Est.*	95% CI	p-value
Time (years)	0.001234	(-0.01556, 0.01803)	0.8847	0.7342	(-0.2578, 1.7261)	0.1457
Age (years)	0.01550	(0.01235, 0.01865)	<.0001	0.02485	(-0.04543, 0.09513)	0.4857
Male	0.1225	(0.03721, 0.2078)	0.0051	-0.1125	(-2.0420, 1.8169)	0.9085
Current smoking status	0.07073	(0.01658, 0.1249)	0.0108	-1.1385	(-2.3578, 0.08092)	0.0671
SBP	0.000726	(-0.00028, 0.001730)	0.1544	-0.02425	(-0.06244, 0.01395)	0.2116
Total:HDL Cholesterol	0.01051	(-0.00392, 0.02494)	0.1520	-0.2449	(-0.6936, 0.2038)	0.2824
Current STATIN use	0.06222	(-0.05335, 0.1778)	0.2893	3.1025	(0.5174, 5.6876)	0.0190
CD4 Count	0.0000009	(-0.00002, 0.000020)	0.9265	0.000085	(-0.00075, 0.000924)	0.8411

NB) *Est. - Estimate.

Addendum to Chapter 2

The purpose of this addendum is to provide information that was not part of the published manuscript based on Chapter 2, but was nonetheless considered essential to clarify some of the important elements of the project.

Baseline characteristics of patients:

Baseline summary data are reported as median (inter-quartile range[IQR]) for all continuous variables, except Carotid IMT in Tables 8, 9 and 10, whereas they were reported as mean (SD) in Tables 1 to 4. At baseline, median (IQR) of FMD was 4.31 (5.38)%, while that of total:HDL cholesterol was 5.16 (1.77). Patients had baseline median (IQR) of 460 (333) cells/mm³, 1.40 (1.39) copies/mL, and 120 (20) mmHg for CD4 count, Log viral load and SBP, respectively (Table 8). Please see Tables 9 and 10 for the summary data of patients in the progression data and patients excluded from fixed-effects models, respectively.

Multiple and fixed effects regression models:

The units of CD4 count and SBP were changed to 100 cells/mm³ and 10 mmHg respectively, to make their regression coefficients more interpretable. Thus, Tables 11 and 12 have been added to provide this additional information for CD4 count and SBP. The only difference between Tables 11-12 and Tables 6-7 is the change in the regression coefficients and associated 95% confidence limits of CD4 count and SBP; the latter being increased by a factor of 10 while the former was increased by a factor of 100. All other coefficients and p-values remain the same.

Relationship between Framingham risk group and Carotid IMT:

In Table 2, an Analysis of Variance model was used to study the relationship between Framingham risk group and IMT. We found a statistically significant dose-response relationship between IMT and risk classification at 5% level of significance ($p < 0.001$). Please note the disproportionate number of patients in the medium-high risk group ($n = 105$) compared to the other groups, especially the low risk group ($n = 64$). An ideal situation is when all groups have the same number of patients; a situation which is possible in experimental studies. One of the limitations of observational data is that categories within a variable may not have the same number of subjects (unbalanced data). Unbalanced data may lead to non-homogenous variances across the subgroups, and thus ANOVA results should be interpreted with caution in such instances. However, two non-parametric alternatives with less restrictive assumptions, the Kruskal-Wallis test and an asymptotic median test, led to similar results ($p < 0.001$ in both tests).

Bivariate associations between all continuous variables:

IMT and FMD - In Chapter 2, the relationship between Carotid IMT and brachial FMD was explored using a scatter plot and Pearson's correlation co-efficient. An alternative metric of linear association, which is robust to outliers, is the Spearman's rank correlation co-efficient. We obtained a Spearman correlation co-efficient of -0.092 ($p\text{-value} = 0.139$), depicting a weak linear association between the two variables. The co-efficient is quite similar to the Pearson correlation co-efficient ($r = -0.126$) obtained earlier, but appears more conservative in the hypothesis of significant linear association.

IMT and other continuous variables – IMT appeared to increase with: increasing age (Figure 3, Spearman's correlation co-efficient = 0.601, p-value < 0.001); increasing total:HDL cholesterol level (Figure 4, Spearman's correlation co-efficient = 0.261, p-value < 0.001) and increasing systolic blood pressure (Figure 5, Spearman's correlation co-efficient = 0.352, p-value < 0.001) at baseline. Notice there were a few potential outliers in the figures, but Spearman's correlation coefficient is quite robust to influential data points. Carotid IMT did not appear to have a strong linear relationship with each of the variables CD4 count and viral load (p-value = 0.125 and 0.064 respectively). Brachial FMD did not appear to have strong linear association with age (p-value = 0.249), total:HDL cholesterol (p-value = 0.584), systolic blood pressure (p-value = 0.184), CD4 count (p-value = 0.715) and viral load (p-value = 0.138).

Perhaps a curvilinear model can be useful to study the relationship between IMT (or FMD) and the variables with which it had no statistically significant linear association. However, for ease of model interpretation and parsimony, we have decided to fit linear models using multiple linear regression and fixed effects linear models. Further, relationships such as between IMT and CD4 count - for instance - often do not exist in isolation. Multiple regression models, which simultaneously adjust for other prognostic factors, in a multi-dimensional space may serve to explain a little more of the IMT trajectory as a result of an increase in CD4 count.

Bivariate relationships between baseline IMT and each continuous variable, stratifying by gender:

From the multiple regression and fixed effects models, gender was strongly associated with increased IMT and thus, it may be of interest to explore the relationship between IMT and other risk factors, stratified by gender. However, there is a limited number of females (n=25; 9.7%) in the cohort. This presents a limitation on the use of parametric models due to the highly disproportionate number of patients in each of the subgroups. Nonetheless, the relationships were explored using scatter plots, with LOESS smoothing in SPSS (Version 17) for each gender subgroup.

Males tended to have a higher increase in IMT with: increasing age (Figure 13); increasing total:HDL cholesterol (Figure 14); and increasing systolic blood pressure (Figure 15). From Figures 13-17, gender seemed to be a moderating factor between IMT and each of age, total:HDL cholesterol, SBP, CD4 count, and viral load. It will be worthwhile to ascertain the potential moderating effects of gender on other CVD risk factors in future studies having larger sample sizes. In Chapter 3, we have explored this likely phenomenon in a meta-analysis consisting of a large number of patients.

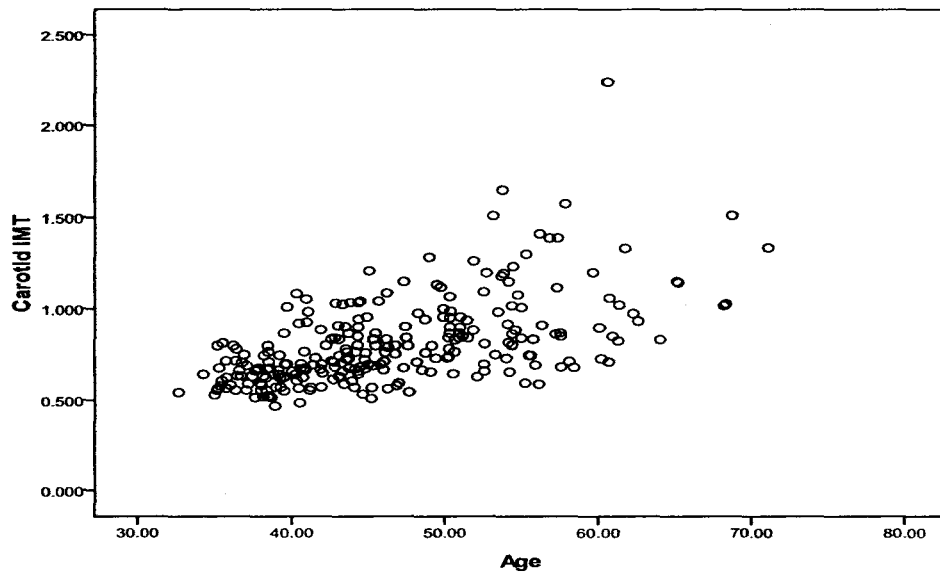
Relationship between IMT/FMD, HIV and associated treatments:

While we note that the contributions of HIV disease and its associated treatments to changes in IMT or FMD are important, this was not our primary objective in the analyses. Our primary goal was to compare the construct validity of IMT versus FMD in a cohort of patients who were HIV positive, by investigating the relationship between IMT/FMD and classical cardiovascular risk factors. In this regard, all other covariates

were considered extraneous. However, we did investigate the relationship between viremia (using Viral Load) and FMD/IMT, but the association was not statistically significant (results not shown in Chapter 2). We decided to drop the variable from the models because the regression co-efficient estimate was very close to zero, similar to the one for CD4 count. Further, adding or removing the variable (Viral Load) did not change the results in *Tables 5 and 6*.

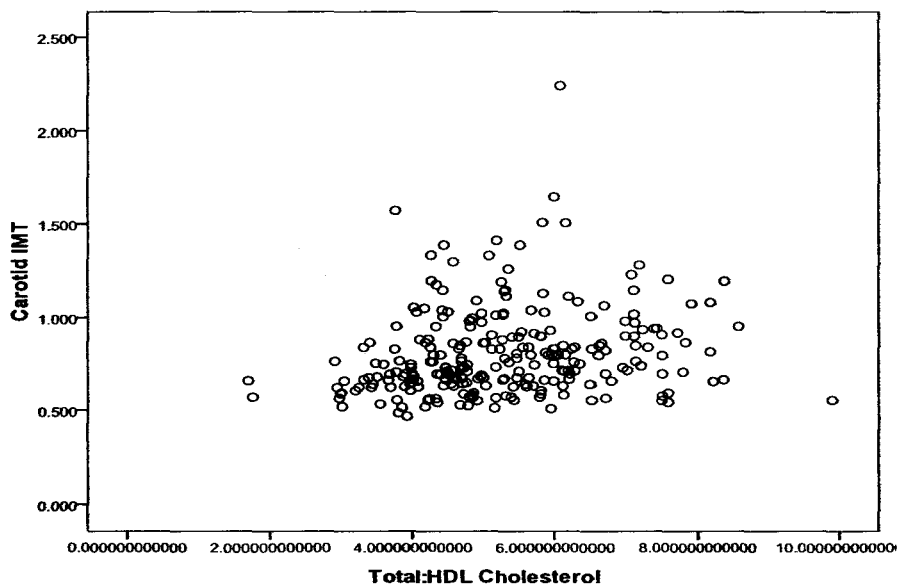
Please note that the association between HIV disease, ART exposure and IMT was the primary objective of Chapter 3, where there was a much larger sample with theoretically bigger statistical power to accommodate more covariates, such as HIV treatment, viremia and treatment duration in the regression models.

Figure 3. Carotid IMT versus age at baseline



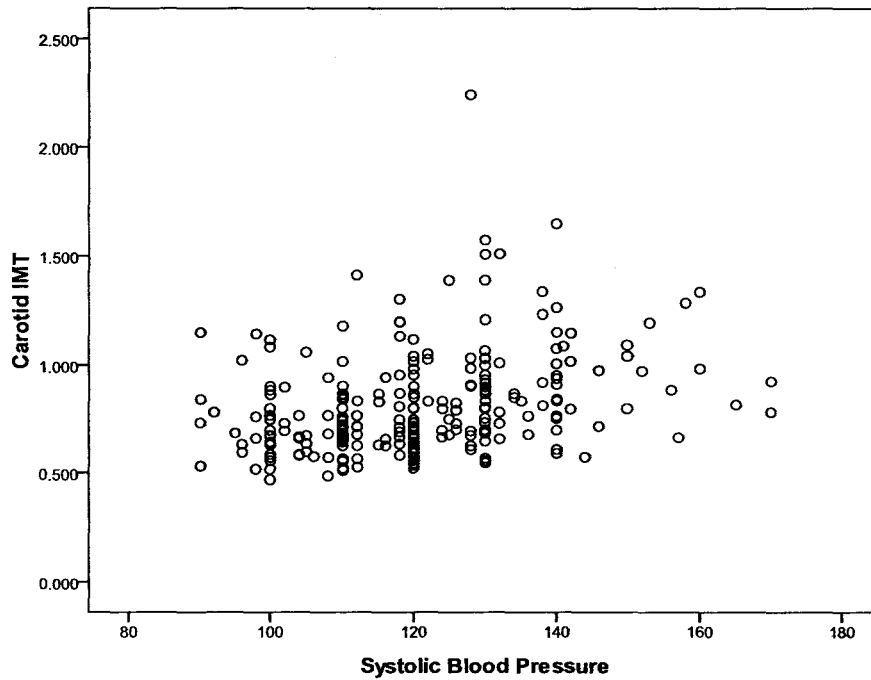
Spearman's Correlation coefficient = 0.601 (p-value < 0.001)

Figure 4. Carotid IMT versus Total:HDL cholesterol at baseline



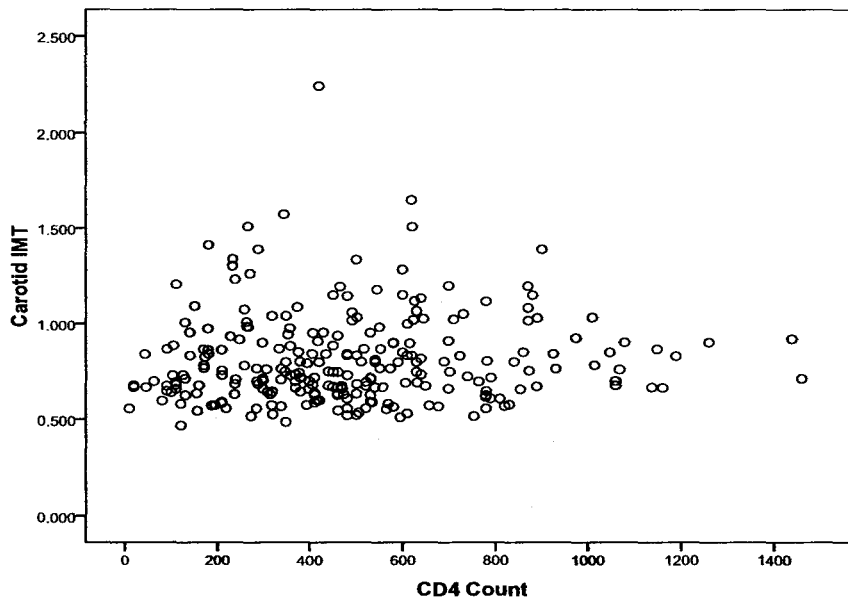
Spearman's Correlation coefficient = 0.261 (p-value < 0.001)

Figure 5. Carotid IMT versus systolic blood pressure at baseline



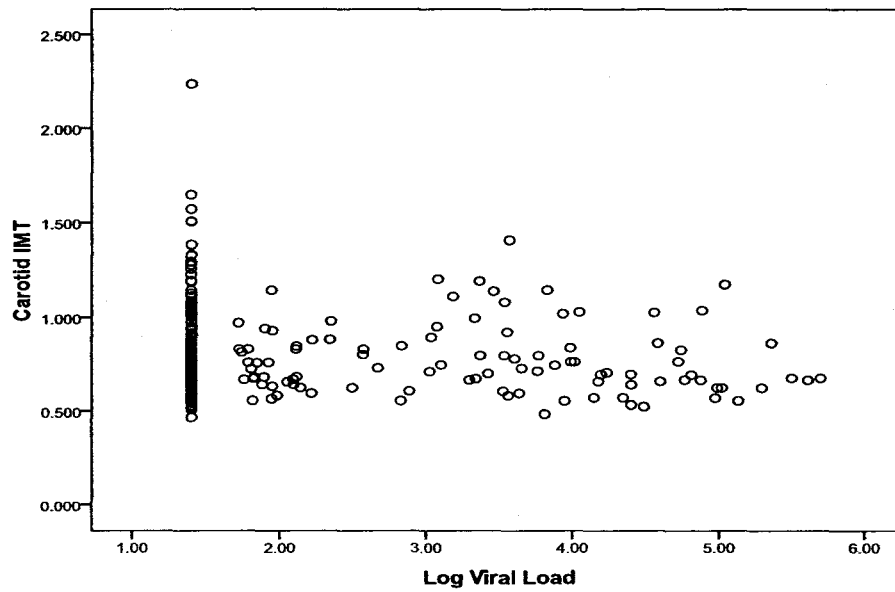
Spearman's Correlation coefficient = 0.352 (p-value < 0.001)

Figure 6. Carotid IMT versus CD4 count at baseline



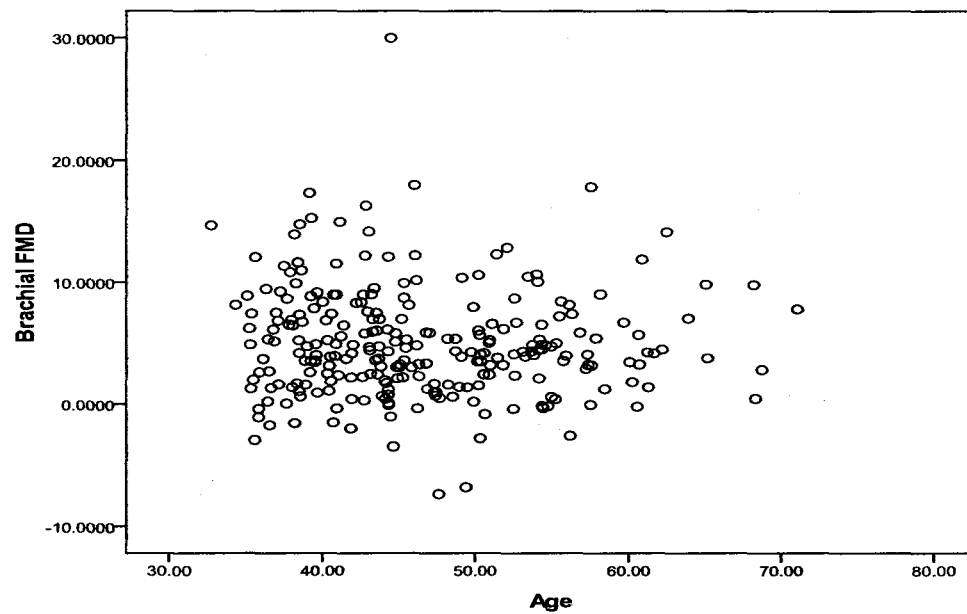
Spearman's Correlation coefficient = 0.096 (p-value = 0.125)

Figure 7. Carotid IMT versus logarithm of viral load at baseline



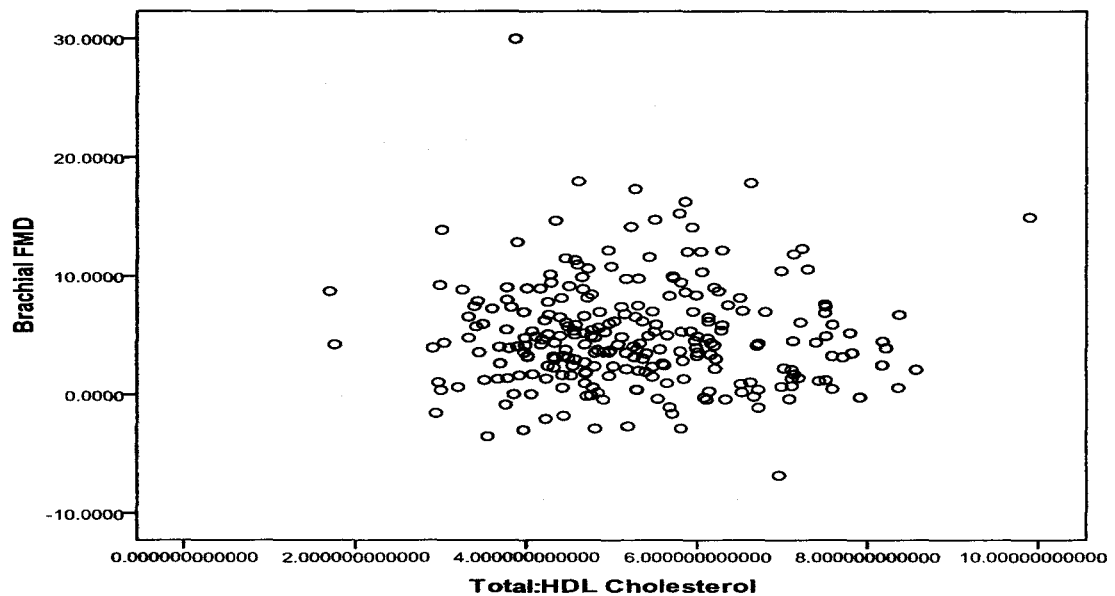
Spearman's Correlation coefficient = -0.116 (p-value = 0.064)

Figure 8. Brachial FMD versus age at baseline



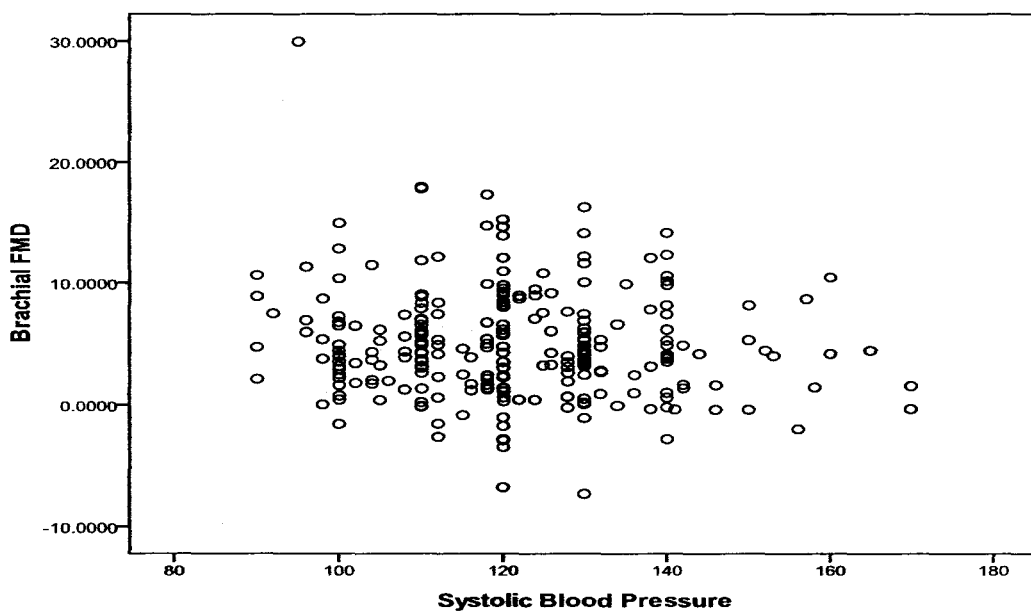
Spearman's Correlation coefficient = -0.072 (p-value = 0.249)

