

VITAMIN D STATUS AND CAROTID ARTERY IMT IN HIV INFECTION

VITAMIN D STATUS
AND
CAROTID ARTERY INTIMA-MEDIA THICKNESS
IN ADULTS LIVING WITH
HIV INFECTION

By
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Abstract

Background: Vitamin D activity is important for the functioning of a broad range of body systems. Some of these, including the skeletal, immune, and cardiovascular systems, are particularly relevant in the management of HIV-infection; thus, and in consideration of evidence that factors associated with the scenario of HIV-infection can disrupt vitamin D metabolism, the assessment of vitamin D status in people living with HIV-infection may be particularly important. In this thesis, I address cardiovascular implications of vitamin D status in HIV-infection. More specifically, and based on a growing body of evidence implicating low vitamin D status in the development of cardiovascular disease (CVD), I hypothesized that in HIV-positive adults low 25-hydroxyvitamin D (25(OH)D) concentration would be associated with increased subclinical vascular disease as measured by carotid intima-medial thickness (IMT).

Methods: Using regression analyses I cross-sectionally studied the relationship between 25(OH)D and carotid IMT in 283 participants of the Canadian HIV Vascular Study, a prospective study of CVD risk among HIV-positive Canadians.

Results: The prevalence of vitamin D deficiency in the Canadian HIV Vascular study was surprisingly low. Plasma 25(OH)D by quartile was not associated with carotid IMT. However, in restricted cubic spline regression analyses designed to accommodate non-linearity there was evidence of an inverted U-shaped 25(OH)D-carotid IMT relationship. In exploratory regression models restricted to participants comprising the suboptimal range

of vitamin D status, lower 25(OH)D concentration was statistically significantly associated with lower carotid IMT after adjustment for known CVD risk factors and other variables hypothesized to potentially confound a 25(OH)D-carotid IMT association.

Main implication: While inference from these exploratory findings requires cautious interpretation, future investigations into the relationship between vitamin D status and vascular disease should consider the problem of non-linearity as a feature of primary analyses; otherwise, such studies might fail to detect a true association.

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List of Abbreviations

1,25(OH) ₂ D	1,25-dihydroxyvitamin D; calcitriol
25(OH)D	25-hydroxyvitamin D
AIDS	Acquired Immunodeficiency Syndrome
ANOVA	Analysis of variance analysis
ART	Antiretroviral therapy
BMI	Body mass index
CHF	Congestive heart failure
CI	Confidence interval
CRP	C-reactive protein
CVD	Cardiovascular disease
CYP	Cytochrome P450
DAGs	Directed acyclic graphs
FMD	Flow mediated vasodilation
HDL	High density lipoprotein
HIV	Human Immunodeficiency Virus
HR	Hazard ratio
ICC	Intraclass correlation coefficient
IL-10	Interleukin-10
IL-6	Interleukin-6
IMT	Intima-medial thickness
INF	Interferon
IU	International Units
LDL	Low density lipoprotein
MAC	<i>Mycobacterium avium complex</i>
MI	Myocardial infarction
MMP9	Matrix metalloproteinase 9

NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside/nucleotide reverse transcriptase inhibitor
OR	Odds ratio
PI	Protease inhibitor
PTH	Parathyroid hormone
r	Correlation coefficient
R ²	Coefficient of determination
RAS	Renin-angiotensin system
RCT	Randomized controlled trial
RIA	Radioimmunoassay analysis
RR	Relative risk
TB	Tuberculosis
TC:HDL	Total cholesterol-to-HDL ratio
TNF α	Tumor necrosis factor-alpha
UVB	Ultraviolet B
VDR	Vitamin D receptor
Vitamin D2	Ergocalciferol
Vitamin D3	Cholecalciferol

Chapter 1

Introduction and literature review: Vitamin D status and its association with cardiovascular disease in people living with HIV infection

1.1. Thesis overview

There is emerging evidence that vitamin D deficiency is associated with the development of cardiovascular disease (CVD).¹⁻⁵ A causal association would have particular relevance to persons living with human immunodeficiency virus (HIV) infection in Canada: HIV-positive adults are at increased risk for CVD⁶⁻⁹, and vitamin D deficiency is regionally prevalent.¹⁰⁻¹² The prospect of vitamin D status as a modifiable risk factor is appealing. Vitamin D repletion among HIV-negative individuals is considered feasible, safe, and attainable.¹³ In the context of HIV infection, however, the clinical relevance of vitamin D status on cardiovascular health is particularly unclear.