Figure 9. Brachial FMD versus Total:HDL cholesterol at baseline



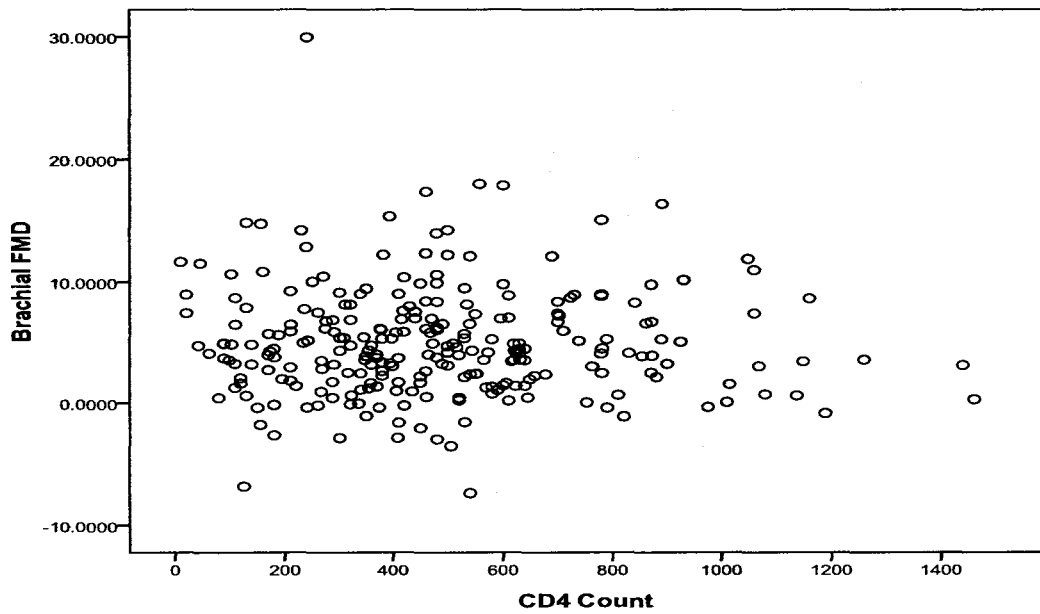
Spearman's Correlation coefficient = -0.035 (p-value = 0.584)

Figure 10. Brachial FMD versus systolic blood pressure at baseline



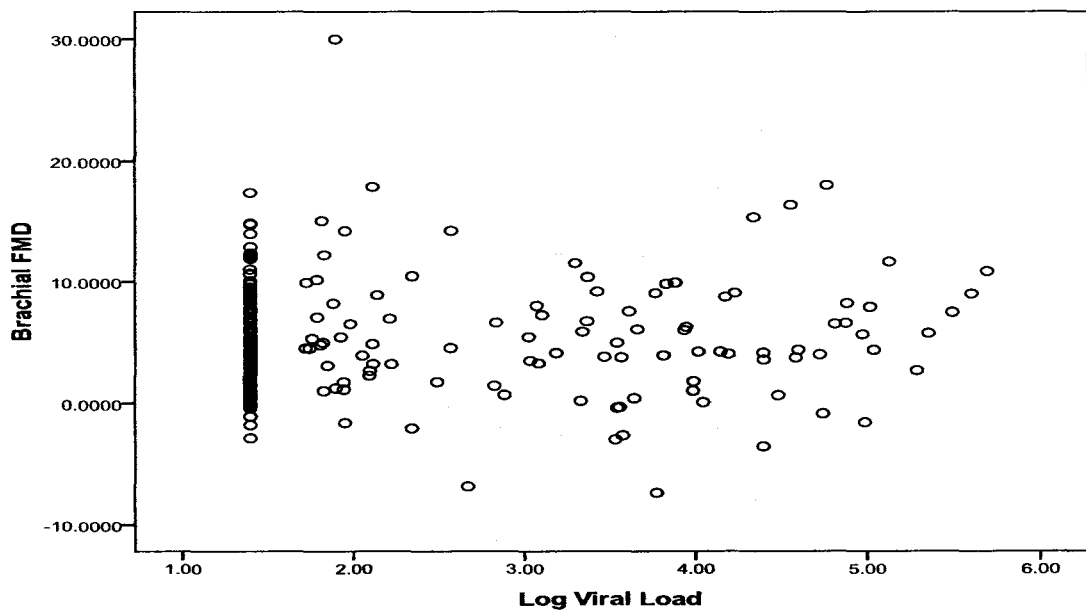
Spearman's Correlation coefficient = -0.083 (p-value = 0.184)

Figure 11. Brachial FMD versus CD4 count at baseline



Spearman's Correlation coefficient = -0.023 (p-value = 0.715)

Figure 12. Brachial FMD versus logarithm of viral load at baseline



Spearman's Correlation coefficient = 0.093 (p-value = 0.138)

Figure 13. Carotid IMT versus age at baseline by gender with loess smoothing

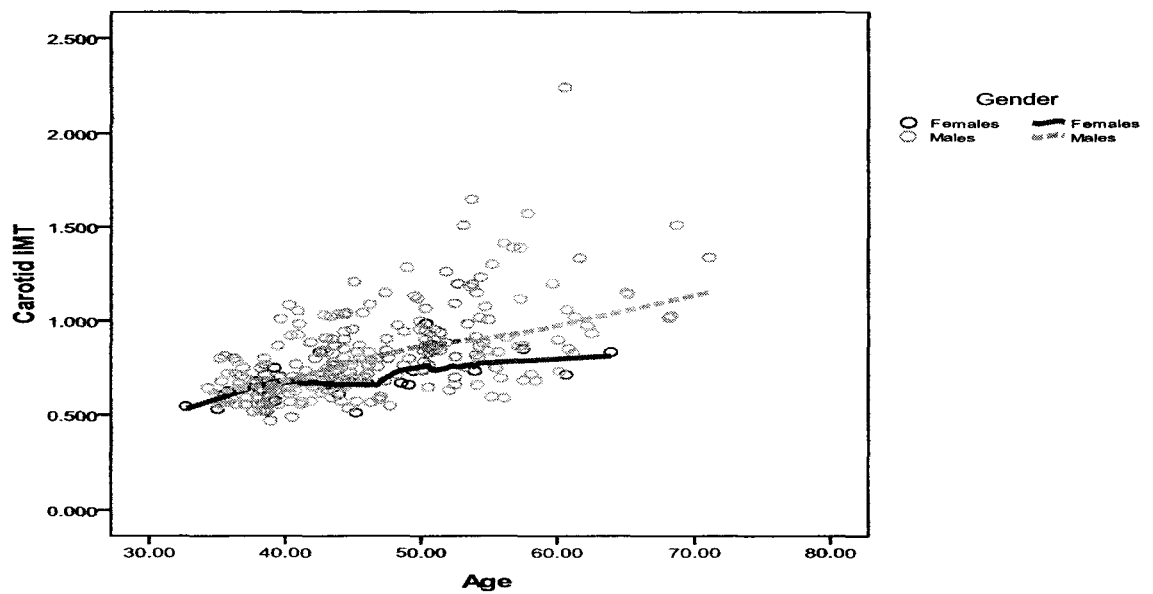


Figure 14. Carotid IMT versus Total:HDL cholesterol at baseline by gender with loess smoothing

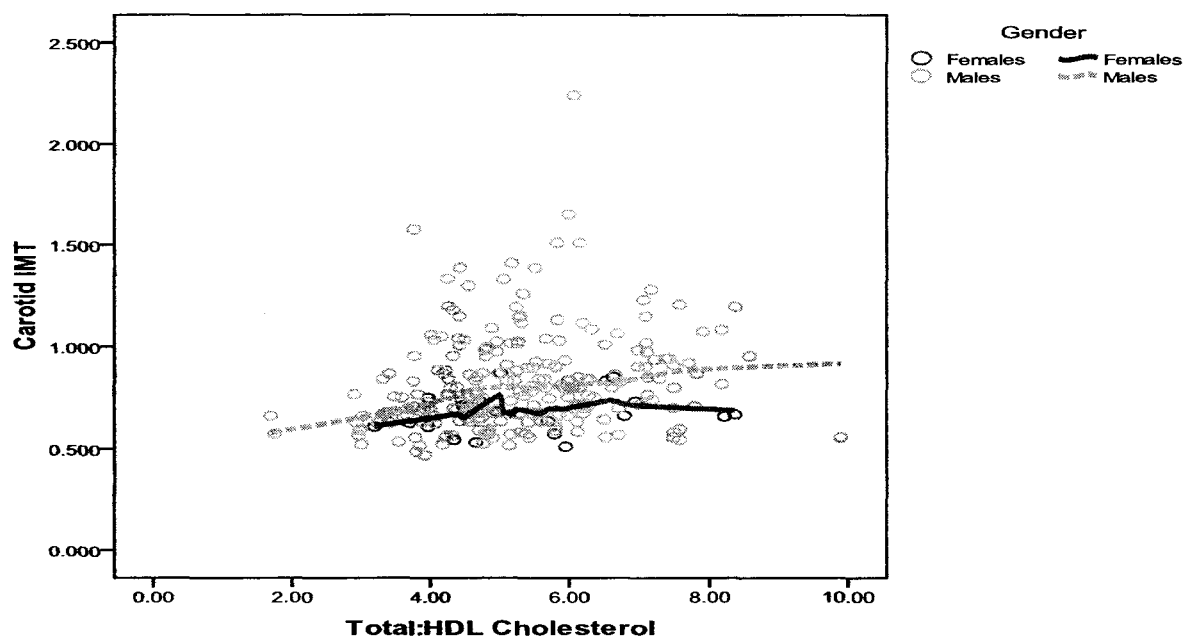


Figure 15. Carotid IMT versus systolic blood pressure at baseline by gender with loess smoothing

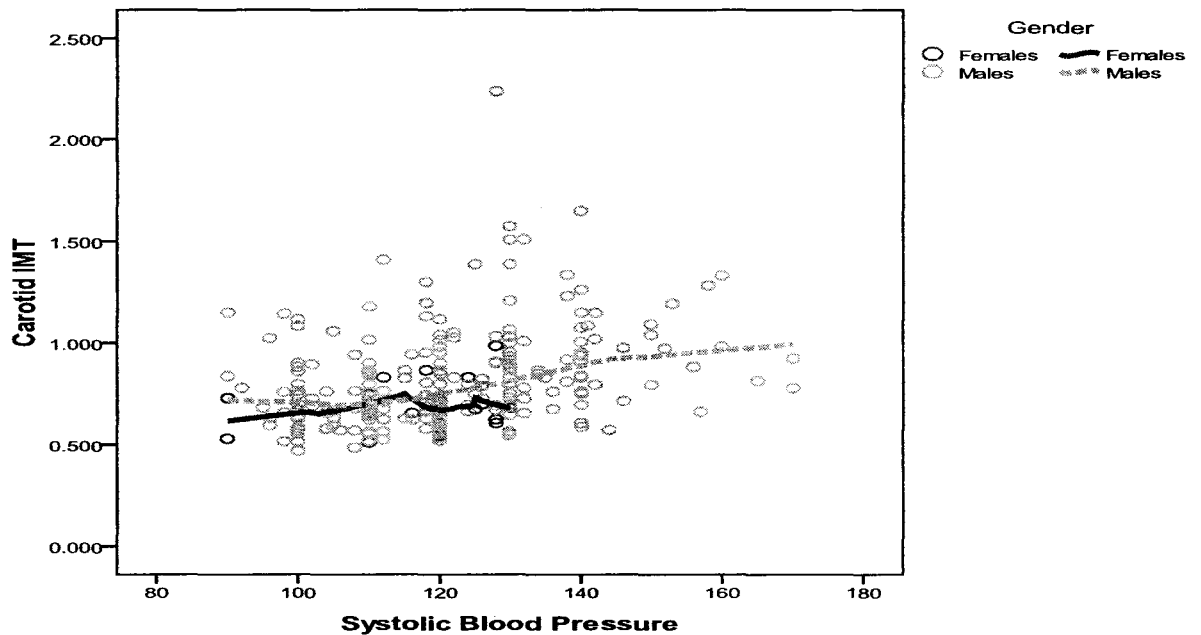


Figure 16. Carotid IMT versus CD4 count at baseline by gender with loess smoothing

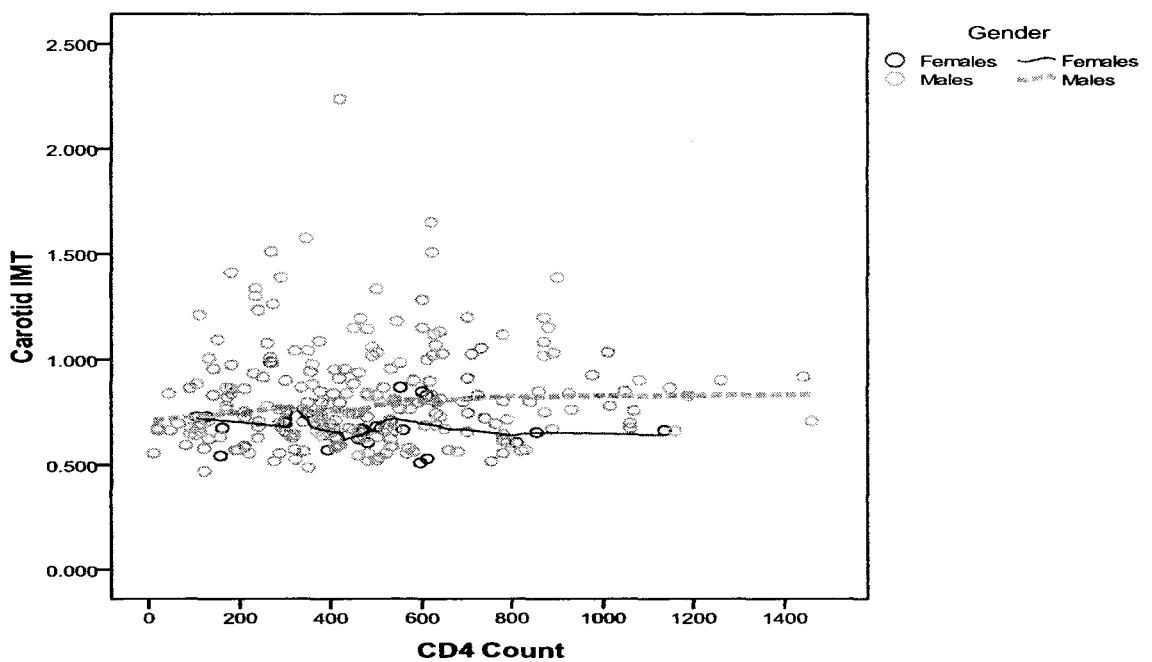


Figure 17. Carotid IMT versus logarithm of viral load at baseline by gender with loess smoothing

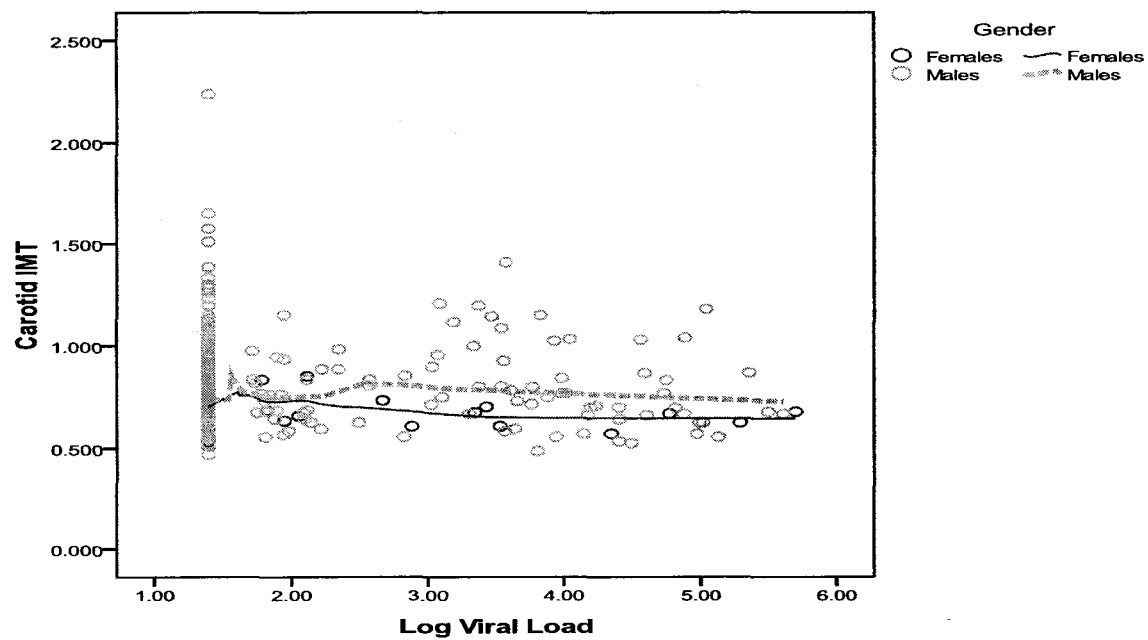


Table 8. Baseline characteristics for extent data

Variable	Summary statistics
Male [*]	232 (90.3)
Age (years) [#]	40.3 (11.7)
Carotid Artery Intima Media Thickness (IMT, mm) ^{&}	0.81 (0.23)
Flow Mediated Vasodilation (FMD, %) [#]	4.31 (5.38)
Total:HDL Cholesterol [#]	5.16 (1.77)
Systolic Blood Pressure (mm Hg) [#]	120 (20)
Current Smoking Status [*]	1 96 (37.5)
Current STATIN use [*]	1 18 (7.0)
CD4 Count [#]	460 (333)
Log ₁₀ Viral Load [#]	1.40 (1.39)

*NB) 1=current smoker/user; * = count(%); # = median(inter-quartile range); &=mean(standard deviation)*

Table 9. Baseline and follow-up characteristics for progression data

Variable	Baseline	Follow-up
Male [*]	151 (89.9)	
AGE (years) [#]	45.6 (13.1)	46.5 (13.3)
IMT (mm) ^{&}	0.82 (0.22)	0.84 (0.23)
FMD (%) [#]	4.21 (4.87)	4.02 (5.59)
SBP (mm Hg) [#]	120 (20)	120 (20)
Total: HDL Cholesterol [#]	5.29 (1.92)	5.05 (1.65)
Current smoking status [*] 1	60 (35.7)	
Current STATIN use [*] 1	9 (5.4)	
CD4 Count [#]	467.50 (338.25)	470.00 (328.50)
Log ₁₀ Viral Load [#]	1.40 (0.64)	1.40 (0.51)

*NB) 1=current smoker/user; * = count(%); # = median(inter-quartile range);
&=mean(standard deviation)*

Table 10. Baseline characteristics of excluded cases (n = 89)

Variable		Baseline Summary Statistics
Male [*]		81(91)
AGE (years) [#]		43.9 (9.7)
IMT (mm) ^{&}		0.79 (0.26)
FMD (%) [#]		4.47 (6.63)
SBP (mm Hg) [#]		120 (20)
Total: HDL Cholesterol [#]		4.88 (1.47)
Current smoking status [*]	1	36 (40.9)
Current STATIN use [*]	1	9 (10.1)
CD4 Count [#]		420.00 (361.25)
Log ₁₀ Viral Load [#]		1.75 (2.11)

*NB) 1=current smoker/user; * = count(%); # = median(inter-quartile range); &=mean(standard deviation)*

Table 11. Estimates from multiple regression models for baseline Carotid IMT and Brachial FMD (%)

PARAMETER	CAROTID IMT			BRACHIAL FMD		
	Est. *	95% CI	p-value	Est. *	95% CI	p-value
Age (years)	0.016	(0.014, 0.019)	<0.001	-0.021	(-0.093, 0.051)	0.569
Male	0.081	(0.006, 0.155)	0.034	-1.738	(-3.601, 0.124)	0.067
Current smoking status	0.096	(0.050, 0.143)	<0.001	0.294	(-0.874, 1.462)	0.620
SBP (10 mmHg)	0.033	(0.018, 0.047)	<0.001	-0.211	(-0.579, 0.158)	0.262
Total:HDL Cholesterol	0.026	(0.008, 0.043)	0.004	0.001	(-0.435, 0.438)	0.995
Current STATIN use	-0.006	(-0.096, 0.085)	0.904	1.578	(-0.683, 3.839)	0.171
CD4 Count (100 cells/mm3)	-0.0004	(-0.0089, 0.0081)	0.929	-0.0710	(-0.2840, 0.1419)	0.5118

NB) *Est. - Estimate.

Table 12. Estimates from fixed effects models for progression of Carotid IMT and Brachial FMD (%)

PARAMETER	CAROTID IMT			BRACHIAL FMD		
	Est.*	95% CI	p-value	Est.*	95% CI	p-value
Time (years)	0.001234	(-0.01556, 0.01803)	0.8847	0.7342	(-0.2578, 1.7261)	0.1457
Age (years)	0.01550	(0.01235, 0.01865)	<.0001	0.02485	(-0.04543, 0.09513)	0.4857
Male	0.1225	(0.03721, 0.2078)	0.0051	-0.1125	(-2.0420, 1.8169)	0.9085
Current smoking status	0.07073	(0.01658, 0.1249)	0.0108	-1.1385	(-2.3578, 0.08092)	0.0671
SBP (10 mmHg)	0.0072	(-0.0028, 0.0173)	0.1544	-0.2425	(-0.6244, 0.1395)	0.2116
Total:HDL Cholesterol	0.01051	(-0.00392, 0.02494)	0.1520	-0.2449	(-0.6936, 0.2038)	0.2824
Current STATIN use	0.06222	(-0.05335, 0.1778)	0.2893	3.1025	(0.5174, 5.6876)	0.0190
CD4 Count (100 cells/mm3)	0.00009	(-0.00185, 0.00203)	0.9265	0.00852	(-0.07537, 0.09241)	0.8411

NB) *Est. - Estimate.

CHAPTER 3

An Individual-Patient Meta-analysis to Study the Association between Anti-Retrovirals and Atherosclerosis

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Abstract

Background: There are conflicting results on the association between anti-retroviral therapies (ARTs) and cardiovascular disease (CVD). Inconsistencies among studies are likely due, in part, to limited sample sizes which can impact precision and statistical power for hypothesis testing. We conducted an individual-patient meta-analysis to study the relationship between ARTs and an ultrasound surrogate of CVD in a large sample of patients recruited to three cohort studies across North America and Europe.

Methods and Results: We obtained baseline, record-level data for 1,032 patients from three HIV cohort studies. Atherosclerosis was quantified using thickness of the left common carotid artery (CCA) obtained from high resolution B-mode ultrasound. We investigated the association of known CVD risk factors and patients' exposure to certain Protease Inhibitors (Saquinavir (INVIRASE/FORTOVASE), Ritonavir (any dose), Amprenavir, Indinavir, Nelfinavir), Nucleoside Reverse Transcriptase Inhibitors (Abacavir, Zidovudine (AZT), Stavudine (D4T), Zalcitabine, Didanosine, Lamivudine (3TC)), and Non-nucleoside Reverse Transcriptase Inhibitors (Delavirdine, Efavirenz, Nevirapine), with CCA using hierarchical linear models (HLMs). We also conducted a secondary analysis of all two-way interactions among covariates in a separate multivariable HLM.

Results: In univariate analysis, factors significantly associated with CCA included: male gender($p=0.002$), age($p<0.001$), diastolic blood pressure(DBP)($p=0.025$), systolic blood pressure(SBP)($p=0.003$), LDL cholesterol($p=0.001$), CVD history($p=0.002$), smoking($p=0.021$), use of AZT, D4T and 3TC($p=0.007$, 0.003 , and <0.001 respectively),

and duration of AZT, D4T, 3TC, Ritonavir and Nelfinavir($p=0.006$, 0.006 , 0.002 , 0.029 , and 0.022 respectively). Only male gender, age, LDL cholesterol, smoking and Ritonavir duration remained significantly associated with CCA in multivariable analysis. In exploratory analyses, we found significant interaction effects for: gender*AZT use($p=0.019$); gender*D4T use($p=0.022$); CVD history*DBP($p=0.020$); CVD history*SBP($p=0.001$); Smoking*AZT($p=0.005$); smoking*age($p=0.002$); and 3TC use*LDL($p=0.025$). The negative impacts of D4T and AZT seemed more pronounced in males than females. The relationship between DBP(or SBP) and atherosclerosis appeared dependent on past history of CVD. Smoking likely moderates the relationship between AZT(or age) and CVD. The drug 3TC may lead to CVD in patients with elevated LDL cholesterol.

Conclusions: From the main-effects only models, ARTs (except Ritonavir duration) were not significantly associated with atherosclerosis, independent of traditional risk factors. Significant interaction effects from adjunct analyses warrant further investigation of potential moderating effects between traditional risk factors and ARTs in the atherosclerotic process. An understanding of these interactions will facilitate a classification of patients to various risk profiles, and assist healthcare providers in prescribing the ‘best’ medication to patients based on risk stratification.

Keywords: Atherosclerosis, ultrasound, HIV, anti-retroviral therapy.

Introduction

The use of anti-retroviral therapies (ARTs) has led to substantial improvements in AIDS-related survival and other beneficial outcomes, such as immune reconstitution, in HIV patients [1]. Along with favorable outcomes, complications such as abnormal lipid profiles, insulin resistance, and lipodystrophy have been associated with ART use [2-6]. Consequently, there is considerable interest in studying the predisposition of HIV patients to cardiovascular disease (CVD), and the specific role of ARTs in the atherosclerotic process [1,2].

Results regarding the association between ARTs and vascular disease have been conflicting [5,7-22]. In the Data Collection on Adverse Events of Anti-HIV Drugs (D:A:D) study, the use of ARTs was associated with an elevated risk of myocardial infarction even after adjusting for traditional cardiovascular risk factors [12]. In an update of the D:A:D study, the investigators found a significant relationship between protease inhibitors (PI) and elevated MI risk, but found no link between non-nucleoside reverse transcriptase inhibitors (NNRTI) and CVD [13]. Results from the D:A:D study have been replicated in a few smaller studies [17,23-25].

However, not all studies have completely supported the hypothesized relationship between ARTs and CVD. Mercie et al [8] in a multi-center prospective cohort study of 423 patients found an association between ARTs and atherosclerosis, but this association disappeared after adjusting for traditional CVD risk factors [8]. Mangili et al [26] also concluded that ARTs are not significantly associated with atherosclerosis, independent of

traditional CVD risk factors [26]. Several smaller studies have reported no association between ARTs (PIs in particular) and atherosclerosis [5,10,11,14,15,20,21,27].

The conflicting results are, in part, likely due to several factors including small sample sizes, type of endpoint (e.g. surrogate *versus* intermediate *versus* direct outcomes), heterogeneity in protocols for ultrasound surrogates of sub-clinical atherosclerosis, investigating effects of drug classes (e.g. PIs) *versus* specific drug regimens (e.g. Kaletra), or inherent differences among populations studied [8,10-17,19-21,24,25,28].

To more reliably quantify the nature of the relationship between ARTs and CVD, we investigated the association between carotid intima media thickness (IMT), a validated and reliable surrogate marker for early atherosclerosis [1,29-34], and certain ARTs in an individual-patient meta-analysis using cross-sectional data from three observational HIV cohort studies in North America and Europe. The increase in sample size should provide more precision and statistical power in studying this relationship. In a novel approach to investigating the vascular impacts of ARTs, we have also conducted a secondary analysis to evaluate potential interaction effects among CVD risk factors.

Methods

Literature search:

We searched for relevant literature in PUBMED using MEDLINE terms “*carotid ultrasound*” OR “*imt*” OR “*carotid imt*” AND “*hiv*”. A total of 61 published articles

were retrieved as of April 12, 2009. We further reduced the pool of retrieved studies using the criteria listed below.

Inclusion/Exclusion Criteria:

A study was retained for consideration in the meta-analysis if it: was conducted in North America or Europe; recruited at least 100 HIV patients; assessed at least 3-segments from the carotid arteries; and reported that Research Ethics Board approval and patients' informed consent were obtained.

Studies were excluded if surrogate measures other than carotid IMT were used to quantify vascular disease; or patients were sampled from the pediatric population. It is worth noting that we have only considered studies having at least 100 patients, to obtain more stable estimates of "within-study" characteristics. Please see Figure 1 for a flow chart of the inclusion/exclusion process. Also see Table 1 for a list of studies retained for further consideration.

Included studies:

From the relevant studies retained, we identified study groups to which we extended formal invitations to participate in an individual patient meta-analysis.

Following our invitation and subsequent drafts of protocol synopsis and memorandum of understanding on data usage, three study groups -- Mercie et al [35], Mangili et al [26] and Smieja et al (Ongoing) -- agreed to participate in this study as of June 30, 2009.

Authors who declined our invitation were excluded from further consideration.

Invitations are still ongoing.

Data management:

We requested for baseline (cross-sectional) data using a uniform template made available to each study group. Patient identifiers such as names, initials, addresses, dates of birth and zip codes were deleted from data cuts available for this meta-analysis.

Participating groups sent data directly to a core center at McMaster University using secure electronic transmission. All data were stored on a controlled-access computer. This study received research ethics approval from the St Joseph's Healthcare Research Ethics Board.

Summary of IMT protocols and segment definitions for included studies:

One of the challenges of pooling data across different studies is the heterogeneity in available carotid IMT techniques [34]. Atherosclerotic burden is often quantified using measures obtained from 1 to 12 arterial segments (1-6 in each of the right and left carotid arteries) [29,30]. The segments are: near and far walls of the common carotid, bifurcation and internal carotid arteries [29,30]. On merging the datasets from the three included studies, only two segments were common to all: left far wall thickness for each of internal carotid arteries (ICA) and common carotid arteries (CCA). The protocol used by each study is summarized below:

Mangili et al [26]: was a prospective cross-sectional analysis of data from a cohort study of 327 HIV patients. This study was conducted in Boston, Massachusetts between 2002 and 2004. The authors sought to investigate the association between IMT, coronary artery calcium and known cardiovascular risk factors. IMT was associated with neither ART class nor individual medications, but was associated with traditional CVD risk factors.

- IMT was measured by centrally trained and certified ultrasonographers using a standardized protocol [36], and interpreted by one person stationed at a central reading site.
- CCA was obtained from a single longitudinal lateral view of the *distal* 1cm of the far wall of the left common carotid artery. ICA was obtained from three longitudinal views (anterior, lateral and posterior) of the left internal carotid artery.
- Both average and maximum thicknesses were recorded for each segment of CCA and ICA. Intra-class correlation coefficients for the common and internal carotid arteries were 0.911 and 0.883 respectively.

Mercie et al [35]: was a prospective cohort study conducted in Bordeaux, France to investigate the relationship between IMT and ART, lipodystrophy and traditional CVD risk factors. Four hundred and twenty three HIV infected subjects were recruited into the study which started in 1999 and is ongoing. Only conventional CVD risk factors were found to have significant association with IMT in a multivariable regression model.

- IMT measurement/reading was conducted by “two experienced examiners” using a standardized protocol [37].
- CCA was obtained from ultrasound images of the *distal* far wall of the left carotid artery along at least 1cm of longitudinal length. The recorded thickness is average of two measurements performed by two examiners. Intraclass correlation coefficient was 0.96.

Smieja et al (Ongoing): is an ongoing 5-year cohort study to investigate the effects of ARTs on CVD. HIV-positive subjects aged 35 years or older, attending university-

affiliated clinics in five Canadian centers (Hamilton, Toronto, Calgary, Montreal and Vancouver) were recruited into a prospective study of cardiovascular risk beginning from year 2000.

- Ultrasound imaging and readings were conducted by trained personnel using high resolution B-mode ultrasonography, standardized protocol and centralized reading [29,30].
- IMT measurements were obtained from longitudinal scans of near and far walls of the *proximal* 1cm of the internal carotid arteries, the carotid bifurcation starting at the tip of the flow divider and extending 1cm above this point, and the segment extending 1cm above the bifurcation in the common carotid arteries. The carotid flow divider was used as a longitudinal marker [30].
- Individual, average and maximum thicknesses from left and right segments were recorded for the bifurcation, internal and common carotid arteries. Intraclass correlation coefficient and coefficient of variation were > 0.90 and $< 5\%$ respectively, for repeat examinations [29,38].

Approximately 59%, 88% and 96% of ICA data are complete for Mercie et al [35], Mangili et al [26] and Smieja et al (Ongoing) respectively. However, CCA data are 100%, 96% and 98% complete for Mercie et al [35], Mangili et al [26] and Smieja et al (Ongoing) respectively. We have thus limited our primary analysis to CCA to maximize the use of data available from all three studies. CCA is a well validated and strong predictor of cardiovascular end points in various patient populations [34]. The definition of CCA appear quite different (e.g. longitudinal lateral view of the *distal* 1cm of the far

wall of the left common carotid artery in Mercie et al [35] and Mangili et al [26] versus *proximal* 1cm of the segment extending 1cm above the bifurcation in the common carotid arteries in Smieja (Ongoing)). Proximal and distal segments are likely to be affected to different degrees by atherosclerosis. The variation in CCA across the three studies will be discussed in the Results Section.

Further details on protocols and data collection techniques for each of the included studies have been published elsewhere [28,35,39].

Statistical methods:

Only variables common to all three studies were included in the statistical analysis. We categorized each patient's baseline ART exposure status as current, previous or never. Summary statistics were expressed as mean (standard deviation [SD]) or median (Inter-quartile range) for continuous variables, and number (percent) for categorical variables. Categorical variables were compared across studies using Chi-Squared test or Fisher's exact test (for cells with frequency less than 5). Continuous variables were compared across centers using one-way ANOVA (Analysis of Variance) or Kruskal-Wallis non-parametric test depending on the skewness of the data distribution.

The primary outcome (i.e. dependent variable) was IMT quantified using CCA. We modeled CCA as a function of exposure to individual PIs (Saquinavir (INVIRASE/FORTOVASE), Ritonavir (any dose), Kaletra, Atazanavir, Amprenavir, Indinavir, Nelfinavir), NRTIs (Abacavir, Zidovudine (AZT), Stavudine (D4T), Zalcitabine, Didanosine, Lamivudine (3TC)), and NNRTIs (Delavirdine, Efavirenz, Nevirapine) and traditional cardiovascular risk factors using hierarchical linear models

(HLMs) [40]. An HLM is essentially a regression model that adjusts for the effect of clustering (within-/between-study variations). Ignoring the multi-level nature of an inherently hierarchical data will likely result in underestimated standard errors and inflated Type I error [40].

In formulating the HLM models, we hypothesized that responses for patients *within* a particular study will be similar, for instance, due to common measurement protocol and ultrasound equipment. Further, responses between studies are likely to differ with respect to important variables, depending on the country of residence and other unmeasured characteristics. Consequently, each study was considered a “cluster” for analyses purposes. We calculated an estimate of intra-class correlation co-efficient (ICC) to quantify the proportion of total variance in response due to clustering of subjects within studies.

Two hierarchical levels were considered: (1) Individuals within study; and (2) Study (see Figure 2 and Appendix A for more details). Level 1 model (within-study) consisted of variables at the individual level, such as age, gender etc. Level 2 model (between-study) contained a random effect (study) to capture potential variations in responses across the three included studies. Level 1 and Level 2 models were combined into a composite model from which the effects of covariates were estimated.

We made a few assumptions in formulating the multi-level models, in particular, we assumed that: (1) the included studies represent a random sample of all available studies that fulfilled the inclusion criteria; (2) individuals within a particular study are more similar than individuals from other studies; (3.) the responses are normally

distributed; and (4) within-cluster residuals are normally distributed with zero mean and some unknown variance [40]. All model parameters were estimated using Restricted Maximum Likelihood Estimation (REML) [41]. REML estimation provides *asymptotically efficient* estimates when sample sizes for clusters are unequal. This means the standard errors approach the true values when sample size is large, consequently controlling for Type I error [41].

The primary hypothesis is that extent of atherosclerosis will be significantly associated with traditional CVD risk factors and ART use [2-6]. As an initial step, we obtained univariate HLMs to test the strength of the relationship between each covariate and CCA. Covariates that were statistically significant ($\alpha = 0.05$) in univariate models were included in subsequent multivariable models. Non-statistically significant variables were excluded from further consideration. We formulated two multivariable models: (a) traditional risk factors and ART exposure status; and (b) traditional risk factors and duration of ART exposure. We did not include both duration and exposure in the same model to avoid collinearity, which often leads to unstable parameter estimates [40].

In a separate *exploratory* analysis of model in (a) above, we included all possible two-way interaction effects among the covariates to test for possible moderating effects on CCA. There is a possibility that traditional CVD risk factors interact with ARTs to initiate undesirable cardiovascular effects. A number of review articles/research studies provided the motivation for exploring these interaction effects [42-44]. Sudano et al [45] proposed that patients are screened for hyperlipidemia before initiating ART, suggesting

that HIV therapy may accelerate CVD in patients with abnormal lipid profiles [45]. Orlando et al [42], Egger & Drewe [43], and Lundgren et al [44] concluded that age-related diseases (like atherosclerosis) are enhanced with ART initiation [42-44]. The foregoing statements suggest potential synergistic effects between traditional cardiovascular risk factors (such as age, LDL) and ARTs on atherosclerosis, but this possibility has not been well explored empirically in previous studies.

The covariance structure for residuals was selected using Akaike Information Criterion (AIC). Smaller AICs indicate better fit for covariance structure [46]. The improvement in model fit resulting from inclusion of interaction terms was assessed by testing the difference in the full likelihood functions of both models using a chi-squared test. Small p-values indicate a statistically significantly better fit for the interaction model [40].

All hypotheses tests were conducted at 5% level of significance (2-sided). Significance level for post-hoc tests was adjusted using Bonferroni correction [47]. Statistical analyses results and graphs were obtained using SAS (Version 9.1) and SPSS (Version 17.0).

Results

Patient characteristics: Summary statistics from the three studies are presented in Tables 2, 3, 4 and 5. Of 1,032 patients, 423 (41%) were contributed by Mercie et al [35], 343 (33%) by Mangili et al [26] and 266 (26%) by Smieja et al (Ongoing). CD4 nadir and CD4 count were statistically similar across the three studies. The mean (SD) of age

varied between 41 (8.8) years and 47 (8.0) years. Males constituted 89.5%, 72.6% and 74.1% of the study sample from Smieja et al (Ongoing), Mercie et al [35] and Mangili et al [26] respectively. Mean (SD) of CCA varied between 0.56 (0.1)mm and 0.63 (0.2)mm across the three studies. Mean (SD) of ICA varied between 0.50 (0.1)mm and 0.68 (0.4) mm across the three studies. Although the statistical tests for between-study comparisons of many variables were significant, the means do not appear markedly different for age, systolic blood pressure (SBP), diastolic blood pressure (DBP), high density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol levels. Only one patient reported being a user of statin medication in Mercie et al [35].

Across the three studies: at least 96% of patients reported never being on Delavirdine medication; at least 79% of patients reported being current/previous users of Lamivudine; the longest duration of exposure was reported for Zidovudine (between 131 and 180 months). The data on duration are reported as minimum to maximum due to high skewness. The highest estimated total person-years of exposure was observed for Lamivudine (2,541 years), while Delavirdine had the lowest (55 years).

The variance components type was selected as covariance structure for residuals after comparing AIC values for different plausible covariance structures. Estimated ICC was 0.04; meaning approximately 4% of the total variation in IMT was attributable to the clustering of subjects within studies. Although the variation attributable to clustering was quite small, our decision to analyze the data using HLM was deemed appropriate due to the multi-level nature of the combined dataset. Further, with an average study sample size of $n = 344$, the variance inflation factor [VIF], a measure of relative increase in variation

from HLM analysis in comparison with standard regression analysis, is approximately 15. This means an approximately 15 fold increase in sample size is required to maintain the same statistical power obtainable using HLM if one is to assume independence for all observations irrespective of cluster membership. This VIF is quite substantial, potentially leading to under-estimated standard errors of regression coefficients and inflated Type I error if the effect of clustering is neglected [48].

Please see Tables 2, 3, 4 and 5 for more information and comparison of patients' characteristics across the three studies.

Relationship between ART exposure status and CCA: Residuals from multi-level models were skewed; hence we applied logarithmic transformation to IMT to obtain a more bell-shaped distribution. Logarithmic transformation provided a better fit judging from the distribution of residuals plotted on a histogram. Only 1 individual reported ever using STATIN in one of the included studies [35]. Consequently, we dropped the STATIN variable from all models because the within-study effect estimate of the drug will be unreliable.

In univariate analysis, variables significantly associated with CCA included: male gender ($p=0.002$), age ($p<0.001$), DBP ($p=0.025$), SBP ($p=0.003$), LDL ($p=0.001$), CVD history ($p=0.002$), smoking ($p=0.021$), use of Zidovudine, Stavudine and Lamivudine ($p=0.007$, 0.003 , and <0.001 respectively). Only male gender ($p=0.026$), age ($p<0.001$), LDL ($p=0.021$) and smoking ($p<0.001$) remained significant predictors of CCA in multivariable analysis (Tables 6, 7 and 8). Post-hoc comparisons, with Bonferroni adjustments, showed that males had significantly higher CCA than females (Table 9).

Both former and current smokers had a significantly higher CCA than non-smokers. CCA for current and former smokers were similar (Table 10).

Relationship between ART duration and CCA: From univariate analysis, duration of Zidovudine, Stavudine, Lamivudine, Ritonavir and Nelfinavir ($p=0.006$, 0.006 , 0.002 , 0.029 , and 0.022) were statistically related to CCA. Only male gender ($p=0.010$), age ($p<0.001$), LDL ($p=0.021$), smoking ($p<0.001$) and Ritonavir duration ($p=0.024$) remained significant predictors of CCA in multivariable analysis (Tables 11 and 12). Ritonavir duration appeared to lead to reduced atherosclerosis.

Interaction analysis: In a secondary analysis including main effects and all two-way interactions in the multivariable model involving ART exposure status, we found significant interaction effects for: gender & AZT use ($p=0.019$); gender & D4T use ($p=0.022$); CVD history & DBP ($p=0.020$); CVD history & SBP ($p=0.001$); Smoking & AZT ($p=0.005$); smoking & age ($p=0.002$); and 3TC use & LDL ($p=0.025$). The negative impacts of D4T and AZT seemed more pronounced in males than females. The relationship between DBP (or SBP) and atherosclerosis appeared dependent on past history of CVD. Smoking likely moderates the relationship between AZT (or age) and atherosclerosis. The drug 3TC may lead to worse vascular outcomes in patients with elevated LDL cholesterol (see Figures 3-10). Please see Tables 13-20 for the number of patients at each combination of variables explored for interaction effects.

Relationship between ART exposure/duration and ICA: In univariate analysis, male gender ($p=0.028$), age ($p<0.001$), CD4 ($p=0.033$), DBP ($p=0.001$), SBP ($p<0.001$), glucose level ($p=0.006$), LDL ($p=0.013$), Total:HDL cholesterol ($p=0.024$), CVD history

($p<0.001$), use and duration of Saquinavir ($p=0.049$, 0.002 respectively), use and duration of D4T ($p=0.011$, 0.004 respectively) and duration of Zidovudine ($p=0.013$) were significantly associated with ICA.

However, in multivariable analysis, only age ($p<0.001$) and CVD history ($p<0.001$) remained significantly associated with ICA (Tables not shown).

Discussion

The use of anti-retroviral therapies (ARTs) has led to significant reduction in AIDS/HIV related mortality. However, there is a growing concern that ARTs, despite their beneficial effects, may also lead to cardiovascular disease [1]. The hypothesized relationship between ARTs and CVD is likely a result of dyslipidemia associated with these medications [1].

The inferences regarding the relationship between ARTs and IMT (as a surrogate of CVD) have been conflicting. Significant adverse ART effects have been found in some [1,12,13,19,22,49-52], but not all studies, mostly after adjusting for traditional cardiovascular risk factors [10,14,15,20,26,27,35,53-55]. Many of the reported studies are limited by sample size and insufficient power to test hypotheses of interest. Thus we conducted an individual-patient meta-analysis to, more precisely, quantify the effects of anti-retroviral therapy on cardiovascular disease in HIV patients recruited across Canada, France and USA.

In univariate analysis, factors significantly associated with CCA included: male gender, age, DBP, SBP, LDL, CVD history, smoking, use of Zidovudine, Stavudine and

Lamivudine, and duration of Zidovudine, Stavudine, Lamivudine, Ritonavir and Nelfinavir. Only male gender, age, LDL cholesterol, smoking and Ritonavir duration remained significant predictors of CCA in “main effects only” multivariable analysis. Ritonavir duration appeared to lead to reduced atherosclerosis. In a secondary analysis/hypothesis generation step, we found significant interaction effects for: (1) Gender & Use of AZT; (2) Gender & Use of D4T; (3) History of cardiovascular disease & DBP; (4) History of CVD & SBP; (5) Smoking & AZT; (6) Smoking & Age; and (7.) Use of 3TC & LDL. In descriptive analysis, we found that male smokers were at a higher risk of atherosclerosis than their female counterparts (See Figure 3). Also, the effects D4T and AZT on atherosclerosis appeared to differ by gender. Males tended to have higher IMT with the use of D4T and AZT than their female counterparts (Figures 4 and 5). Being a smoker (versus non-smoker) likely determined the extent of atherosclerosis based on age (Figure 6). Non-smokers using AZT appeared to have better IMT than smokers on the drug (Figure 9). The therapy, 3TC, may lead to CVD in patients with elevated LDL cholesterol (Figure 10).