In this thesis, I examine the hypotheses that suboptimal vitamin D status is prevalent within the Canadian HIV Vascular Study cohort and that there is greater vascular disease, as determined by baseline carotid intima-media thickness (IMT), in participants with lower versus higher vitamin D status.

This work is divided into four chapters. In this first chapter, I provide a brief overview of issues relevant to the study of vitamin D and its influence on human health. In

Chapter Two I summarize the background evidence upon which the current investigation is based. This consists of a literature review of studies that have assessed prevalence and

determinants of vitamin D deficiency among HIV-infected populations, and an overview of evidence regarding the relationship between vitamin D status and risk of cardiovascular disease. This literature review is intended to guide development of cross-sectional analysis of the relationship between vitamin D status and vascular disease in persons living with HIV infection. As there are substantial gaps in the evidence concerning a vitamin D-CVD association among HIV-infected individuals, this background overview is fortified by discussion of findings drawn from HIV-negative populations.

In addition to informing development of the current cross-sectional study, this background review is intended to address fundamental questions pertinent to my rationale for undertaking this preliminary-level investigation. First, do factors associated with HIV infection, including the infection itself, its management, and particulars of the population—or sub-populations within—increase the value of screening for suboptimal vitamin D status in HIV-infected adults. In other words, irrespective of the clinical relevance of vitamin D status in the general community, does the the scenario of HIV infection warrant specified or enhanced surveillance for vitamin D deficiency? Second, in lieu of sufficient evidence in support of a causal association between exposure to suboptimal vitamin D status and development of cardiovascular disease, what further investigations are justified based on the available data?

In **Chapter Three** I discuss methodological considerations and issues related to accomplishment of my study objectives and present the cross-sectional study in detail.

Study results are presented and discussed in **Chapter Four**.

1.2. Overview of vitamin D biochemistry, physiology, and supply

Vitamin D is a nutrient with pleiotrophic potential. When activated—that is once it has been metabolized to its active metabolite, calcitriol—vitamin D is capable of wide-spread influence spanning most tissues across most body systems. In this respect, a number of non-classic actions of vitamin D have only recently become more broadly appreciated.¹⁴ While the classic actions of vitamin D, which pertain primarily to calcium homeostasis via effects in bone, kidney and gut tissue, are essential to human health and have justifiably received much attention, a range of more subtle vitamin D effects have more recently received a more equitable balance of attention. The vitamin D receptor (VDR) has been found in most tissues¹⁴ and the cellular machinery necessary to activate and deactivate vitamin D is more widespread than initially thought. Accordingly, the clinical relevance of vitamin D nutritional status, and by implication the potential benefit of repletion, may correspond to a range of clinical disorders that by tissue specificity span VDR distribution. These would include disorders of bone, immunology, oncology, and cardiology, all of which have some relevance to the scenario of HIV infection.

1.2.1. Vitamin D synthesis and nutritional supply

Vitamin D exists as two forms: ergocalciferol or D₂, derived from the plant sterol ergosterol; and cholecalciferol or D₃, derived from the animal sterol 7-dehydrocholesterol.

1.2.1.1: Vitamin D: oral input

Vitamin D is not naturally abundant in the human food supply.¹¹ The best natural sources are oily fish and fish liver oil.¹⁵

According to estimates presented by Lu et al.¹⁶, a typical dinner serving of wild salmon (100 g) would contribute approximately 1000 International Units (IU) of dietary vitamin D₃. Next highest sources, bluefish and farmed trout, contained less than half, and farmed salmon less than 25%, the vitamin D₃ content of wild salmon.

A historically utilized source of vitamin D has been cod liver oil, typically dispensed by the *teaspoon* dose providing 400 IU vitamin D₃. Thus, for all but a few individuals willing to adhere to frequent consumption of salmon, higher recommended intakes of vitamin D cannot efficiently be attained without food fortification or nutritional supplementation.

To prevent conditions associated with severe vitamin D deficiency, specifically rickets and osteomalacia, cow's milk is fortified with vitamin D₃. Milk alternatives, such

as soy and rice milk, are typically fortified with vitamin D₂. Additionally, some Canadians consume vitamin D₃ supplements, as treatment for low bone density, or as a preventative measure during seasons where exposure to sunlight is minimal.