Our results from the main effects models are similar to the results obtained by Mercie et al [35] and Mangili et al [26] in which ARTs did not predict atherosclerosis after adjusting for risk factors [26,35]. However, in a cross-sectional study involving 130 patients, Sankatsing et al [1] concluded that at least 2 years exposure to PIs led to a significant increase in IMT independent of traditional risk factors. NNRTIs, on the other hand, were not associated with increased IMT [1]. Depairon et al [15] found no association between the use of PIs and atherosclerosis in a sample of 168 patients. Jerico

et al [17] concluded that exposure to ART was significantly associated with the development of subclinical atherosclerosis, even after adjusting for traditional CVD risk factors. At present, there appears to be no definitive answers regarding the specific effects of ARTs on cardiovascular health.

Perhaps the current practice of merely testing ‘main effects’ may not be sufficient to help investigators understand the inter-relationships among risk factors on one hand, and how this affects cardiovascular health on the other hand. Risk factors do not act in isolation and may interact to cause CVD. While we make no definitive statements about the nature of these interactions and their effects on atherosclerosis, we believe that the results warrant more research into this likely phenomenon [42-45]. Interpretation of main effects without recourse to significant interaction effects may lead to incomplete inferences [56]. However, these interactions need to be replicated in other studies before any substantive conclusion can be drawn.

Apart from limited sample sizes, other plausible explanations for the conflicting results in the literature include: type of endpoint (e.g. surrogate versus intermediate versus direct outcomes), heterogeneity in protocols for ultrasound surrogates of sub-clinical atherosclerosis (e.g. arterial thickness), effects of drug classes (e.g. PIs) versus individual drugs (e.g. Kaletra), not accounting for duration of drug use, or inherent differences among populations studied [9-12,28,35,57].

Our study has numerous strengths relative to previous studies. We have studied the effects of specific drug regimens, rather than drug classes. Consequently we were able to quantify the specific contribution of individual drugs to the atherosclerotic

process. The relationship between drug classes and CVD is likely influenced by the distribution of patients on particular drug regimens [5]. This fact may have also added to the confusion regarding the role of ARTs on CVD. Take for instance, a study with 90% of patients on indinavir and 10% on Kaletra will classify all patients as being PI users, although the proportion of indinavir users is much higher and this may disproportionately affect the hypothesis being studied.

We used two-level HLMs to account for the hierarchical nature of the meta-analysis (patients nested within each study). Subjects within a study are likely different from subjects in another study due to reasons like inclusion criteria, study environment, IMT protocol etc. An HLM treats each study as a cluster and helps to reduce Type I error in statistical hypothesis, due to under-estimated standard errors [40].

We have also studied potential moderating effects of risk factors in the development of atherosclerosis. Most investigations on the relationship between ARTs and CVD have overlooked this important phenomenon. Main effects may not be entirely meaningful if there are interaction effects that need to be accounted for in regression models [56].

The sample size of 1,032 is one of the largest to date to study the relationship between IMT and CVD risk factors. However, we do plan to update the meta-analysis as more data are accumulated from other studies that in future, agree to participate in this project.

We note here that the risk of CVD in HIV patients may not be entirely due to ARTs alone. Some studies have suggested that HIV infection, marked by

immunodeficiency and inflammation, may be a contributing factor to CVD in HIV patients [9,11,15,19,52]. In our study, CD4 count was not significantly associated with atherosclerosis.

Our study also has several key limitations. We have only considered cross sectional (baseline) data in this meta-analysis. Longitudinal data will, more adequately, allow the investigation of the effect of cumulative exposure to ARTs on atherosclerotic progression.

Our analysis was restricted to measurements from only two individual segments (left CCA and ICA), whereas there are 12 possible segments from which atherosclerosis can be quantified [29,30]. It is possible to obtain slightly different results depending on the segment used as outcome, as evidenced in the non-convergent results from literature [58]. In our analysis, CCA was significantly associated with male gender, age, LDL cholesterol, smoking and Ritonavir duration, while ICA was significantly associated with only age and CVD history. However, we note that there was substantial missingness for ICA across the three included studies, which could have affected the results.

Ritonavir dosage information was unavailable for one of the studies included in the meta-analysis. Thus, exposure to *any dose* of Ritonavir was used as a covariate in the models. Note that Ritonavir is often used an adjunct therapy, in low doses, to other ARTs and it will be of interest to study the effects of low *versus* high dosage on IMT. The results presented here on the effect of Ritonavir exposure/duration should be interpreted with caution.

Due to the exploratory nature of the interactions, we have created only one interaction model having traditional risk factors and ART exposure status as covariates. Secondly, this interaction model only included variables that were statistically significant from univariate analyses (*Tables 6 and 7*). Thus in total, only 10 covariates were included, with 45 possible two-way interactions (10 *combination 2* when using combinatorics). Please note that these results could have arisen by chance or may be specific to the data analyzed for this study, and thus should be interpreted with caution.

IMT protocol variation is a major constraint in conducting meta-analyses across studies involving ultrasound measures [34]. Every investigator provides a justification for preferring a particular metric of atherosclerosis over another, which often leads to difficulties when comparing or combining results across studies. It may not be appropriate to compare results from a study that quantified IMT using all 12 segments of carotid arteries, to a study using one or a limited number of segments because the measures have implicitly different definitions [34]. For instance, Oduyungbo et al [28] found a statistically significant association between 12-segment-mean-maximal IMT and two other variables (SBP and total:HDL cholesterol ratio), in addition to the significant associations we found in the present analysis using CCA.

What is the most valid ultrasound measure of sub-clinical atherosclerosis? Which of the 12 segments is most useful for quantifying this disease? What is the validity of a measure calculated from 12-segments compared to one obtained from less than 12 segments? There is a need for validity studies to compare different approaches for quantifying atherosclerosis and subsequent adoption of a unified strategy by all HIV

researchers. Such tasks will require ongoing and effective collaborative efforts from all involved. Adopting a standardized strategy for assessing IMT in high risk populations will facilitate the comparison of results across studies and encourage information sharing among researchers. Corrective analytical steps, such as we took in this study, are at best ad hoc. No degree of sophistication in statistical techniques will completely eliminate the need for harmonized IMT protocols.

Conclusions

From the main-effects only models, ARTs (except Ritonavir duration) were not associated with atherosclerosis, independent of traditional risk factors. Significant interaction effects from secondary analysis warrant further investigation of potential moderating effects between traditional risk factors and ARTs in the atherosclerotic process. An understanding of these interactions will facilitate a classification of patients to various risk profiles and assist healthcare providers in prescribing the ‘best’ medication to patients based on risk stratification.

This cross-sectional study provides the first step towards understanding the long-term effects of ARTs on CVD. We plan to update the meta-analysis as more data become available from future collaborators. The ultimate goal would be to use longitudinal data to quantify the effects of long-term use of ARTs on CVD.

Acknowledgement

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Appendix A

Level 1 (WITHIN STUDY MODEL):

Let i =STUDY and j =PATIENT (patient is nested within study). We expressed IMT as a function of pre-selected covariates using a regression equation of the form:

$$IMT_{ij} = \pi_0 + \pi_1(GENDER_{ij}) + \pi_2(AGE_{ij}) + \dots + \pi_p(SMOKE_{ij}) + \pi_i(STUDY_{ij}) + \varepsilon_{ij} \quad (1.0)$$

where

- IMT_{ij} is the IMT of the j th patient from study i ;
- SMOKE and GENDER are indicators of patient's smoking status and biological gender respectively, and AGE is the chronological age in years;
- the ellipses (...) are given to avoid a long listing of other covariates that will be considered;
- the π 's are regression coefficients. We have assumed similar effects of each covariate on IMT across all studies. The slope for study variable, π_i , has subscript i to indicate that IMT for individuals within a study are more similar than those of individuals in another study (e.g. due to measurement protocol, country of residence etc.). Thus, STUDY is a random effect.
- p is the number of covariates; and
- ε_{ij} is a normally distributed random error term with zero mean and constant variance.

Level 2 (BETWEEN STUDIES MODEL):

Here we add a *random effect* to accommodate potential differences in an average patient's IMT across centers. This is necessary to adjust for sources of between-study heterogeneity like: measurement protocols, patient population (e.g. French versus Americans), and other unobserved characteristics. Thus for π_i in (1.0) above, we have:

$$\pi_i = \gamma + \xi_i \quad (2.0)$$

where ξ_i is a random effect.

- ξ is the difference between γ and π . If ξ equals zero, then there's no difference between γ and π , meaning we can assume the same regression equation for all three studies (i.e. no random effect). A "Variance Components Test" will be used to test if ξ_0 equals zero or otherwise.

Appendix B

SPSS Syntax for multivariable multi-level model: Drug exposure

```
MIXED LOG_CCA BY Study_ID MALE HIST_CAD SMOKE AZT D4T 3TC WITH  
AGE DBP LDL SBP **MODEL**  
/CRITERIA=CIN(95) MXITER(100) MXSTEP(5) SCORING(1)  
SINGULAR(0.000000000001) HCONVERGE(0, ABSOLUTE) LCONVERGE(0,  
ABSOLUTE) PCONVERGE(0.000001, ABSOLUTE) **Criteria for convergence**  
/FIXED=MALE HIST_CAD SMOKE AZT D4T 3TC AGE DBP LDL SBP |  
SSTYPE(3) **To obtain estimates of Type III parameter estimates**  
/METHOD=REML **Method of estimation is restricted Maximum Likelihood  
Method**  
/PRINT=SOLUTION TESTCOV  
/RANDOM=Study_ID | SUBJECT(Study_ID) COVTYPE(VC) ** Each study is a  
random effect, with variance components as covariance structure**  
  
**Post-hoc test for all categorical variables, adjusting multiple testing using  
Bonferroni correction**  
/EMMEANS=TABLES(MALE) COMPARE ADJ(BONFERRONI)  
/EMMEANS=TABLES(HIST_CAD) COMPARE ADJ(BONFERRONI)  
/EMMEANS=TABLES(SMOKE) COMPARE ADJ(BONFERRONI)  
/EMMEANS=TABLES(AZT) COMPARE ADJ(BONFERRONI)  
/EMMEANS=TABLES(D4T) COMPARE ADJ(BONFERRONI)  
/EMMEANS=TABLES(3TC) COMPARE ADJ(BONFERRONI).
```

SPSS Syntax for multivariable multi-level model: Drug duration

```
MIXED LOG_CCA BY Study_ID MALE HIST_CAD SMOKE WITH AGE DBP LDL  
SBP AZT_T D4T_T 3TC_T NFV_T RTV_T  
/CRITERIA=CIN(95) MXITER(100) MXSTEP(5) SCORING(1)  
SINGULAR(0.000000000001) HCONVERGE(0,  
ABSOLUTE) LCONVERGE(0, ABSOLUTE) PCONVERGE(0.000001,  
ABSOLUTE)  
/FIXED=MALE HIST_CAD SMOKE AZT_T D4T_T 3TC_T NFV_T RTV_T AGE  
DBP LDL SBP | SSTYPE(3)  
/METHOD=REML  
/PRINT=SOLUTION TESTCOV  
/RANDOM=Study_ID | SUBJECT(Study_ID) COVTYPE(VC)  
/EMMEANS=TABLES(MALE) COMPARE ADJ(BONFERRONI)  
/EMMEANS=TABLES(HIST_CAD) COMPARE ADJ(BONFERRONI)  
/EMMEANS=TABLES(SMOKE) COMPARE ADJ(BONFERRONI).
```