The contribution of vitamin D intake to circulating vitamin D supply is dependent on intact small intestinal, pancreatic, and hepatic function. Vitamin D insufficiency has been reported in an number of gastrointestinal disorders including celiac and Crohn's disease, and can occur secondary to post-gastrectomy.¹⁷ Low vitamin D status has also been reported in some hepatic diseases including chronic cholestatic disorders, viral and chronic active hepatitis, and alcoholic cirrhosis.¹⁷

1.2.1.2: Vitamin D: cutaneous input

In humans, epidermal 7-dehydrocholesterol, upon exposure to ultraviolet B (UVB) radiation, is transformed to previtamin D₃. Under the influence of body heat previtamin D₃ is then converted to vitamin D₃.¹⁸ Presumably to prevent toxicity, maximum vitamin D production is limited at a threshold setpoint approximate to 10 - 15% of the original 7-dehydrocholesterol concentration¹⁹. According to estimates presented by Hollis et al.²⁰, light skinned Caucasian individuals attain their synthesis threshold, releasing ~10,000 to 20,000 IU of vitamin D into general circulation, within 10-12 minutes of full-body UVB exposure.

The skin's natural pigment, melanin, reduces the efficiency of dermal vitamin D production. Accordingly, darker skin is a risk factor for vitamin D deficiency as darker

skinned individuals require a longer duration of UVB exposure than lighter skinned individuals to attain maximum production.²¹ Compared to Caucasians, South Asians or dark skinned African Americans require 2.5 and 10 times as much sun exposure, respectively, to attain maximum vitamin D₃ production.¹⁹

Advanced age is associated with reduced cutaneous levels of 7-dihydrocholesterol.^{18,22}; thus, vitamin D deficiency found in elderly populations may in part be explained by reduced capacity for synthesis.

1.2.2. Risk factors for suboptimal vitamin D status

Risk factors for low vitamin D status include female gender; darker skin colour; advancing distance from the equatorial latitudes; winter season; cultural and lifestyle barriers to sunlight, including sunscreen use; and absence of food fortification.²³ Cultural factors appear to be particularly relevant to vitamin D status and may obscure the influence of regional differences in the availability of sunlight. For instance, vitamin D deficiency is particularly abundant in South Asia and the Middle East despite each possessing a climate and latitude favourable to abundant UVB-radiation exposure.²³

Health related factors associated with deficiency include advanced age; institutionalized care; obesity; malabsorption, renal and liver disease; and anticonvulsant use.²⁴

1.2.3. Measurement of vitamin D status

Circulating 25(OH)D, in plasma or serum, is considered the best available biomarker of vitamin D supply.²⁵ Unlike circulating 1,25(OH)₂D₂, which is tightly regulated and has a short half-life, the 25(OH)D concentration more directly reflects the level of cutaneous and dietary input. A dose-response relationship was recently confirmed by meta-regression analysis.²⁶ Each 1 IU/d increase in oral vitamin D intake was associated with a corresponding increase in serum 25(OH)D concentration of 0.013 nmol/L over, at minimum, a 6-week period. Of note, the dose-response analysis was limited by large heterogeneity, a finding consistent with known validity issues¹⁵ associated with 25(OH)D measurement. Furthermore, factors associated with known risks of 25(OH)D deficiency such as age, adiposity, and gender may alter 25(OH)D status independent of vitamin D input.^{27,28}

While 25(OH)D concentration is considered a reasonable marker of vitamin D input, its utility as a biomarker of vitamin D function is less clear.²⁹ The biological activity of vitamin D via interaction with VDR is dependent on a sufficient level of 25(OH)D as substrate but also on a number of other factors unrelated to 25(OH)D concentration.²⁸ For instance, as I describe in section 2.2.5.2, severe 1,25(OH)₂D deficiency has been documented in individuals living with advanced HIV infection, yet this occurs without commensurately severe 25(OH)D deficiency.³⁰

However, despite some notable limitations in the utility of 25(OH)D as a measure of vitamin D status, a multitude of health outcomes correlate strongest with circulating 25(OH)D, but less so or not at all with plasma calcitriol.³¹ Plasma 1,25(OH)₂D levels typically fall only in certain pathological conditions and states of severe 24(OH)D deficiency. Furthermore, 25(OH)D reflects substrate availability for intracellular calcitriol synthesis across a range of tissues whereas circulating 1,25(OH)₂D directly reflects the biological compartment under renal control.³¹