Figure 1. Inclusion/exclusion criteria

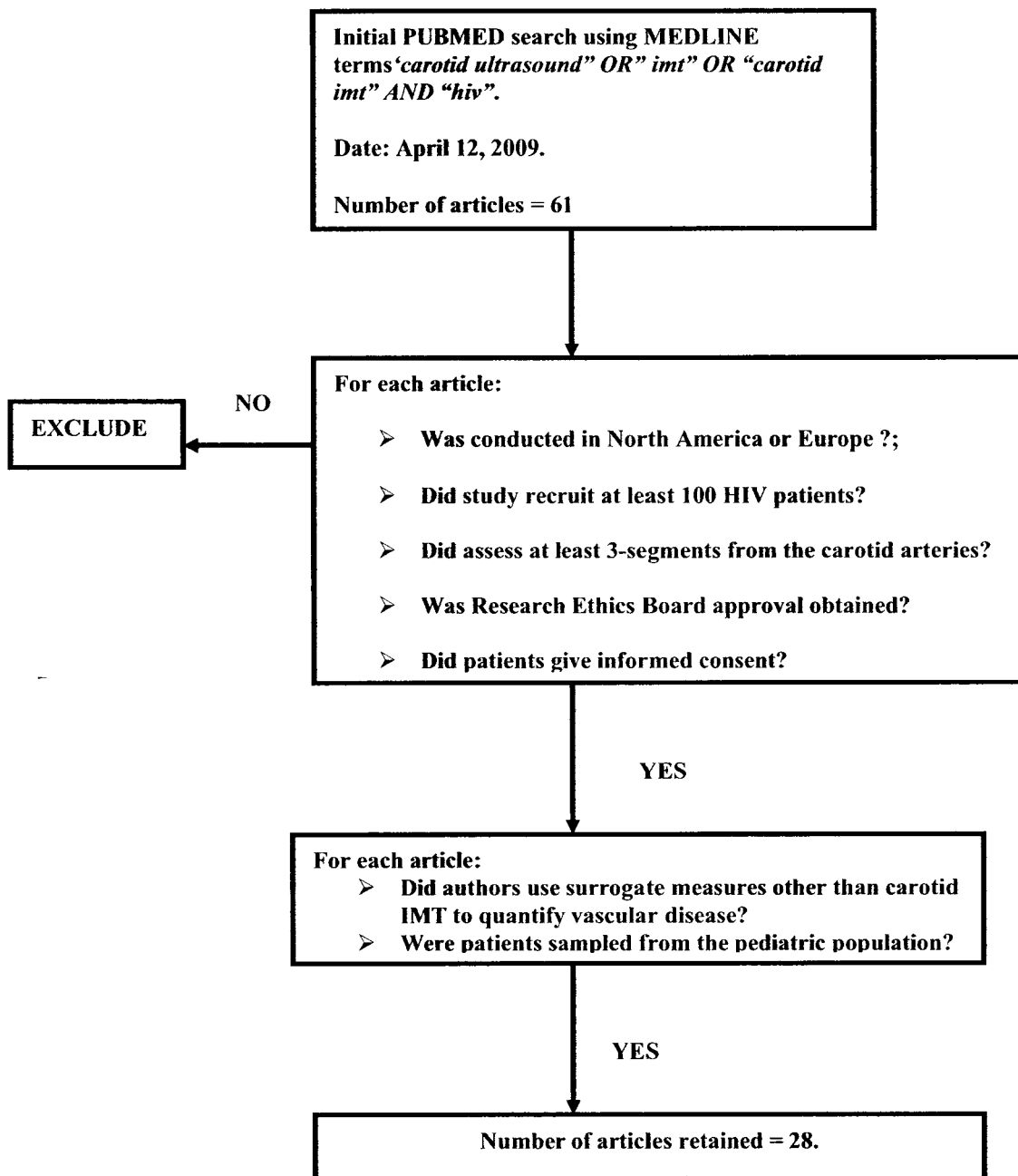


Figure 2. Multi-level modelling

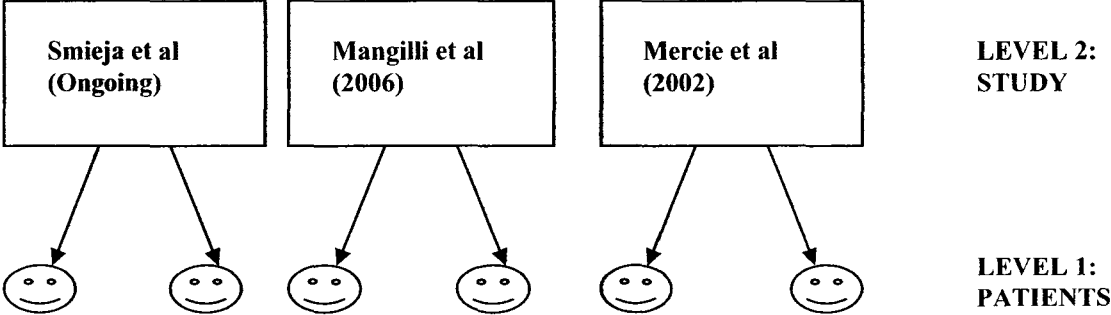


Figure 3. Relationship between gender and smoking status

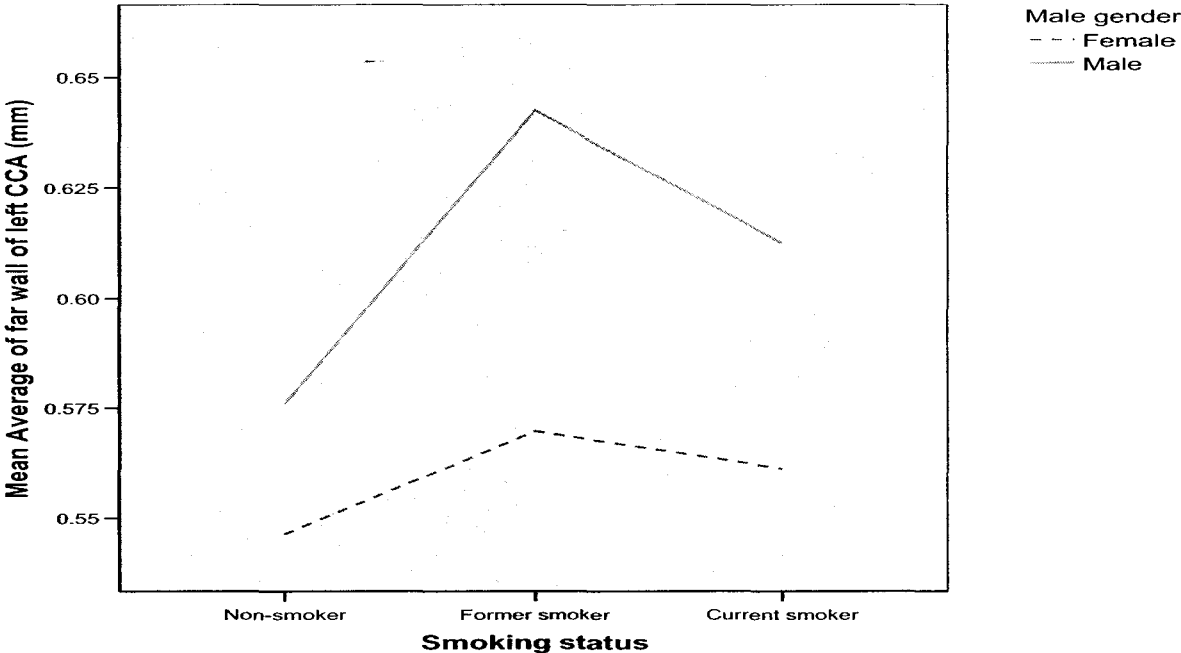


Figure 4. Interaction between gender and use of stavudine

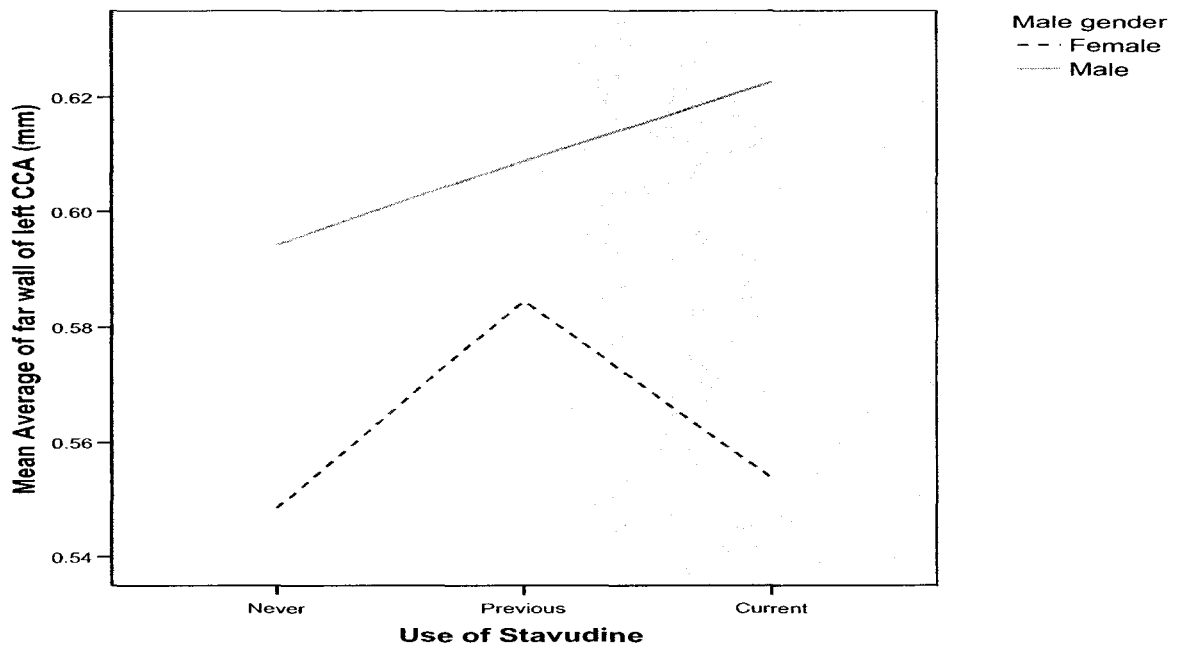


Figure 5. Interaction between gender and use of zidovudine

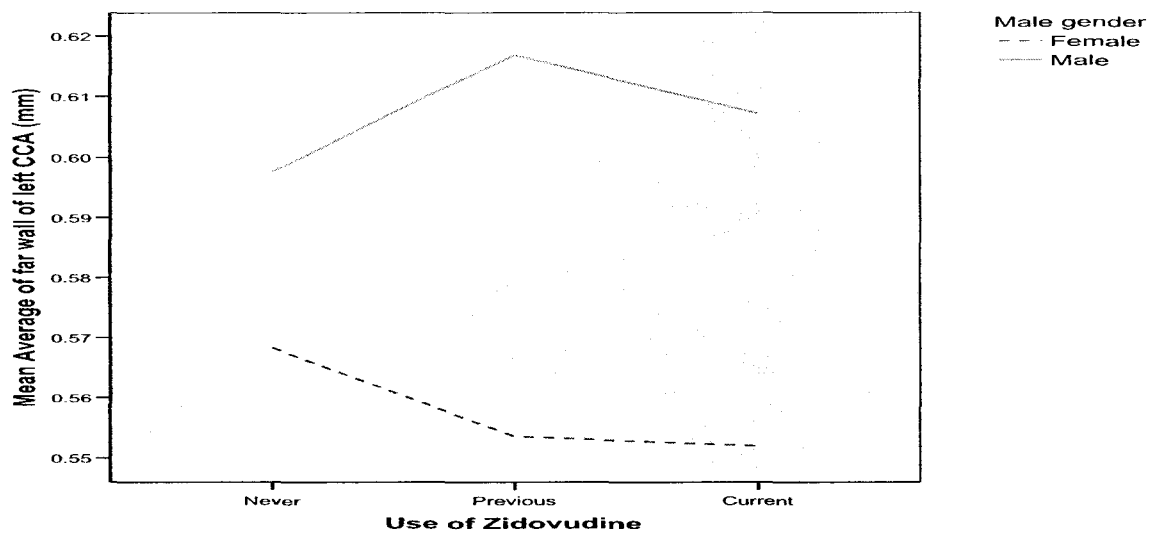


Figure 6. Interaction between age and smoking status

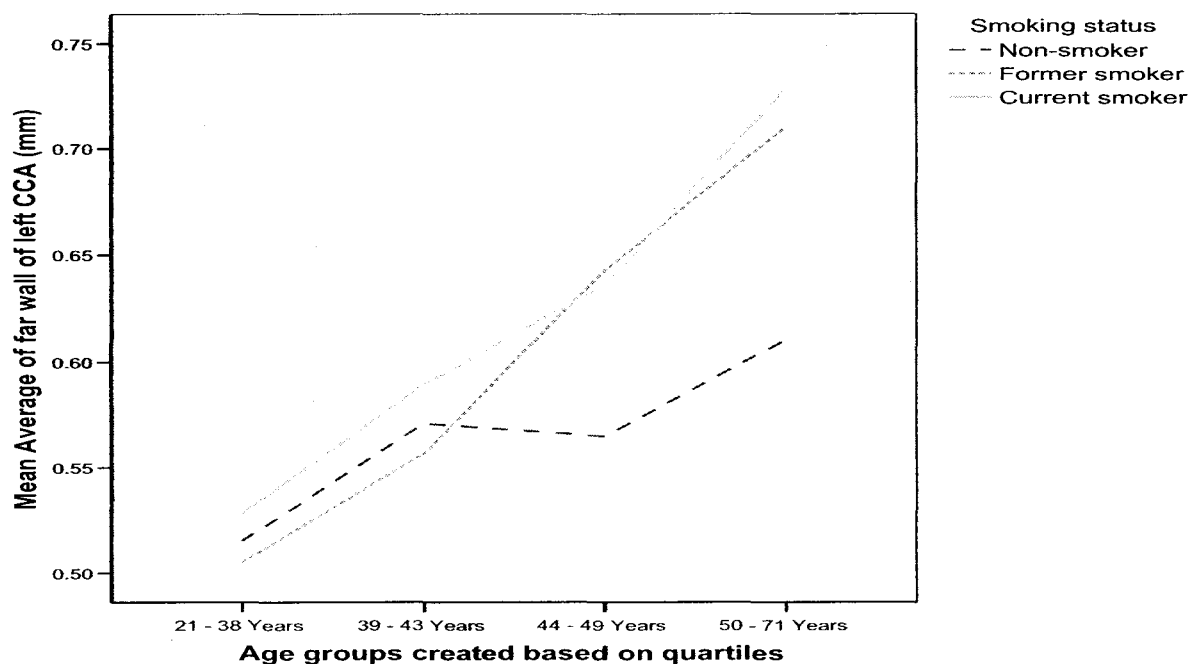
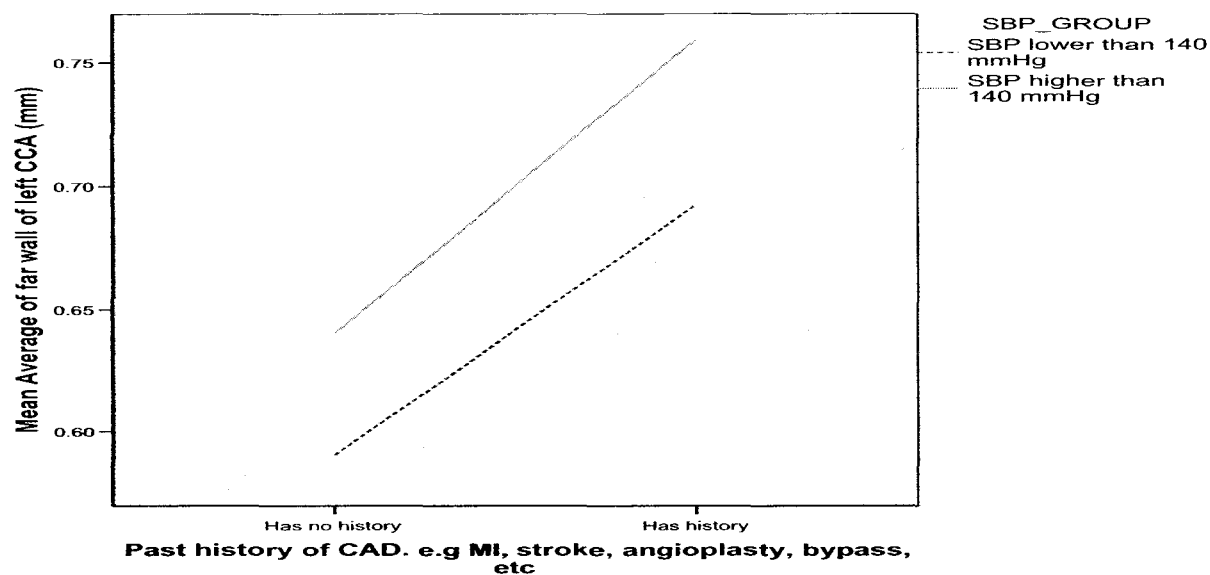
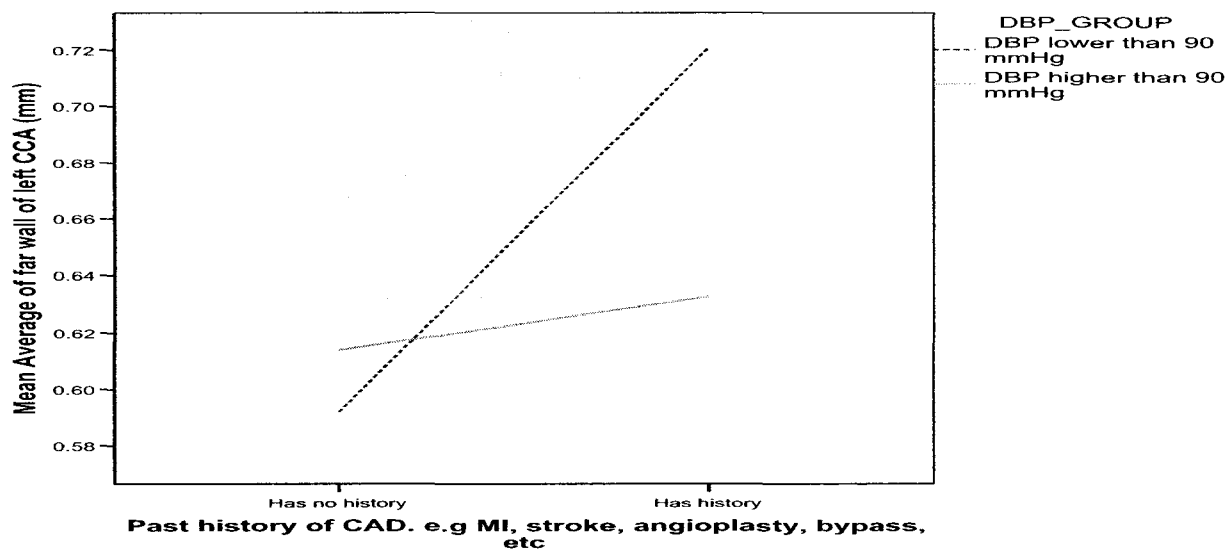


Figure 7. Interaction between SBP and past history of CVD



*CVD – Cardiovascular disease; SBP – Systolic blood pressure.

Figure 8. Interaction between DBP and past history of CVD



* CVD – Cardiovascular disease; DBP – Diastolic blood pressure.

Figure 9. Interaction between Smoking and Use of Zidovudine

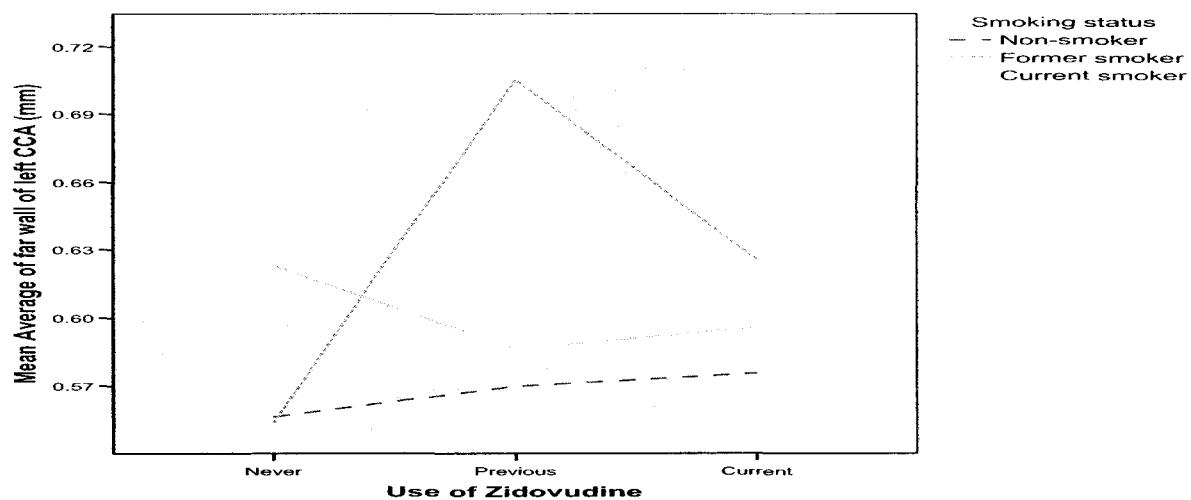
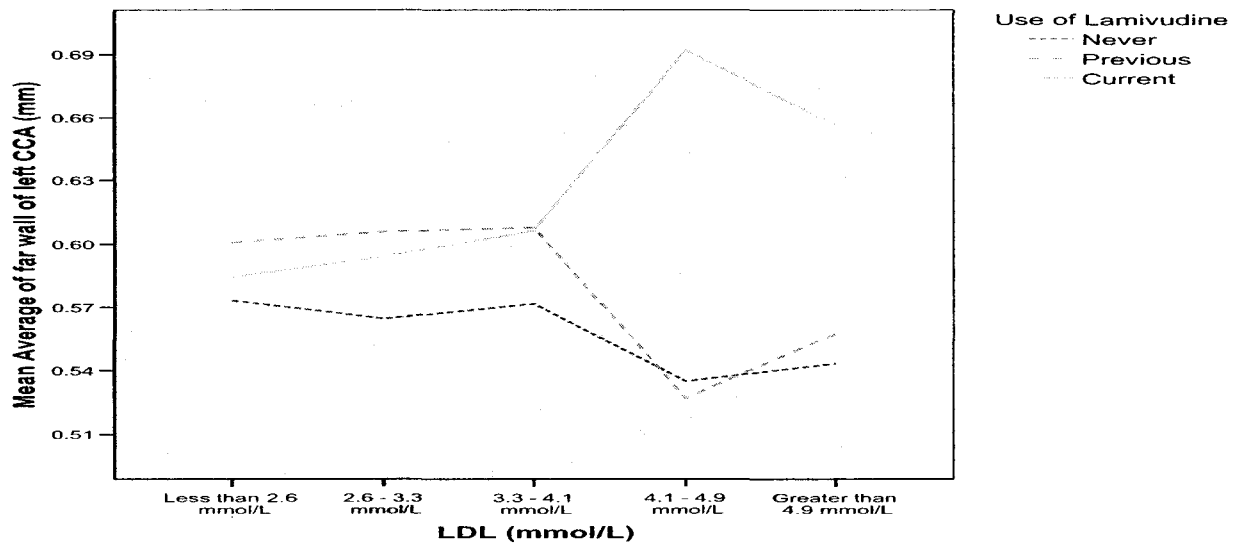


Figure 10. Interaction between Lamivudine use and LDL Cholesterol



* LDL is Low Density Lipoprotein. Groupings are based on the American Heart Association guidelines for fasting LDL-Cholesterol levels, estimated or measured, and risk for heart disease (<http://www.americanheart.org/presenter.jhtml?identifier=4500>).

Table 1. Potentially relevant studies

Study	Country	Sample size*	Carotid Ultrasound Method/Arterial Segments
Johnsen et al (2006)	USA	97	Left carotid arteries.
Hsue et al (2004; 2006)	USA	363	All 12 segments from right and left carotid arteries.
Smieja et al (Ongoing)	Canada	266	All 12 segments from right and left carotid arteries.
Lorenz et al (2008)	Germany	292	Far wall of left and right arterial segments
Mangili et al (2006; 2007)	USA	314	Left and far walls of right and left CCA and ICA
Mercie et al (2002; 2005)	France	346	Distal left CCA
Iacobellis et al (2007; 2007)	Canada	103	Not clear
Alonso-Villaverde et al (2004)	Spain	183	Far wall of CCA, ICA and bifurcation.
Vellossi et al (2008)	USA	700	“Carotid artery ultrasound measuring intima-medial thickness” (Specific measure not stated)
Maggi et al (2000; 2004; 2006; 2007; 2007; 2008)	Italy	133	Not clear
Kaplan et al (2008)	USA	1931	Far wall of right CCA, ICA and carotid bulb.
Sankatsing et al (2009)	Netherland/ Germany	130	Left and right CCA, ICA and carotid bulb.
Coll et al (2005; 2006; 2007)	Spain	141	CCA, Carotid bulb, ICA and Common femoral artery
Norata et al (2006)			
Jerico et al (2006)	Spain	132	Not clear
de Saint Martin et al (2006; 2007)	France	154	Far wall of left and right CCA
Depairon et al (2001)	Switzerland	168	Not clear

* HIV patients only

Table 2. Comparison of patient characteristics across included studies

Measure	Smieja et al (Ongoing)	Mercie et al (2002)	Mangili et al (2006)	Statistical Comparison at 5% level
Number of patients	266	423	343	
Male	238 (89.5)	307 (72.6)	254 (74.1)	<0.001 ^a
Age (Years)	46.5 (8.0)	41.0 (8.8)	44.3 (7.3)	<0.001 ^c
CD4 Count (cells/mm3)	478.8 (268.2)	442.6 (252.9)	452.2 (296.9)	0.233 ^c
**CD4 Nadir (cells/mm3)	174.5 (234.7)	180.0 (225)	211.5 (210.03)	0.268 ^d
DBP (mmHg)	76.9 (10.5)	75.1 (9.6)	75.6 (10.5)	0.060 ^c
SBP (mmHg)	120.5 (15.9)	122.0 (13.5)	118.2 (16.6)	0.003 ^c
Glucose level (mmol/L)	5.51 (1.4)	5.01 (0.9)	4.70 (1.2)	<0.001 ^c
HDL (mmol/L)	1.09 (0.32)	1.19 (0.5)	1.08 (0.5)	0.001 ^c
LDL (mmol/L)	2.89 (0.9)	3.23 (1.1)	2.85 (1.0)	<0.001 ^c
Total Cholesterol (mmol/L)	5.20 (1.3)	5.28 (1.3)	4.89 (1.5)	<0.001 ^c
Log ₁₀ Maximum Viral Load	4.60 (0.9)	4.46 (0.9)	3.86 (1.2)	<0.001 ^c
**Triglycerides level	2.1 I (2.0)	1.52 (1.5)	1.53 (1.6)	<0.001 ^d
Has cardiovascular disease history	15 (5.6)	3 (0.7)	24 (7.0)	<0.001 ^b
Current/previous use of Fibrates	24 (9.2)	4 (0.9)	4 (1.2)	<0.001 ^b
Current/previous use of Statins	24 (9.6)	1 (0.2)	23 (6.7)	<0.001 ^b
Current smokers	100 (37.9)	261 (61.7)	169 (49.3)	<0.001 ^a
Current/Former smokers	170 (64.4)	285 (67.5)	258 (75.2)	<0.001 ^a
CCA	0.63 (0.2)	0.56 (0.1)	0.61 (0.2)	<0.001 ^c
ICA	0.66 (0.3)	0.50 (0.1)	0.68 (0.4)	<0.001 ^c

* CCA – Common Carotid Artery; ICA – Internal Carotid Artery; IQR – Inter-quartile range; DBP – Diastolic blood pressure; SBP – Systolic blood pressure; HDL – High density lipoprotein; LDL – Low density lipoprotein. Categorical variables reported as number (percent); Continuous variables reported as mean (standard deviation) unless otherwise stated.

** Reported as median (IQR) due to highly skewed data.

^a Chi-squared test.

^b Fisher's exact test.

^c Analysis of Variance test.

^d Kruskal-Wallis test.

Table 3. Available Segments and comparison of IMT reproducibility across all studies

	Smieja et al (Ongoing)	Mercie et al (2002)	Mangili (2006)
Available segments	Individual, average and maximum thicknesses from each of the 12 segments. ICC and coefficient of variation were > 0.90 and < 5% respectively.	Average thickness of the far walls of each of the left bifurcation, internal carotid and common carotid (3 Segments)	Average and maximum thickness for far wall and near wall of each of the left common and internal carotid arteries (4 segments)
CCA information	Longitudinal view of the segment extending 1cm above the bifurcation in the CCA.	Obtained from the distal far wall of the left carotid artery along at least 1cm of longitudinal length. ICC of 0.96.	Obtained from three longitudinal views (anterior, lateral and posterior) of the left and right internal carotid artery. ICC of 0.0883

* CCA – Common Carotid Artery; ICC – Intra-class correlation coefficient.

Table 4. Information on the use of anti-retroviral therapy

Drug Class	Drug	Smieja et al (Ongoing)			Mercie et al (2002)			Mangilli et al (2006)		
		N	P	C	N	P	C	N	P	C
Protease Inhibitors	Saquinavir	169 (64)	80 (30)	17 (6)	299 (71)	72 (17)	52 (12)	273 (80)	38 (11)	30 (9)
	Ritonavir	195 (73)	46 (17)	25 (9)	321 (76)	55 (13)	47 (11)	268 (79)	41 (12)	32 (9)
	Amprenavir	237 (89)	14 (5)	15 (6)	414 (98)	1 (0.2)	8 (2)	315 (92)	8 (2)	18 (5)
	Indinavir	175 (66)	84 (32)	7 (3)	241 (57)	110 (26)	72 (17)	249 (73)	68 (20)	24 (7)
	Nelfinavir	187 (70)	58 (22)	21 (8)	263 (62)	69 (16)	91 (22)	234 (69)	62 (18)	45 (13)
Nucleoside Reverse Transcriptase Inhibitors	Abacavir	142 (53)	34 (13)	90 (34)	378 (89)	8 (2)	37 (9)	245 (72)	17 (5)	79 (23)
	Zidovudine	62 (23)	98 (37)	106 (40)	78 (18)	171 (40)	174 (41)	149 (44)	81 (24)	111 (33)
	Stavudine	118 (44)	87 (33)	61 (23)	129 (31)	76 (18)	218 (52)	178 (52)	79 (23)	84 (25)
	Zalcitabine	223 (84)	40 (15)	3 (1)	253 (60)	153 (36)	17 (4)	323 (95)	15 (4)	3 (1)
	Didanosine	201 (76)	44 (17)	21 (8)	190 (45)	132 (31)	101 (24)	252 (74)	43 (13)	46 (14)
	Lamivudine	30 (11)	41 (15)	195 (73)	71 (17)	65 (15)	287 (68)	70 (21)	92 (27)	179 (53)
Non-Nucleoside Reverse Transcriptase Inhibitor	Delavirdine	256 (96)	4 (2)	6 (2)	411 (97)	7 (2)	5 (1)	331 (97)	7 (2)	3 (1)
	Efavirenz	117 (44)	46 (17)	103 (39)	346 (82)	8 (2)	69 (16)	238 (70)	32 (9)	71 (21)
	Nevirapine	233 (88)	12 (5)	21 (8)	283 (67)	42 (10)	98 (23)	273 (80)	29 (9)	39 (11)

(*) P, C and N are categorical variables to identify previous, current and non-users of each drug and are reported as number (percent). The total percentage may not be equal to 100 due to rounding.

Table 5. Duration of anti-retroviral therapy

Drug Class	Drug	PYE	Smieja et al (Ongoing)	Mercie et al (2002)	Mangilli et al (2006)
			Duration	Duration	Duration
Protease Inhibitors	Saquinavir	604.96	(0, 104)	(0, 49.6)	(0, 82.9)
	Ritonavir	427.84	(0, 96)	(0, 38.8)	(0, 78.2)
	Amprenavir	104.37	(0, 72)	(0, 5.5)	(0, 44.8)
	Indinavir	699.26	(0, 89)	(0, 42.1)	(0, 82.7)
	Nelfinavir	606.69	(0, 81)	(0, 38.2)	(0, 160.8)
Nucleoside Reverse Transcriptase Inhibitors	Abacavir	434.20	(0, 96)	(0, 23.6)	(0, 68.8)
	Zidovudine	2153.81	(0, 180)	(0, 130.8)	(0, 159.7)
	Stavudine	1538.47	(0, 83)	(0, 66.6)	(0, 95.5)
	Zalcitabine	358.74	(0, 86)	(0, 63.5)	(0, 82.6)
	Didanosine	684.71	(0, 158)	(0, 87.2)	(0, 80.5)
	Lamivudine	2541.29	(0, 162)	(0, 60.4)	(0, 144.0)
Non-Nucleoside Reverse Transcriptase Inhibitor	Delavirdine	54.98	(0, 64)	(0, 9.6)	(0, 84.0)
	Efavirenz	554.94	(0, 84)	(0, 15.9)	(0, 62.7)
	Nevirapine	282.39	(0, 72)	(0, 21.7)	(0, 96.0)

(*) PYE is Person-Years of Exposure to each drug, for all three studies combined. Duration reported as (minimum, maximum) in months.