1.2.4. Defining vitamin D status

The 25(OH)D cutoffs for discriminating commonly used vitamin D status categories (*deficiency*, *insufficiency*, and *optimal*) are a matter of some debate.^{20,23,32} Classic vitamin D deficiency (often categorized as *severe* deficiency) is typically diagnosed by a 25(OH)D level of < 25 nmol/L (10 ng/mL) and it is below this threshold where frank presentations of vitamin D malnutrition (rickets in infants and osteomalacia in adults) become most evident. Less severe deficiency, characterized by associations with an emerging list of multiple health outcomes has been more difficult to define.

Optimal vitamin D status is often defined by minimum and maximum 25(OH)D levels of 75 nmol/L (30 ng/mL), or sometimes 80 nmol/L (32 ng/mL), and 250 nmol/L (100 ng/mL). The lower bound of this range is derived from estimation of a 25(OH)D-PTH threshold above which PTH is maximally suppressed. The validity of this threshold as a marker of physiological stability has recently been questioned. According to a 2009

systematic review, there is great variability in threshold estimates across studies that have examined the 25(OH)D-PTH relationship.³³ Moreover, plasma PTH concentration depends on a number of factors independent of 25(OH)D status including age, calcium intake, ethnicity, substance abuse, and kidney function;²⁸ and likely on factors yet to be fully elucidated such as exposure to HIV-therapy (this is described in section 2.2.5.1). For these reasons, estimation of a single clinically relevant threshold upon which to define vitamin D adequacy is problematic.^{28,33}

Although expert consensus has yet to be reached, there is some convention in categorizing vitamin D status as deficient, insufficient or optimal (or desirable), differentiated by the 25(OH)D cutpoints 50 nmol/L (20 ng/mL) and 75 nmol/L (30 ng/mL). Alternatively, a number of studies have defined vitamin D deficiency as a 25(OH)D level of ≤ 37.5 nmol/L (15 ng/mL).³⁴⁻³⁸ To convert nmol/L 25(OH)D to ng/mL 25(OH)D multiply by the conversion factor 2.496.

Some researchers have argued that optimal status may be closer to the upper threshold of a normal range defined by minimum and maximum 25(OH)D levels of 80 nmol/L and 250 nmol/L, respectively.²⁰ To my knowledge, there is no clinical trial data upon which to substantiate purported health benefits of 25(OH)D targets beyond correction of vitamin D insufficiency.

As definitions of vitamin D status have not been uniform across studies I use the term *suboptimal vitamin D status* in reference to a broader category of status, encom-

passing both vitamin D insufficiency and deficiency, where the condition of status presumably represents a state where 25(OH)D levels are insufficient to fully accommodate functional needs.

1.2.5. Vitamin D metabolism

Bioactivation of vitamin D involves a two-step process facilitated by a variety of cytochrome P450 (CYP) enzymes. Step one entails synthesis of 25(OH)D by CYP isoenzymes capable of vitamin D 25-hydroxylase activity. In step two 25(OH)D is 1 α -hydroxylated to 1,25(OH)₂D.

Calcitriol and 25(OH)D are inactivated by 24-hydroxylase (CYP24A1). VDRs are present in most tissues and likewise most cells require the capacity to inactivate calcitriol. This is mainly accomplished through expression of CYP24A1. In addition to its catabolic effect on 1,25(OH)₂D, 24-hydroxylase can divert 25(OH)D metabolism down an alternative pathway, towards the inactive metabolite 24,25-(OH)D.

1.2.6. Vitamin D: endocrine, autocrine and paracrine activities

Vitamin D homeostasis reflects the relative activity of all three of the aforementioned vitamin D hydroxylases. Under normal circumstances, for instance barring impaired hepatocyte function as occurs in liver disease, the rate of 25(OH)D production is substrate dependent and likewise 25(OH)D levels generally reflect the availability of vitamin D derived from oral and cutaneous inputs.