Table 6. Predictors of CCA in univariate mixed linear models (Traditional risk factors)

Predictor variable	Univariate model estimate (95% CI)	p-value
Male	0.076 (0.0282, 0.1232)	0.002
Age (10 years)	0.095 (0.072, 0.118)	<0.001
CD4 Nadir (100 cells/mm3)	-0.003 (-0.013, 0.008)	0.630
CD4 (100 cells/mm3)	0.003 (-0.004, 0.010)	0.432
DBP (10 mmHg)	0.022 (0.003, 0.042)	0.025
SBP (10 mmHg)	0.020 (0.007, 0.033)	0.003
Glucose level (mmol/L)	0.012 (-0.0039, 0.0287)	0.135
HDL (mmol/L)	-0.011 (-0.0524, 0.0310)	0.614
LDL (mmol/L)	0.030 (0.0129, 0.0476)	0.001
Total Cholesterol (mmol/L)	0.022 (0.0091, 0.0348)	0.001
Total:HDL Cholesterol	0.005 (-0.0020, 0.0119)	0.161
Triglycerides level	-0.0005 (-0.0056, 0.0045)	0.839
Cardiovascular disease history	0.161 (0.0589, 0.2633)	0.002
Use of Fibrates	0.105 (-0.0088, 0.2186)	0.071
Smoking	0.026 (0.0039, 0.0487)	0.021
Log ₁₀ Maximum Viral Load	-0.0012 (-0.0203, 0.0179)	0.899

* Only variables that were statistically significant (p -value < 0.05) were included in multivariable models.
DBP – Diastolic blood pressure; SBP – Systolic blood pressure; HDL – High density lipoprotein; LDL – Low density lipoprotein; CI – Confidence Interval.

Table 7. Predictors of CCA in univariate mixed linear models (Drug exposure)

Predictor variable	Univariate model estimate (95% CI)	p-value
Use of Saquinavir	0.026 (-0.0042, 0.0559)	0.092
Use of Ritonavir	-0.025 (-0.0553, 0.0047)	0.099
Use of Amprenavir	-0.012 (-0.0603, 0.0357)	0.615
Use of Indinavir	0.021 (-0.0085, 0.0509)	0.161
Use of Nelfinavir	0.018 (-0.0081, 0.0451)	0.172
Use of Abacavir	0.003 (-0.0220, 0.0287)	0.796
Use of Zidovudine	0.034 (0.0090, 0.0585)	0.007
Use of Stavudine	0.035 (0.0124, 0.0584)	0.003
Use of Zalcitabine	0.002 (-0.0406, 0.0456)	0.910
Use of Didanosine	-0.006 (-0.0321, 0.0210)	0.681
Use of Lamivudine	0.05 (0.0226, 0.0745)	<0.001
Use of Delavirdine	-0.046 (-0.1196, 0.0278)	0.222
Use of Efavirenz	-0.001 (-0.0253, 0.0224)	0.905
Use of Nevirapine	0.01 (-0.0167, 0.0375)	0.450

* Only variables that were statistically significant ($p\text{-value} < 0.05$) were included in multivariable models.
CI – Confidence Interval.

Table 8. Predictors of CCA in multivariable mixed linear models (Drug exposure)

Predictor variable	Multivariable model estimate (95% CI)	p-value
Male	0.048 (0.0056, 0.0898)	0.026
Age (10 years)	0.084 (0.062, 0.107)	<0.001
LDL (mmol/L)	0.0198 (0.0029, 0.0366)	0.021
Smoking	0.0502 (0.0301, 0.0704)	<0.001
DBP (10 mmHg)	-0.002 (-0.024, 0.020)	0.871
SBP (10 mmHg)	0.008 (-0.007, 0.023)	0.288
Cardiovascular disease history	0.0762 (-0.0145, 0.1669)	0.100
Use of Zidovudine	0.0136 (-0.0137, 0.0409)	0.329
Use of Stavudine	0.0141 (-0.0094, 0.0376)	0.240
Use of Lamivudine	0.0047 (-0.0225, 0.0320)	0.733

* DBP – Diastolic blood pressure; SBP – Systolic blood pressure; LDL – Low density lipoprotein ; CI – Confidence Interval.

Table 9. Post-hoc test for gender

Post-hoc Pairwise Comparisons ^b (Male Gender)							
(I) Male gender	(J) Male gender	Mean Difference (I-J)	Std. Error	df	Sig. ^a	95% Confidence Interval for Difference ^a	
						Lower Bound	Upper Bound
Female	Male	-.046*	.022	884.471	.033	-.088	-.004
Male	Female	.046*	.022	884.471	.033	.004	.088
Based on estimated marginal means							
*. The mean difference is significant at the .05 level.							
a. Adjustment for multiple comparisons: Bonferroni.							
b. Dependent Variable: Natural logarithm of CCA (Common Carotid Arteries).							

Table 10. Post-hoc test for smoking

Pairwise Comparisons ^b (Smoking)							
(I) Smoking status	(J) Smoking status	Mean Difference (I-J)	Std. Error	df	Sig. ^a	95% Confidence Interval for Difference ^a	
						Lower Bound	Upper Bound
Non-smoker	Former smoker	-.081*	.027	858.278	.009	-.146	-.015
	Current smoker	-.102*	.021	887.950	.000	-.151	-.052
Former smoker	Non-smoker	.081*	.027	858.278	.009	.015	.146
	Current smoker	-.021	.025	824.510	1.000	-.082	.040
Current smoker	Non-smoker	.102*	.021	887.950	.000	.052	.151
	Former smoker	.021	.025	824.510	1.000	-.040	.082
Based on estimated marginal means							
*. The mean difference is significant at the .05 level.							
a. Adjustment for multiple comparisons: Bonferroni.							
b. Dependent Variable: Natural logarithm of CCA (Common Carotid Arteries)..							

Table 11. Predictors of CCA in univariate mixed linear models (Duration of drug exposure)

Predictor variable	Univariate model estimate (95% CI)	p-value
Duration on Saquinavir (years)	0.0109 (-0.0051, 0.0269)	0.180
Duration on Ritonavir (years)	-0.0214 (-0.0405, -0.0022)	0.029
Duration on Amprenavir (years)	-0.0022 (-0.0405, 0.0361)	0.912
Duration on Indinavir (years)	0.0147 (0.0002, 0.0296)	0.054
Duration on Nelfinavir (years)	0.0196 (0.0028, 0.0364)	0.022
Duration on Abacavir (years)	-0.0071 (-0.0263, 0.0122)	0.471
Duration on Zidovudine (years)	0.0111 (0.0032, 0.0190)	0.006
Duration on Stavudine (years)	0.0156 (0.0044, 0.0268)	0.006
Duration on Zalcitabine (years)	0.0015 (-0.0206, 0.0237)	0.891
Duration on Didanosine (years)	0.0019 (-0.0133, 0.0171)	0.806
Duration on Lamivudine (years)	0.0146 (0.0052, 0.0239)	0.002
Duration on Delavirdine (years)	-0.0097 (-0.0545, 0.0351)	0.671
Duration on Efavirenz (years)	-0.0069 (0.0252, 0.0114)	0.459
Duration on Nevirapine (years)	0.0091 (-0.0161, 0.0344)	0.479

* Only variables that were statistically significant (p-value < 0.05) were included in multivariable models.

CI – Confidence Interval.

Table 12. Predictors of CCA in multivariable mixed linear models (Duration of drug exposure)

Predictor variable	Multivariable model estimate, mean (95% CI)	p-value
Male	0.0530 (0.0125, 0.0935)	0.010
Age (10 years)	0.0850 (0.0632, 0.1067)	<0.001
LDL (mmol/L)	0.0189 (0.0028, 0.0350)	0.021
Smoking	0.0521 (0.0327, 0.0715)	<0.001
Duration on Ritonavir (years)	-0.0197 (-0.0367, -0.0027)	0.024
DBP (10 mmHg)	-0.0029 (-0.0241, 0.0183)	0.789
SBP (10 mmHg)	0.0070 (-0.0076, 0.0215)	0.349
Cardiovascular disease history	0.0654 (-0.0217, 0.1525)	0.141
Duration on Nelfinavir (years)	0.0023 (-0.0129, 0.0174)	0.771
Duration on Zidovudine (years)	0.0047 (-0.0038, 0.0133)	0.279
Duration on Stavudine (years)	0.0098 (-0.0014, 0.0210)	0.086
Duration on Lamivudine (years)	0.0005 (-0.0103, 0.0114)	0.922

* DBP – Diastolic blood pressure; SBP – Systolic blood pressure; LDL – Low density lipoprotein; CI – Confidence Interval.

Table 13. Cross-tabulation showing gender and smoking status

		Smoking status			
		Non-smoker	Former smoker	Current smoker	Total
		Number of patients	Number of patients	Number of patients	Number of patients
Gender	Female	83	23	126	232
	Male	233	160	404	797
	Total	316	183	530	1029

Table 14. Cross-tabulation showing Stavudine exposure and gender

		Use of Stavudine			
		Never	Previous	Current	Total
		Number of patients	Number of patients	Number of patients	Number of patients
Gender	Female	100	43	88	231
	Male	325	199	275	799
	Total	425	242	363	1030

Table 15. Cross-tabulation showing Zidovudine exposure and gender

		Use of Zidovudine			
		Never	Previous	Current	Total
		Number of patients	Number of patients	Number of patients	Number of patients
Gender	Female	69	82	80	231
	Male	220	268	311	799
	Total	289	350	391	1030

Table 16. Cross-tabulation showing age groups and gender

		Age groups created based on quartiles				
		21 - 38 Years	39 - 43 Years	44 - 49 Years	50 - 71 Years	Total
		Number of patients	Number of patients	Number of patients	Number of patients	Number of patients
Gender	Female	94	55	40	44	233
	Male	183	210	197	209	799
	Total	277	265	237	253	1032

Table 17. Cross-tabulation showing systolic blood pressure and history of cardiovascular disease

		SBP_GROUP		
		SBP lower than 140 mmHg	SBP higher than 140 mmHg	Total
		Number of patients	Number of patients	Number of patients
Past history of CAD. e.g MI, stroke, angioplasty, bypass, etc	Has no history	908	61	969
	Has history	36	6	42
	Total	944	67	1011

Table 18. Cross-tabulation showing diastolic blood pressure and history of cardiovascular disease

		DBP_GROUP		
		DBP lower than 90 mmHg	DBP higher than 90 mmHg	Total
		Number of patients	Number of patients	Number of patients
Past history of CAD. e.g MI, stroke, angioplasty, bypass, etc	Has no history	925	45	970
	Has history	34	8	42
	Total	959	53	1012

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Table 19. Cross-tabulation showing Zidovudine exposure and smoking status

		DBP_GROUP		
		DBP lower than 90 mmHg	DBP higher than 90 mmHg	Total
		Number of patients	Number of patients	Number of patients
Past history of CAD. e.g MI, stroke, angioplasty, bypass, etc	Has no history	925	45	970
	Has history	34	8	42
	Total	959	53	1012

Table 20. Cross-tabulation showing Lamivudine exposure by LDL group

		Use of Lamivudine			
		Never	Previous	Current	Total
		Number of patients	Number of patients	Number of patients	Number of patients
LDL_GROUP	Less than 2.6 mmol/L	71	71	201	343
	2.6 - 3.3 mmol/L	39	47	175	261
	3.3 - 4.1 mmol/L	34	35	142	211
	4.1 - 4.9 mmol/L	8	19	52	79
	Greater than 4.9 mmol/L	6	6	35	47
	Total	158	178	605	941

CHAPTER 4

An inclusive strategy for imputing missing outcome in multiple imputation can enhance statistical efficiency in longitudinal data analysis: a simulation study

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Abstract

Background: Regression models are used to study relationships between outcomes and covariates in clinical research. Many models discard incomplete data. In instances where this is undesirable, multiple imputation (MI) can help to generate plausible values for missing items. In MI, missing values are randomly generated from pre-selected variables included in an imputation model. One approach is to include all final analysis model variables (inclusive strategy) in the imputation model, but penalties associated with exceptions to this strategy are not well studied for longitudinal data. Further, an inclusive strategy may be impractical when computing resources are inadequate and there are many analysis variables. We conducted a simulation study to investigate performance - measured using a quantity incorporating bias and variability - associated with estimated regression parameters after MI: (1) when some final analysis variables are excluded (restrictive strategy) from the imputation model; and (2) under different percentages of missingness.

Methods: 10,000 replicate longitudinal samples, each having 80 observations - with baseline assessment and two follow-up time points - were simulated for two covariates and an outcome, using summary statistics from an ultrasound longitudinal study. Of the two covariates, one had a stronger linear association with the outcome than the other. For each sample, subsets of the outcome were assumed to be missing-completely-at-random and then imputed using MI. The performance of estimated regression coefficients from generalized estimating equations was quantified using percentage root mean square error (%RMSE).

Results: We obtained the smallest %RMSE when both covariates were used in the imputation model, in contrast to when only one was used. The variable having stronger linear association with missing outcome resulted in lower %RMSE when not excluded from the imputation model. There was more than two-fold increase in %RMSE when percentage of missing outcome increased from 40% to 80%.

Conclusion: Whenever practically possible, an inclusive strategy where all final analysis variables are included in the imputation model *may* provide the most efficient parameter estimation. When computing resources are inadequate, variables strongly associated with outcome having missingness should be given priority in the imputation model. Further, imputation is questionable when percentage missingness is very high.

Background

Missing data constitute a major problem in the statistical analysis of prospective clinical studies. Inferences may not be valid (or reliable) when missing values are completely ignored or not properly handled, especially when missingness is related to the outcome of interest [1]. Likely sources of missing data include, among others: loss to follow-up; drop-outs resulting from untoward effects of study intervention; or transcription error by data entry clerks.

A formal framework for categorizing types of missing data involves identifying the underlying mechanisms responsible for missingness. Consequently, unavailable data can be: *missing completely at random - MCAR* (missingness is neither related to values of the variable with missing items nor to any other variable in the dataset, but may be related to some “unmeasured/unknown” auxiliary variable); *missing at random - MAR* (missingness is unrelated to values of the variable with missing items, but to other observed variables in the dataset); or *non-ignorable (informative) – NI* (missingness depends on values of the variable with missing items and possibly other variables in the dataset) [1]. More extensive discussion on missingness mechanisms can be found in Schafer [2]. Depending on putative (or theorized) missingness mechanism, methods for handling missing data include, among others: complete case analysis (CCA); unbalanced data analysis (UDA); and data imputation [3]. These strategies work by deleting (CCA), optimizing (UDA) or imputing incomplete data prior to, or in the statistical modeling process.

Briefly, data imputation involves generating plausible values for missing information based on some rule or procedure using either a single imputation or multiple imputations. Simulation studies have shown that imputation methods based on multiple iterations produce estimates that are closer to population values under different missingness mechanisms in contrast to methods using single imputation/iteration [4-6]. In one simulation study that compared multiple imputation (MI) to single imputation methods such as single regression, individual mean replacement, overall mean replacement and last observation carried forward, MI appeared to produce estimates closest to parameter values in the context studied [7].

Often in practice, statistical models (e.g. simple linear regression) discard or optimize incomplete data using CCA or UDA respectively, without recourse to data imputation. In situations where CCA and UDA are undesirable - as is often the case - data imputation may provide a viable option for the analyst. In multiple imputation (MI) for instance, missing values (and associated uncertainties) can be generated from pre-selected variables (imputation variables) that are included in an imputation model. Inclusion of imputation variables may help to increase the precision of MI, as well as improve the chance of satisfying a key assumption of *MAR* (SAS Institute, 2002). Imputation variables are not necessarily part of the final analysis model. One approach is to include all final analysis model variables (inclusive strategy) in the imputation model, but penalties associated with exceptions to this strategy are not well studied for longitudinal data. Further, an inclusive strategy may not be practical in instances where

computing resources are inadequate and there are a large number of analysis variables [8,9].

We conducted a simulation study to investigate performance - measured using a quantity incorporating bias and variability - associated with estimated regression parameters after MI: (1) when some final analysis variables are excluded (restrictive strategy) from the imputation model; and (2) under different percentages of missingness. These questions are investigated using simulated data based on summary statistics from a prospective cohort study of HIV patients undergoing carotid ultrasound assessment [10].

Methods

Literature search:

A literature search was conducted in PUBMED, JSTOR (Journal Storage), PsycINFO and WOS (Web of Science) to identify studies that have investigated the same or similar questions, with a primary focus on health research. No year or language restrictions were used in the search. We used the search terms “*multiple imputation*” AND “*simulation*” AND “*missing data*”. We scanned through the title and abstract of each paper to identify relevant articles. Of the fairly large body of literature retrieved (PUBMED=82; JSTOR=273; PsycINFO=103; WOS=124), we identified only one simulation study by Collins et al [8] (retrieved through PUBMED, PsycINFO and WOS) that investigated similar questions.

Collins et al’s [8] simulation study was conducted using completely artificial cross-sectional data, and post MI analysis was done using cross-sectional statistical

methods. Collins et al [8] concluded that an imputation strategy that includes as many variables that are likely associated with missing outcome is superior to one that excludes such variables [8]. While the conclusions by the authors may be generalizable to cross-sectional studies, it is unclear if these results also apply to longitudinal studies using appropriate analytical methods like generalized estimating equations (GEE). There are structural and analytical differences between longitudinal and cross-sectional data: (1) correlation between repeated observations which should be incorporated into analysis models; (2) while covariates in cross-sectional data are not necessarily dependent, there is often dependency within covariates measured over time in longitudinal data. Will these structural differences affect the results from MI? Our study attempts to fill this knowledge gap by conducting a simulation based on summary longitudinal data, partly extrapolated from an original research, with post MI analysis using GEE.

Below, we provide a description of the context that motivated this research.

Application context:

Carotid intima media thickness (IMT) is a non-invasive ultrasound measure for assessing sub-clinical atherosclerosis in individuals [11,12]. IMT is calculated from high resolution B-mode ultrasound images of one or more segments of the left and right carotid arteries. Arterial segments used in the calculation may include: near and far wall of the common carotid, the bifurcation and the internal carotid [11,12].

The problems and inferential consequences of incomplete data in carotid ultrasound research are highlighted in Espeland et al [13] and Probstfield et al [14]. Studies involving carotid IMT assessment may encounter missing data due to many

factors, three of which are: (i) patients are lost to follow-up; (ii) unreadable ultrasound scans of measurements from individual segments, subsequently rendering overall IMT unobtainable; or (iii) certain segments missing but overall IMT still calculable [13,14]. Espeland et al [13] explored the use of likelihood-based (non-imputation) methods in instances where data from individual segments are missing, but this is outside the scope of our study. In this project, we have only considered factors (ii) and (iii) from a data imputation perspective.

A simulation study:

Suppose we wish to investigate the impact of two explanatory variables on IMT over the course of three time points (e.g. 3 years). Suppose also, these two variables are chosen such that, empirically or theoretically, one has a stronger linear relationship with IMT than the other. Two such explanatory variables could be age and total:HDL (high density lipoprotein) cholesterol. Oduyungbo et al [10] showed that age had a much stronger linear relationship with IMT ($p < 0.001$) than total:HDL cholesterol ($p = 0.147$). Consequently, we simulated longitudinal samples based on plausible population parameters for IMT, age and total:HDL cholesterol using summary data obtained from Oduyungbo et al [10]. Estimates of mean, covariance and regression coefficients of the three variables (from Oduyungbo et al [10]) were assumed to be “population” parameters on which 10,000 samples were simulated. We simulated 10,000 samples - each having 80 observations - to obtain *stable* probability distributions of estimates. Stable probability distributions do not change markedly with increase in the number of simulations, thus leading to more reliable results. Sample size of 80 was chosen

arbitrarily. We also accounted for the stronger effect of age in the simulation; an important detail we will return to in subsequent sections of this article. A more detailed description of the simulation process is given in Appendix A.

We conducted the simulation using interactive matrix programming language procedure (PROC IML) in SAS 9.1 (SAS Institute Inc., Cary, NC, USA) running under a WINDOWS XP platform (Microsoft Corp., Washington, USA). SAS codes/macros used in the implementation are provided in Appendix B. Graphs were obtained using MINITAB 14.0 (MINITAB Inc., PA, USA).

Missing data mechanism and patterns:

We simulated missing values for *only* the outcome, to avoid an intractable simulation algorithm. For each sample, we simulated an *MCAR* mechanism of missingness. We theorized that patients are more likely to drop-out as a result of reasons unrelated to the study protocol, since this simulation was based on an observational study. Further, burden on patients was minimal as they were only required to have assessments done once a year in the original study. However, we note that the MCAR assumption may not be entirely applicable when ultrasound scans are unreadable or when patients drop-out due to severity of disease [13].

Pattern of missingness may be *monotone* or *arbitrary*. In monotone missingness, subjects with missing values at first follow-up will have missing values at subsequent follow-ups. Patients with monotone missing data are considered lost to follow-up. For arbitrary (intermittent) missingness, patients may miss a scheduled assessment for

specific reasons, but return at subsequent follow-up visits [15]. Missing data simulation was based on a mixture of arbitrary and monotone patterns.

Simulation Scenarios:

We considered three scenarios by which one may conduct MI on a dataset with one outcome and two covariates. For each plausible scenario, the imputation model may contain:

- A. outcome (IMT) *and* one covariate having a strong linear association with it (i.e. AGE); or
- B. outcome (IMT) *and* one covariate having a weak linear association with it (i.e. TOTAL:HDL); or
- C. outcome (IMT) *and* both covariates (i.e. AGE and TOTAL:HDL).

In each of scenarios A, B and C, we simulated situations in which 10%, 20%, 40% and 80% of data are missing for: (1) last follow-up outcome only; (2) penultimate and last follow-up outcome. The percentages of missingness were chosen partly based on experience and convenience. Percentage missingness for IMT was approximately 37% after one-year follow-up in Oduyungbo et al [10].

We have restricted our study to only two covariates to simplify the simulation procedure. As noted earlier, we also systematically selected the two variables such that one has a stronger association (AGE) with IMT than the other (TOTAL:HDL).

Multiple imputation and subsequent data analysis:

We used the Markov Chain Monte Carlo (MCMC) method of multiple imputation available in SAS 9.1 under the PROC MI procedure (please see Appendix B). This

method is particularly useful when missingness pattern is both monotone and arbitrary, as in our simulation. The reader can find more technical details on the MCMC method in Schafer [2].