The so-called "classic" action of vitamin D pertains primarily to the endocrine effects of circulating calcitriol. In response to decreasing plasma calcium, $1,25(\text{OH})_2\text{D}$ is synthesized in the distal tubule cells of the kidney, and circulated as a message primarily to enhance calcium absorption across intestinal cells. Renal production of $1,25(\text{OH})_2\text{D}$ is regulated by a homeostatic mechanism involving serum calcium, phosphorus, and parathyroid hormone (PTH).

Non-classic actions of circulating $1,25(\text{OH})_2\text{D}$ are only partially understood. Calcitriol synthesis can occur in macrophages and various tissues including parathyroid, skin, bone, cartilage, and prostate.^{14,39,40} Extrarenal 1α -hydroxylase activity is not regulated by PTH and is thought to contribute little to the circulating $1,25(\text{OH})_2\text{D}$ supply. Rather, its influence is likely regional serving autocrine or paracrine effects on local VDRs. Unlike renal 1α -hydroxylase activity, extrarenal $1,25(\text{OH})_2\text{D}$ production may be more substrate dependent. In this respect, the influence of circulating $25(\text{OH})\text{D}$ may be greater than what was originally thought. Macrophage 1α -hydroxylase expression is predominately under immunological control, up-regulated by inflammatory cytokines such as interferon (IFN)- γ , and by lipopolysaccharides.⁴⁰

Chapter 2

The relevance of vitamin D status in HIV infection: review of the evidence

Since its inception in 1996, access to highly active antiretroviral therapy (ART) has meant considerable improvement in the long-term outlook for HIV-positive persons. For the substantial majority of individuals living in high income countries HIV infection has become a chronic manageable condition. In this emergent scenario, HIV-positive adults are exposed over many years to the combined effects of HIV infection, ART toxicity, and aging. Evidence is accumulating that risk for some of these chronic age-related conditions might vary by HIV status.⁴¹ This is becoming particularly evident for risk of CVD.

Particular features of the scenario of HIV infection including those related to HIV-1 exposure, to ART, and to the population at risk for infection, may individually or in concert contribute to elevate CVD risk.⁴² It is certainly plausible, given known biological effects and emergent epidemiological findings, that suboptimal vitamin D status may intertwine with these various features of HIV infection to increase incident CVD in adults living with HIV infection. However, the clinical importance of this scenario has not been established in an HIV-positive cohort.

This chapter is divided into two main sections. In section 2.1 I overview evidence related to the prevalence of suboptimal vitamin D status in HIV-positive individuals. In section 2.2 I overview evidence relevant to investigation of the relationship between vitamin D status and CVD, in adults living with HIV infection.

This literature review is based on a search strategy designed to identify and retrieve relevant primary reports and reviews of investigations that have assessed vitamin D status and vitamin D effects in HIV infection. The specific strategy applied to OVID Medline (R) (1956 to April 2009) and modified to accommodate EMBASE, is included in appendix A. To enhance the sensitivity of this search strategy I scanned reference lists of all relevant articles and retrieved additional reports whenever appropriate. I conducted a supplementary search strategy (see appendix B) not limited to HIV-positive populations, designed to identify research relevant to investigation of a vitamin D-CVD association. Several articles are discussed that have emerged subsequent to when the initial searches were conducted.

2.1. The prevalence of suboptimal vitamin D status in HIV-positive populations

In this section I overview evidence related to the prevalence of suboptimal vitamin D status in HIV-positive individuals and address the question: is the prevalence of suboptimal vitamin D status higher in HIV-positive adults compared with the general population. In the discussion that follows, emphasis is placed on those investigations with

greatest generalizability to the Canadian setting. My literature search identified 22 studies that reported measurement of 25(OH)D in people living with HIV infection.^{34,43-63} These investigations are summarized in Table 1.

Table 1. Studies Reporting 25-hydroxyvitamin D Status in HIV-positive Individuals