We generated missing values for IMT in each sample. Then, for each incomplete sample data, 10 multiply imputed datasets were created based on an iterative process using the Bayesian framework [2,16]. Results from theory and simulation studies suggest that 5 to 10 imputations are adequate for producing stable inferences [1,8,16].

Next, for each sample, each of the 10 imputed datasets was analyzed using Generalized Estimating Equations (GEE) [17] with IMT as outcome, and TOTAL:HDL cholesterol and AGE as covariates (final analysis model). GEE is a statistical method for analyzing longitudinal (clustered) data, which has been demonstrated to have superior power for detecting “statistically significant intervention effects” in clustered data, when compared to other methods such as random effects or hierarchical linear models [18]. It involves specifying a regression equation to model the relationship between an outcome and corresponding covariates, while simultaneously adjusting for correlation among repeated observations. An introduction to GEE can be found in Sheu [19]. We specified autoregressive correlation (AR(1)) structures for IMT (Please see Appendix A for more technical details). For each sample, the regression coefficients and precision estimates obtained from analyzing each imputed dataset were combined using Rubin’s rules [20] (PROC MIANALYZE in SAS 9.1) to calculate overall *pooled* estimates for inference purposes. Thus for 10,000 samples, we obtained ten thousand regression coefficients for each covariate.

Assessment of estimates:

Let's denote the population regression coefficients of TOTAL:HDL and AGE as β_A and β_B , with corresponding sample estimates $\hat{\beta}_A$ and $\hat{\beta}_B$ respectively. We obtained mean square error (MSE) of each estimate as $\left(\hat{\beta}_A - \beta_A\right)^2$ and $\left(\hat{\beta}_B - \beta_B\right)^2$ averaged over all 10,000 samples. MSE measures the magnitude by which an estimate differs from its population value [21]. We chose MSE as the index of overall performance/efficiency because it incorporates both *bias* and *variability*, which are two desirable qualities of parameter estimators [21]. MSE has also been used as a measure of performance in similar simulation studies [8].

To assess the difference between estimates and their respective population values for each covariate, the percentage root mean squared error (%RMSE) was obtained as:

$$\%RMSE = \frac{RMSE}{true\ value} * 100$$

where RMSE is simply the square root of MSE and is in the same unit as the quantity being estimated. Lower %RMSE indicates more efficient parameter estimates.

Please refer to Appendix A and Figure 1 for more details.

Results

Missingness for last follow-up outcome only:

We simulated 10%, 20%, 40% and 80% missingness for the last follow-up outcome, imputed missing values using MI, and calculated %RMSE of regression estimates for AGE and TOTAL:HDL obtained from GEE under each of Scenarios A to C.

Figure 2 shows a plot of %RMSE versus percentage missingness given each scenario, for each of AGE and TOTAL:HDL in separate panels. In each scenario, %RMSE was smaller for the estimator of AGE than for TOTAL:HDL.

Of the three scenarios, Scenario C (both covariates in imputation model) led to the least %RMSE for regression coefficients of AGE and TOTAL:HDL. The worst %RMSE were obtained from Scenario B. Regression estimates from Scenario A were better (lower %RMSE) than those from Scenario B, but worse than those from Scenario C.

More specifically at 10% missingness for outcome, Scenario C is approximately four times more efficient than Scenario B, and approximately twice more efficient than Scenario A in estimating the effect of AGE. For TOTAL:HDL, Scenario C is approximately four times more efficient than Scenario B, and approximately twice more efficient than Scenario A. The efficiency of Scenario C - relative to other Scenarios - increased with increasing outcome missingness. Thus, the imputation strategy that included all final analysis model (GEE) variables provided the highest efficiency for each covariate estimated. The strategy that excluded the covariate having higher correlation with missing outcome (Scenario B) produced the worst efficiency.

As anticipated, higher percentage of missingness led to considerably worse efficiency for estimated effects. For instance, in each scenario, %RMSE for both AGE and TOTAL:HDL at 80% outcome missingness was more than double the %RMSEs at 40% missingness (Figure 2). Thus with very high percentage missingness, an analyst may want to explore other analytical approaches for handling incomplete data.

Equal percentage missingness for penultimate and last follow-up:

The simulation was replicated for situations where there was an equal percentage of missingness (10%, 20% and 40%) for penultimate and last follow-up outcomes. Of the three scenarios, Scenario C provided the best parameter estimates for AGE and TOTAL:HDL (Figure 3). As before, %RMSE was smaller for AGE than TOTAL:HDL in all three scenarios. Also, regression estimates from Scenario A were better than those from Scenario B, but worse than those from Scenario C. Higher percentage missingness also led to worse efficiency.

Unequal percentage missingness for penultimate and last outcomes:

The simulation was replicated for situations where there was unequal percentage missingness for penultimate and last follow-up outcomes (i.e. 10%-20%, 20%-40% and 40%-80%). The results are similar to previous ones.

Result patterns appear to be consistent across Figures 2 to 4.

Discussion

Statistical analysis of data from prospective clinical studies may present an array of challenges when there are incomplete observations from research subjects. In many

statistical procedures, such as linear regression, subjects with incomplete data are ignored in the calculation of covariate effects on outcome. This approach is often not desirable as it results in a waste of limited resources, and reduction in statistical power for detecting important covariate-outcome relationships. Depending on the mechanism and pattern of incompleteness, an analyst may employ any, in a range of strategies to deal with the missing data problem. Multiple imputation is one of such strategies.

Multiple imputation (MI) can be used for obtaining plausible values for missing items, especially when the *missing at random* (MAR) assumption is satisfied. This prediction is done through an imputation model that includes observed variables likely to be related to missing outcome or variable with missing items. One approach for conducting MI is to include all variables that are (presumably) related to the variable having missingness in the imputation model [8,9] but penalties associated with exceptions to this rule are not well studied for longitudinal HIV data. In this paper, we conducted a simulation study to investigate the statistical efficiency of estimated regression parameters after MI: (1) when variables strongly associated with the missing outcome are included/excluded from imputation model; and (2) under different percentages of missingness.

Our results show that an inclusive strategy that incorporates variables that are weakly and strongly associated with the variable having missingness leads to the least combined variability and bias in regression estimates. Further, variables having stronger linear association with missing outcome may lead to more efficient parameter estimates when included in the imputation model. In instances where computing resources are

inadequate and there are many variables in the dataset, the analyst may want to start the process by hierarchically selecting variables into an MI model based on how strongly they are related to the variable having missing data. This may help to avoid an intractable and slow MI procedure, and also lead to relatively more efficient estimates. As expected, higher percentage of missingness led to worse estimates after MI.

Our results are similar to those obtained by Collins et al [8] in a simulation study involving artificial cross-sectional data consisting of 1000 samples, each having 500 observations. Collins et al [8] concluded that an imputation strategy that includes as many variables that are likely associated with missing outcome is superior to one that excludes such variables. They also found slightly higher RMSE with percentage missingness of 50% compared to 25% missingness [8]. While the study by Collins et al [8] involved completely artificial cross-sectional data and associated analytic methods, our study involved longitudinal HIV-related data based on descriptive statistics partly extrapolated from an original study, with analysis method appropriate for longitudinal clinical studies.

The use of MI comes with caveats. MI is not the *perfect* fix for every missing data problem. The MAR/MCAR assumption is not applicable to all studies, thus it is important to always investigate reasons for missingness before deciding on an approach going forward. Sophisticated methods such as pattern-mixture models, where a probability distribution is assumed for missingness, can be used when missingness appears non-ignorable [3]. As stated in the Results Section, when percentage of missing data is very high, any imputation strategy is questionable since efficiency decreases substantially with increasing missingness as evidenced in Figure 3. %RMSE for both

AGE and TOTAL:HDL at 80% outcome missingness (for last follow-up) was more than double the %RMSEs at 40% missingness.

Our simulation study has some key limitations. We have taken certain steps to make the simulation algorithm more tractable. For instance, we considered situations in which only the outcome is missing. In practice, missingness can occur in both outcome and explanatory variables. The extent to which this affects our results is not known. We also considered only two explanatory variables, but analysis involving outcomes like carotid IMT usually include more than two covariates. We have used the Markov Chain Monte-Carlo (MCMC) method of simulation in this paper, although other methods such as propensity scoring are also possible [22]. The sample size of 80 was arbitrary. However, between 500 and 10,000 simulations appear to be the standard in statistical simulation studies [8,23]. Further simulation may be needed to ascertain if the results we have presented can be replicated under different statistical models, and to what extent we can generalize to other plausible scenarios.

Theorizing and selecting a threshold RMSE, in the context of clinical significance, will be useful for many practical purposes in medical research. However, we have refrained from selecting a threshold RMSE because it will be, at best, arbitrary. Please note that our primary aim was to *explore* the magnitude of changes in RMSE when certain covariates are included/excluded from multiple imputation models.

We have used ratios to compare %RMSE for different percentages of missingness. Absolute difference in %RMSE can also be used to assess relative

efficiency. Note that *ratios* and *absolute differences* are on different numerical scales and will have distinct interpretations.

Ideally, looking at various missingness mechanisms and patterns, a host of missingness scenarios, longer time points, and more covariates is a worthy research endeavor. Nonetheless, this investigation gives us a glimpse of the potential impact of missingness on inferences if one was willing to make the kind of assumptions used in the simulations. We see this as a beginning of a potential research program to explore the issues further. Thus, additional research would still be necessary to investigate the findings under other scenarios and assumptions which may be more realistic than the ones considered here – but of course, much more difficult to simulate. From our experience in this study, considerable amount of computer resources and time will be required for such inquests. Obtaining Figure 1 for instance, took approximately one month of simulation on a PC running Windows XP 2002 (Service Pack 2) with central processing speed (CPU) of 3.2GHz (Giga Hertz), random access memory (RAM) of 2GB (Giga Bytes), and available hard disk space of 223GB.

Conclusion

Whenever practically possible, an inclusive strategy where all final analysis variables are included in the imputation model *may* provide the most efficient parameter estimation. In instances where computing resources are inadequate and there are many variables being considered, effort should be made not to exclude variables strongly

associated with outcome having missingness in the imputation model. Further, imputation is questionable when percentage missingness is very high.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

AO and LT conceived of the study. Simulation was conducted by AO, with inputs from LT and MS. AO wrote the first draft of manuscript. Subsequent drafts were severally edited by AO, LT, MS, JB and EL to ensure content accuracy. All authors reviewed and approved the final version of manuscript.

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Appendix A: Technical overview of simulation

Longitudinal model:

The population model assumed is -

$$y_{it} = \beta_A * x_{it}^A + \beta_B * x_{it}^B + \varepsilon_{it} \quad (1.1)$$

where

- $i = 1, \dots, 80$ is the number of observations;
- $t = 1, 2, 3$ is the number of equally-spaced observation time-points;
- ε_{it} is independently and identically distributed error terms with mean 0 and variance 1;
- Each y_i (vector of observations for subject i) is the outcome of interest (carotid IMT), having an auto-regressive correlation structure of order 1 (AR(1)) ;
- x^A (AGE) and x^B (TOTAL:HDL cholesterol) have multivariate normal distributions with mean vectors μ_A and μ_B , and covariance matrices Σ_A and Σ_B respectively.

Steps to simulate longitudinal model:

1. x^A, x^B are independently simulated from multivariate normal distributions, each having a specified covariance structure and mean vector. Multivariate normal distributions were simulated using the Cholesky Root Transformation in SAS PROC IML. We will assume x^A to be AGE (years), while x^B is TOTAL:HDL cholesterol ratio. The outcome (Y) is carotid IMT (intima-media thickness)

measured in millimeter (mm). Using summary data from Oduyungbo et al (2009, under review), we assumed the following population parameters:

$$\boldsymbol{\mu}_A = \begin{pmatrix} 47.19 \\ 48.19 \\ 49.19 \end{pmatrix} \quad \text{and} \quad \mathbf{R}_A = \begin{pmatrix} 1 & 0.99 & 0.98 \\ 0.99 & 1 & 0.99 \\ 0.98 & 0.99 & 1 \end{pmatrix} \text{ for AGE}$$

and

$$\boldsymbol{\mu}_B = \begin{pmatrix} 5.40 \\ 5.18 \\ 5.12 \end{pmatrix} \quad \text{and} \quad \mathbf{R}_B = \begin{pmatrix} 1 & 0.81 & 0.66 \\ 0.81 & 1 & 0.81 \\ 0.66 & 0.81 & 1 \end{pmatrix} \text{ for TOTAL:HDL}$$

where $\mathbf{R}_A$ and $\mathbf{R}_B$ are correlation matrices for AGE and TOTAL:HDL respectively.

Vector of population variances for AGE and TOTAL:HDL are assumed to be:

$$\boldsymbol{\sigma}_A^2 = \begin{pmatrix} 68.78 \\ 69.56 \\ 69.01 \end{pmatrix} \quad \text{and} \quad \boldsymbol{\sigma}_B^2 = \begin{pmatrix} 1.92 \\ 1.37 \\ 1.63 \end{pmatrix}$$

NOTE: Data from Oduyungbo et al (2009, under review) only covered baseline and one-year follow-up. Mean AGE for time 3 was extrapolated from that of time 2. TOTAL:HDL cholesterol for time 3 was assumed to be the arithmetic difference between values for time 1 and time 2, *plus* 5 to give 5.12. For $\mathbf{R}_A$ and $\mathbf{R}_B$, we simply calculated the square root of correlation for observations separated by 1 time unit, to obtain correlation for observations separated by 2 time units. This reflects an AR(1) correlation structure.

2. Generate random error terms ε_{it} having mean 0 and variance 1. Specifically,

$$\boldsymbol{\mu}_\varepsilon = \begin{pmatrix} 0 \\ 0 \\ 0 \end{pmatrix} \quad \text{and} \quad \mathbf{R}_\varepsilon = \begin{pmatrix} 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{pmatrix}$$

where $\boldsymbol{\mu}_\varepsilon$ is the mean vector for 3 time points and $\mathbf{R}_\varepsilon$ is the correlation matrix.

Note that the correlation matrix is the same as the covariance matrix (identity matrix) in this instance.

3. Next, simulate 10,000 longitudinal samples (80 observation each) satisfying model 1.1 and fixing the beta parameters as $\beta_A = 0.016$ and $\beta_B = 0.010$. The specified beta coefficients were obtained from the coefficients of AGE (β_A) and TOTAL:HDL cholesterol (β_B) from Oduyungbo et al (2009). The two coefficients measure the change in IMT per unit change in AGE and TOTAL:HDL cholesterol respectively. AGE was statistically significantly related to carotid IMT ($p < 0.001$) while TOTAL:HDL cholesterol was not ($p = 0.147$) (Oduyungbo et al (2009)).

4. The outcome (carotid IMT or Y) will thus have a normal distribution with mean

$$\boldsymbol{\mu}_y = \begin{pmatrix} 0.81 \\ 0.83 \\ 0.84 \end{pmatrix}$$

and an auto-regressive correlation structure. $\boldsymbol{\mu}_y$ follows directly from substituting

$\beta_A = 0.016$ and $\beta_B = 0.010$ into equation (1.1) and taking expected values for each

time point. The AR(1) correlation structure for outcome follows from structures

specified for both covariates. Observe that the mean vector μ_y is close to what obtains in carotid IMT studies in HIV patients, and quite close to values from Oduyungbo et al (2009, under review). Carotid IMT for baseline and 1 year follow-up in the Canadian HIV Vascular study was 0.82mm and 0.84mm respectively (Oduyungbo et al, 2009, under review).

5. Randomly set a percentage of last follow-up of response variable ($t = 3$) as “missing” for each sample. Thus we assume data is missing completely at random (MCAR).
6. Conduct multiple imputations (10 imputations) on each sample.
7. Conduct GEE analysis using AR(1) correlation structure on each sample to estimate β_A and β_B .
8. Calculate MSE from each estimate as $\left(\hat{\beta}_A - \beta_A\right)^2$ and $\left(\hat{\beta}_B - \beta_B\right)^2$ averaged over all 10,000 samples. Take the square root of MSE to obtain root mean square error (RMSE).
9. Divide RMSE by original parameter value (the betas), and multiply by 100 to obtain percentage RMSE. This expresses RMSE as a percentage of the true parameter value.
10. Next, randomly set a percentage of last follow-up ($t = 3$) and penultimate ($t = 2$) of response variable as “missing” for each sample. Proceed with steps (6) to (10) above.

Appendix B: SAS codes for simulation

Macro to simulate longitudinal data:

```
/* Generate the multivariate normal data in SAS/IML */
/* non-macro version */

ODS HTML body=      "MVNGen.html"
      headtext="<title>Simulated Multivariate Normal Data</title>"
      anchor="MVNGen";
Options PS=55 LS=80 PageNo=1 NoDate
      FORMCHAR='|----|+|---+|=|-\<>*';

/*defining various macros to be used in the simulation*/

      %let n = 80; /*number of observations */
      %let nsamples =10000; /*number of samples */

      %let betaA = 16; /*Population parameter A */
      %let betaB = 10; /*Population parameter B */

data MVN_par; /* data for the parameter for the multivariate normal
data */
/* the r's are contents of correlation matrix, means are mean vector
and vars are variances */

input rA1 rA2 rA3 rB1 rB2 rB3 meansA meansB varsA varsB;
cards;
1 0.99 0.98 1 0.81 0.66 47.19 15.40 8.29 1.39
0.99 1 0.99 0.81 1 0.81 48.19 15.18 8.34 1.17
0.98 0.99 1 0.66 0.81 1 49.19 15.12 8.31 1.28
;
run;

proc iml;
use MVN_par;
read all var {rA1 rA2 rA3} into RA;
read all var {rB1 rB2 rB3} into RB;
read all var {meansA} into muA;
read all var {meansB} into muB;
read all var {varsA} into sigmaA;
read all var {varsB} into sigmaB;

/* p is number of columns in correlation matrix */
p = ncol(RA);

/* Make sigma into a diagonal matrix */
diag_sigA = diag( sigmaA );
diag_sigB = diag( sigmaB );

/* Calculate covariance matrix */
DRDA = diag_sigA * RA * diag_sigA`;
DRDB = diag_sigB * RB * diag_sigB`;
```

```

/* obtain Cholesky root of covariance matrix*/
UA = half(DRDA);
UB = half(DRDB);

Sample = j(&n*&nsamples,1,0); /* Initialize a variable to record sample
number */
Subject = j(&n*&nsamples,1,0); /* Initialize a variable to record
subject ID */

do g = 1 to &n*&nsamples;
Subject[g] = g;
end;

do k = 1 to &nsamples;
Sample[((&n*k)-(&n-1)):(&n*k)] = k; /* Store sample number in
initialized variable */

do i = 1 to &n;

/* Generate independent standard normals with p rows (time frame), 1
column and seed=1234*/
zA = rannor( j(p,1,1234));
zB = rannor( j(p,1,1454));

/* Random error matrix */
E = rannor( j(p,1,1654));

/* compute multivariate distribution with mean=mu and covariance=DRD */
XA = muA + UA` * zA;
XB = muB + UB` * zB;

/*Transpose X*/
XprimeA = XA`;
XprimeB = XB`;
ErrPrim = E`;

XallA = XallA // XprimeA; /*.... // is just like SET in Data
step...ADD data vertically */
XallB = XallB // XprimeB; /*....// is just like SET in Data
step...ADD data vertically */
ErrAll = ErrAll // ErrPrim;
end;
end;

YData = j(&n*&nsamples,p,0);

XData = XallA*&betaA || XallB*&betaB || ErrAll; /* Merge all data
horizontally to create model

This gives the right
hand side of model equation */
/* Also multiply each X variable by it's beta parameter.
We need to Calculate the Y's using this info */

```

```

YData[,1]=XData[,1]+XData[,4]+XData[,7]; /* The Y's are calculated for
each time period */
YData[,2]=XData[,2]+XData[,5]+XData[,8];
YData[,3]=XData[,3]+XData[,6]+XData[,9];

XData2 = Sample || Subject || YData || XallA || XallB || ErrAll; /*
Full dataset with responses */

IMPORTANT!! Also leave the X variables without multiplying by betas */

varnames = { Sample Subject Y1 Y2 Y3 XA1 XA2 XA3 XB1 XB2 XB3 Err1 Err2
Err3};
create XDataALL from XData2 (|colname = varnames|);
append from XData2;

quit;
run;

```

Macro to generate missing values, conduct multiple imputation and generalized estimating equations:

```

%macro SMac(betaA=, betaB=, nseq= );

/* Reshaping FULL Data to Long Format Using SAS Macros "tolong" */
%include 'C:\tolong.sas';
%tolong(XDataALL,XDataALL_long,Sample Subject,time,1,3,Y XA XB
Err,types=N N N N);

/* MULTIPLE IMPUTATION MODULE */

/* To select a random proportion (approximate) of observations from
dataset and set specific variables as missing
for the random selection*/
Data Reduced;
Set XDataAll;
rand = ranuni(692) * 100; /*Generate random numbers between 1and 100
for each sample */
by Sample;
proc sort ; by Sample rand; /* Sort data by SAMPLE and then by RAND */
run;

/* Rank each RAND from least to highest */
proc rank data=Reduced out=Reduced2;
var rand;
ranks Sequential; by Sample;
run;

/* Select first nseq of each random generation. Each cumulative nseq
is a random sample of n */
data Reduced3; set reduced2;

```

```

if Sequential<=&nseq then Y3=. ; /* Randomly select exactly nseq of n
data as missing for variable Y3, XA3 and XB3*/
by sample;
run;

data reduced4;
set reduced3;
drop Sequential rand;
proc sort ; by Sample Subject;
run;

/* Multiple imputation on REDUCED dataset by SAMPLE */
proc mi data =reduced4 out=reduced_mil
seed = 172296
nimpute = 10
minimum= 0 0 0 0 0 0 0 0 0
maximum= 1700 1700 1700 100 100 100 30 30 30;
var Y1 Y2 Y3 XA1 XA2 XA3 XB1 XB2 XB3;
mcmc chain=multiple initial=EM impute=full nbiter=200 acfplot(wlf)
timeplot(wlf);
by Sample; /* Conduct multiple imputation for EACH sample SEPARATELY!!!
Thus, 10 imputations for each sample*/
run;

/* Reshaping REDUCED(IMPURED) Data to Long Format Using SAS Macros
"tolong" */
%include 'C:\tolong.sas';
%tolong(reduced_mil,Reduced_long,Sample Subject,time,1,3,Y XA XB
Err,types=N N N N);

/* GEE on IMPUTED LONG Data */
proc genmod data=reduced_long;
class Subject;
model Y = XA XB /covb noint; /* Model with no intercept */
by Sample _imputation_ ; /* Obtain GEE estimates by SAMPLE,
and by IMPUTATION within each sample */
repeated subject = Subject /type=ar(1) modelse;
ods output ParameterEstimates=gmparms CovB=gmcovb parminfo=gmpinfo;
Title1 'GEE on IMPUTED DATA';
run;

data gmpinfo;
set gmpinfo;
if Parameter = "Prml" then delete; /* Need to delete INTERCEPT from
parameters */
run;

/* Within each sample, combine estimates from each imputation */
proc mianalyze parms=gmparms Covb=gmcovb parminfo=gmpinfo;
MODELEFFECTS XA XB;
by Sample;

```



```

ods output ParameterEstimates = MI_Estimates; /*Read MI estimates into
a table */
run;

/* Extract only the required variables from output and Subtract
parameter values from estimates */
data MI_Estimates2;
set MI_Estimates;
If Parm='XA' then Diff = Estimate-&beta;A;
Else if Parm='XB' then Diff = Estimate-&beta;B;
DiffSq = Diff*Diff;
run;

data MI_Estimates3;
set MI_Estimates2;
keep SAMPLE Parm Estimate Diff DiffSq;
run;

/* Calculate MSE for all data*/
proc means data=MI_Estimates3 SUM MEAN;
var DiffSq;
Title1 'Mean of DiffSq is MSE for all data';
run;

/* Subset XA of data*/
data MI_EstimatesA;
set MI_Estimates3;
IF Parm='XA';
run;

/* Subset XB of data*/
data MI_EstimatesB;
set MI_Estimates3;
IF Parm='XB';
run;

/* Means etc for each subset */
proc means data = MI_EstimatesA VARDEF=n SUM MEAN VAR STDDEV;
var Estimate Diff DiffSq;
ods output Summary=summaryA;
Title1 'MSE for Variable A is Mean of DiffSQ';
run;

proc means data = MI_EstimatesB VARDEF=n SUM MEAN VAR STDDEV;
var Estimate Diff DiffSq;
ods output Summary=summaryB;
Title1 'MSE for Variable B is Mean of DiffSQ';
run;

Data summaryAA;
set summaryA;
MSE_A = DiffSq_Mean;

```

```

MAERR_A = abs(Diff_Mean);
Var_A = Estimate_Var;
BiasA_SQ = MSE_A - Var_A;
Bias_A = (BiasA_SQ)**0.5;
run;

Data summaryBB;
set summaryB;
MSE_B = DiffSq_Mean;
MAERR_B = abs(Diff_Mean);
Var_B = Estimate_Var;
BiasB_SQ = MSE_B - Var_B;
Bias_B = (BiasB_SQ)**0.5;
run;

proc print data=summaryBB;
run;

/* Merge all results from MI*/
Data summaryAAA;
set summaryAA;
keep MSE_A Var_A Bias_A;
run;

Data summaryBBB;
set summaryBB;
keep MSE_B Var_B Bias_B;
run;

/* merge all */
Data Combo;
set summaryAAA summaryBBB;
Title1 "Bias, Variance and MSE from MI";
run;

proc print data=Combo;
run;

%mend;
ODS HTML Close;

```

Main program interface for executing macros:

```

/* Save Log file for later viewing*/
filename mylog 'C:\first.sas';
proc printto log=mylog;
run;

/* Import simulated longitudinal data previously saved as a database
file */
PROC IMPORT OUT= WORK.XDataAll
            DATAFILE= "XDataAll.dbf"
            DBMS=DBF REPLACE;

```

```

                                GETDELETED=NO;
RUN;

Data XDataAll;
    set WORK.XDataAll;
    run;

/* Code to execute SMAC macro */
%include 'C:\Documents and Settings\odueyuao\Desktop\MCMaster\PhD
Thesis Proposal\Thesis papers\Thesis Paper 3\NEW_NEW PROG\SMac.sas';
%SMac(betaA=, betaB=, nseq= );

```

Figure 1. Flow Chart of Simulation

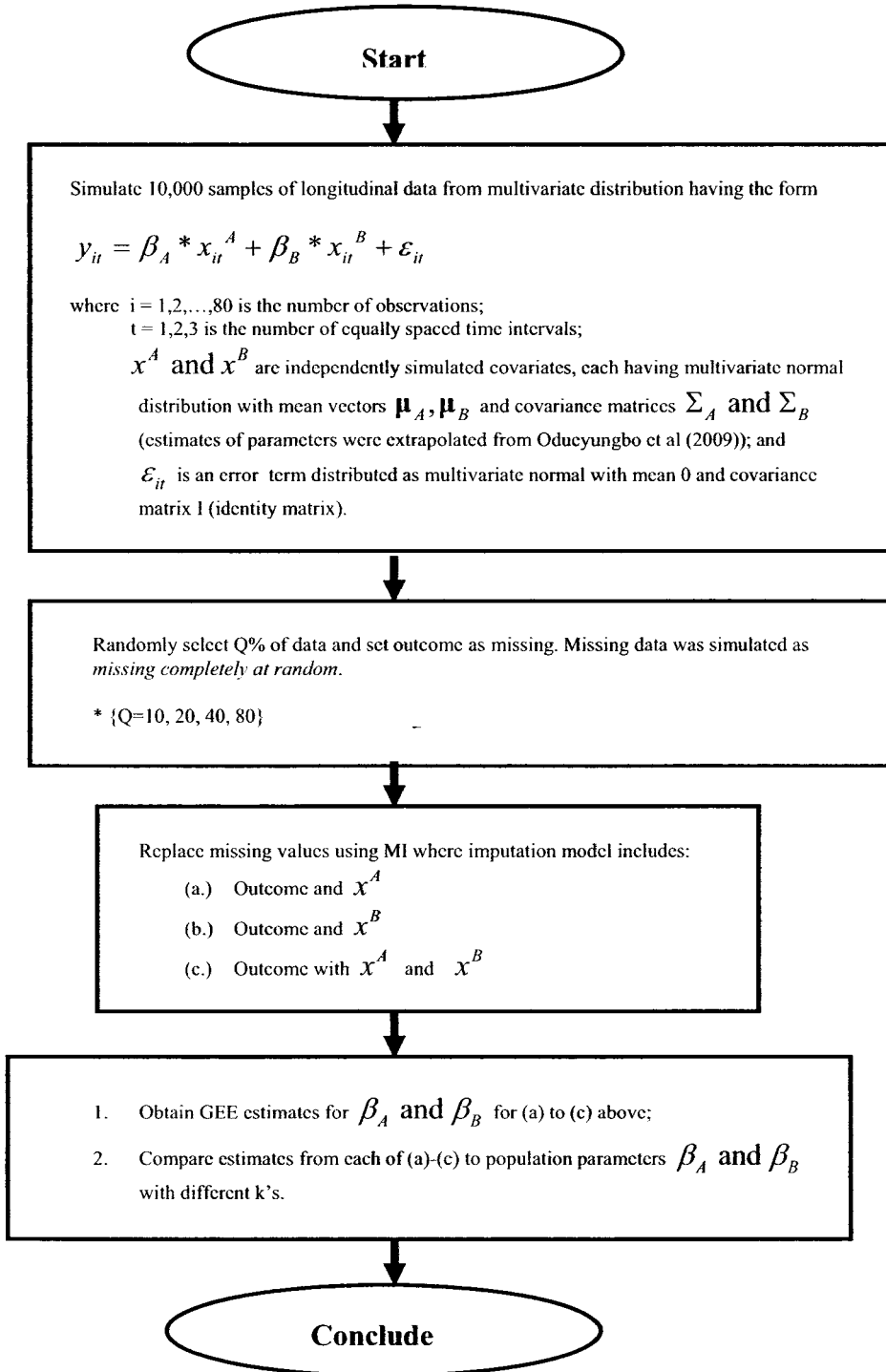
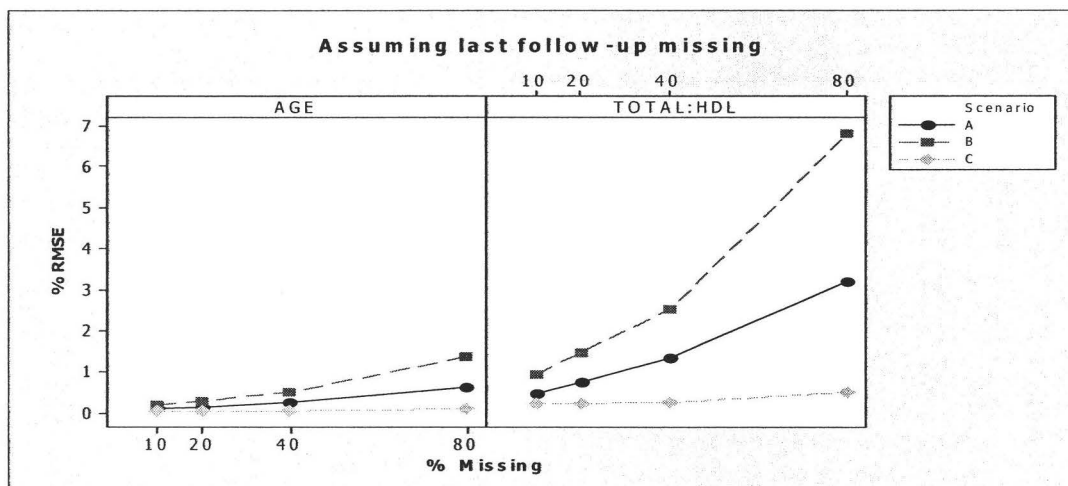
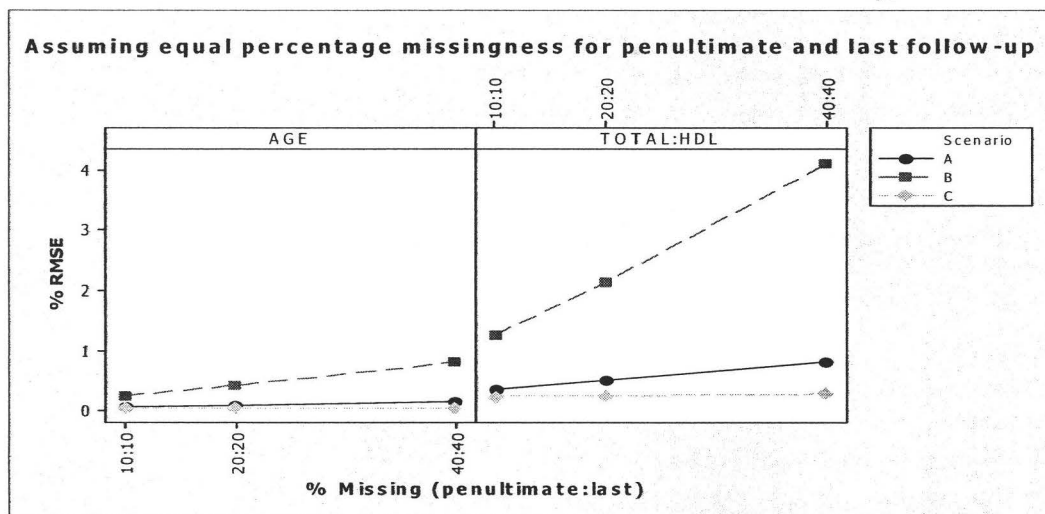


Figure 2. %RMSE as a function of percentage missingness for variables AGE and TOTAL:HDL cholesterol



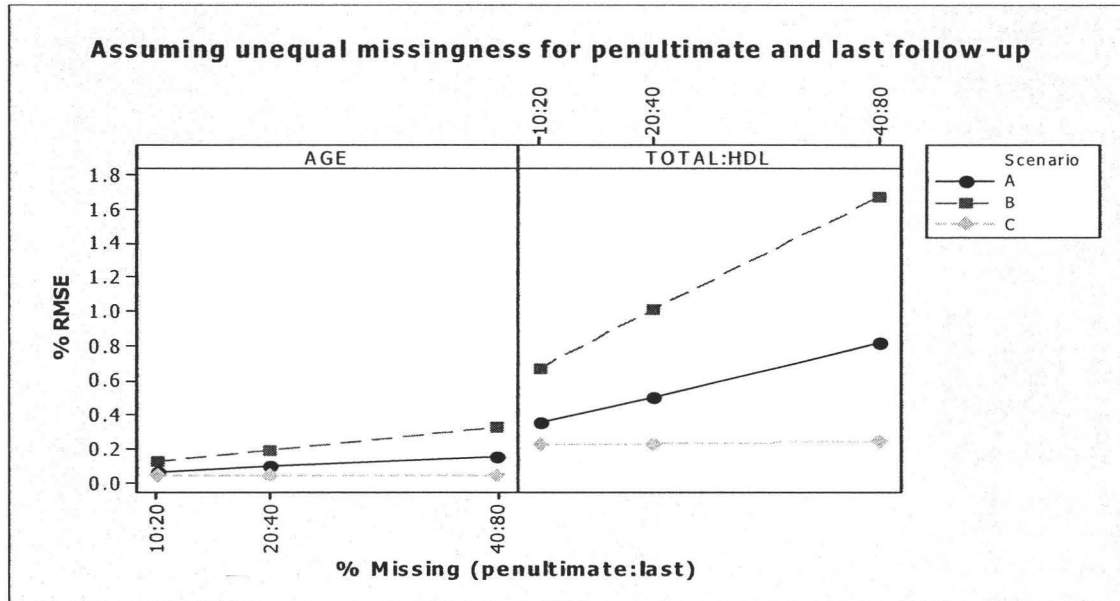
* total:HDL(High density lipoprotein) cholesterol level; %RMSE (Percentage root mean square error)

Figure 3. %RMSE as a function of percentage missingness for variables AGE and TOTAL:HDL cholesterol



* total:HDL(High density lipoprotein) cholesterol level; %RMSE (Percentage root mean square error)

Figure 4. %RMSE as a function of percentage missingness for variables AGE and TOTAL:HDL cholesterol



* total:HDL(High density lipoprotein) cholesterol level; %RMSE (Percentage root mean square error)

Table 1: Correlation matrix for simulated outcomes at times 1, 2 and 3 for one randomly selected sample.

Correlations		y1	y2	y3
y1	Pearson Correlation	1	.990**	.980**
	Sig. (2-tailed)		.000	.000
	N	80	80	80
y2	Pearson Correlation	.990**	1	.990**
	Sig. (2-tailed)	.000		.000
	N	80	80	80
y3	Pearson Correlation	.980**	.990**	1
	Sig. (2-tailed)	.000	.000	
	N	80	80	80

** . Correlation is significant at the 0.01 level (2-tailed).

* The matrix shows an auto-regressive correlation structure for outcome y

CHAPTER 5

CONCLUSIONS OF THE THESIS

There are many methodological and clinical issues that present challenges to HIV researchers. A subset of such issues include: missing data, conflicting results on the nature of the relationship between anti-retroviral therapies (ARTs) and cardiovascular disease (CVD), heterogeneity of ultrasound protocols to quantify atherosclerosis, to mention a few. We have conducted research on these important topics in a “sandwich” thesis, with each chapter dedicated to investigating each of the issues. In this chapter, the findings of the research in this thesis are summarized and we discuss their implications.

In Chapter 2 [1], we compared the construct validity of two ultrasound techniques (Carotid intima media thickness [IMT] and Brachial flow mediated vasodilation [FMD]) used in measuring the extent of atherosclerosis. We obtained baseline and one-year follow-up data for 257 HIV patients recruited across five clinical centers in Canada. A technique was adjudged to have good construct validity if it had statistically significant relationship with established CVD risk factors such as male gender, age, systolic blood pressure and cholesterol level. Cross-sectional data were analyzed using multiple regression analysis, while one-year longitudinal data were analyzed using fixed effect models to adjust for correlation between repeated responses. From our results, baseline IMT was significantly associated with age ($p<0.001$), male gender ($p=0.034$), current smoking status ($p<0.001$), systolic blood pressure ($p<0.001$) and total:HDL cholesterol ratio ($p=0.004$), but not statin use ($p=0.904$) and CD4 count ($p=0.929$). One-year IMT

progression was significantly associated with age ($p<0.001$), male gender ($p=0.0051$) and current smoking status ($p=0.011$), but not statin use ($p=0.289$) and CD4 count ($p=0.927$). One-year FMD progression was significantly associated with current statin use ($p=0.019$), but not CD4 count ($p=0.84$). Neither extent nor progression of FMD was significantly associated with any of the known CVD risk factors. Of the two techniques, we concluded that IMT had better construct validity since it correlated better than FMD with established CVD risk factors in the cohort of patients studied. We hope the results from Chapter 2 [1] will bring researchers a step closer to adopting a universal methodology for assessing atherosclerosis in HIV patients. Standardization of protocols for assessing atherosclerosis will facilitate the comparison of results across studies and further encourage collaborative work among researchers. Unfortunately, our results may not be *directly* comparable to those from other studies due to heterogeneity in ultrasound protocols.

In Chapter 3, the relationship between certain ART regimens and carotid IMT (a surrogate measure for CVD) was studied in a meta-analysis of 1,032 record-level baseline data across three studies from Canada, USA and France. The specific ARTs studied included: Protease Inhibitors (Saquinavir (INVIRASE/FORTOVASE), Ritonavir (any dose), Amprenavir, Indinavir, Nelfinavir), Nucleoside Reverse Transcriptase Inhibitors (Abacavir, Zidovudine (AZT), Stavudine (D4T), Zalcitabine, Didanosine, Lamivudine (3TC)), and Non-nucleoside Reverse Transcriptase Inhibitors (Delavirdine, Efavirenz, Nevirapine). Univariate and multivariable hierarchical linear models were used in investigating the research questions. All multivariable models were adjusted for

traditional CVD risk factors. In univariate analysis, factors significantly associated with the outcome (IMT) included: male gender ($p=0.002$), age ($p<0.001$), diastolic blood pressure (DBP) ($p=0.025$), systolic blood pressure (SBP) ($p=0.003$), LDL cholesterol ($p=0.001$), CVD history ($p=0.002$), smoking ($p=0.021$), use of AZT, D4T and 3TC ($p=0.007$, 0.003 , and <0.001 respectively), and duration of AZT, D4T, 3TC, Ritonavir and Nelfinavir ($p=0.006$, 0.006 , 0.002 , 0.029 , and 0.022 respectively). Only male gender, age, LDL cholesterol, smoking and Ritonavir duration remained significantly associated with CCA in multivariable analysis. When all two-way interaction effects were considered, we found significant interaction effects for: gender*AZT use($p=0.019$); gender*D4T use($p=0.022$); CVD history*DBP($p=0.020$); CVD history*SBP($p=0.001$); Smoking*AZT($p=0.005$); smoking*age($p=0.002$); and 3TC use*LDL($p=0.025$). The negative impacts of D4T and AZT appeared more pronounced in males than females. The relationship between DBP(or SBP) and atherosclerosis seemed dependent on past CVD history. Cigarette smoking likely moderates the relationship between use of AZT(or age) and CVD. The drug 3TC may lead to CVD in patients with elevated LDL cholesterol. Our results and conclusions from the main effects models are similar to some (not all) previous studies in which ARTs were not significantly associated with atherosclerosis, independent of traditional risk factors. Nonetheless, significant interaction effects from the exploratory analysis warrant further investigation of potential moderating effects between traditional risk factors and ARTs in the atherosclerotic process. An understanding of these interactions will facilitate a classification of patients to various risk profiles, and assist healthcare providers in prescribing the ‘best’ medication to

patients based on risk stratification. One key limitation of this study is that we have only considered cross sectional (baseline) data in the meta-analysis. Longitudinal data will, more adequately, allow the investigation of the effect of cumulative exposure to ARTs on atherosclerotic progression.

In the third paper presented in Chapter 4, we investigated the efficiency of a strategy for conducting multiple imputation (MI) in longitudinal carotid IMT studies with missing data using Monte-Carlo simulation. In MI, missing values are predicted from pre-selected variables included in an assumed multivariate normal (imputation) model. A common approach is to include all final analysis model variables (inclusive strategy) in the imputation model [2], but penalties associated with exceptions to this strategy are not well studied for longitudinal HIV data. We investigated the impact of a deviant MI strategy on the variability and bias of estimated regression parameters. We concluded that an inclusive strategy, where all final analysis variables are included in the imputation model, provided the least combined bias and variability for estimated regression coefficients. Our study provides further empirical evidence to support an inclusive, rather than restrictive strategy in MI. It is important to obtain optimal regression estimates because inferences made from these estimates will likely guide clinical judgment in practice. Also, effort should be made to include variables strongly associated with outcome having missingness in the imputation model. Further, our results showed that imputation can be a questionable strategy for handling missing data when percentage of missingness is very high. A limitation of this simulation study is that we have made certain assumptions, for simplicity, which may not be obtainable in real world. For

instance, we have assumed that only the outcome data was incomplete, whereas in most practical situations, both outcome and covariates may be missing. It is not clear how much the simplifying assumptions will affect the generalizability of our conclusions.

Our results and conclusions have key implications for future research in HIV. We have established the construct validity of carotid IMT and brachial FMD to assist investigators in selecting useful outcome measures in HIV vascular research involving ultrasound [1].

In one of the largest studies to date, we have studied the relationship between ARTs and carotid IMT in a meta-analysis of 1,032 patient records. Although we found no evidence to support untoward cardiovascular effects of ARTs independent of known CVD risk factors in multivariable main effects models, we have challenged researchers to initiate investigations into potential interactions between ART exposure and other risk factors in the atherosclerotic process. There may be reasons to believe that risk factors do not act in isolation, but may interact with each other to increase atherosclerotic degeneration. A number of review articles/research studies provided the motivation for exploring these interaction effects [3-5]. Sudano et al [6] proposed that patients are screened for hyperlipidemia before initiating ART, suggesting that HIV therapy may accelerate CVD in patients with abnormal lipid profiles [6]. Orlando et al [3], Egger & Drewe [4], and Lundgren et al [5] concluded that age-related diseases (like atherosclerosis) are enhanced with ART initiation [3-5]. The foregoing statements suggest potential synergistic effects between traditional cardiovascular risk factors (such as age, LDL) and ARTs on atherosclerosis.

This cross-sectional study provides the first step towards understanding the long-term effect of ARTs on CVD. We plan to update the meta-analysis as more data become available from future collaborators. The ultimate goal would be to use longitudinal data to quantify the effect of long-term use of ARTs on CVD.

Lastly, we have also provided empirical evidence to justify the theoretical rationale for including all final analysis model variables in the imputation model to increase the performance of regression estimates when conducting MI. As stated earlier, inferences from regression coefficients may ultimately affect clinical practice, hence the need for strategies that reduce bias and variability whenever data imputation is a necessity.

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