Study/Year Region	Sex Age ¹	Cohort ²	Baseline 25(OH)D Status			Notable Findings/Comments
			Severe Deficiency < 25 nmol/L	Deficiency < 50 nmol/L	Insufficiency ≤ 75 or 80 nmol/L	
Mehta et al. ³ 2009 Tanzania	F 25	884 HIV+ pregnant woman			39%	In multivariable analysis low maternal vitamin D status (< 80 nmol/L) associated with higher risk of mother-to-child HIV transmission by 6 weeks postpartum (RR 1.50, 95% CI 1.02-2.20), transmission via breast-feeding (RR 2.03, 95% CI 1.08-3.82) and infant mortality during 24 months follow-up (RR 1.58, 95% CI 1.26-1.97)
Wejse et al. ⁴ 2009 Guinea- Bissau, West Africa	M/F 37	365 adults with TB infection (95 were HIV+) RCT 100,000 IU D ₃ at baseline, 5, and 8 months, compared to placebo at 12 months follow- up.		9.0%	46.5%	25(OH)D levels by HIV-status were not reported. Mortality did not differ between treatment and placebo groups (RR 1.19, 95% CI 0.58-1.95); but, in an HIV+ subgroup the hazard ratio estimate was in the direction of harm (HR 1.8, 95% CI 0.8-4.1). Intervention and control groups experienced equal rises in 25(OH)D concentrations suggesting possible contamination bias.

Arpaali et al. ⁶¹ 2009	M/F 6-16	61 HIV+ children and adolescents	7.8% (< 30 nmol/L)	46.8%		Mean baseline 25(OH)D, after exclusion of individuals with severe deficiency, was 61.2 nmol/L.
		New York City, U.S.				
Rodriguez et al. ⁶² 2009	M/F 46	57 HIV+ adults	10.5%	47.3%	74%	25(OH)D deficiency associated with lack of regular exercise (OR 3.1, 95% CI 0.9-11.1); lower dietary vitamin D intake: (OR 3.3, 95% CI 1.0-10.3); reported lactose intolerance (OR 7.8, 95% CI 1.0-61.5); and NNRTI use (OR 3.03, 95% CI 0.99-9.27). ARV use and low calcium intake were significantly associated with elevated PTH.
		Boston, U.S.				
Rubin et al. ⁶³ 2009	M 48	62 HIV+ adults	11.3%	41.9%	75.8%	Median 25(OH)D levels were 42.4 nmol/L and 64.9 nmol/L for participants exposed to NNRTIs and PIs, respectively (P = .002). NNRTI- compared with PI-use was associated with a sixfold increased risk of vitamin D deficiency (OR 6.62, 95% CI 1.91-22.89). Suboptimal status not associated with ethnicity, current CD4 count, history of wasting, current nutritional parameters, and status groups did not differ by age or by duration of HIV infection. Severe deficiency was associated with tobacco use (P = .032).
		New York City, U.S.				

Van Den Bout-Van Beukel et al. ¹¹⁸ 2008 The Netherlands	M/F 41	252 HIV+ adults	29% (M: 25%, F: 58%)	Deficiency defined as April- Sept < 25 nmol/L; Oct-Mar < 35 nmol/L.	Prevalence of 25(OH)D deficiency and median levels by skin colour groups were 19% and 59.5 nmol/L in white; 33% and 57 nmol/L in Mediterranean; 44% and 32 nmol/L in Asian; and 62% and 27.1 nmol/L in black patients. Black skin colour and NNRTI-use were associated with increased risk of vitamin D deficiency (OR 6.22, 95% CI 3.20-12.08; and OR 1.86, 95% CI 1.07-3.22, respectively). In univariable analysis, deficiency was associated with female gender, younger age, lower CD8 cell count, black skin colour, NNRTI treatment, higher PTH, lower calcium, and lower albumin; after adjustment, only skin colour remained significant (OR 5.4, 95% CI 2.3-12.2). PTH was elevated in 17% of all participants and in 37% of those with vitamin D deficiency. PTH was higher in NNRTI- and PI-treated compared with untreated participants.
Fris et al. et al. ¹¹⁹ 2008 Tanzania	M/F 36	655 adults with pulmonary TB (96 (47%) were HIV+)	0.8% 10.8% 41.8%		Mean 25(OH)D was 86 nmol/L and levels did not differ by HIV status. Serum 25(OH)D was positively associated with the harvest season, BMI and serum TIR (a measure of tissue iron deficiency).
Tomazič et al. ¹²⁰ 2007 Slovenia	M 42		8.2%		Mean 25(OH)D levels did not differ by treatment groups (naïve, PI-based ART, and non-PI-based ART)

Curtis et al. ¹⁰ 2006	M/F	44 ethnically diverse adults with lipodystrophy		79% (< 85 nmol/L)	Mean 25(OH)D concentrations and total D intakes were 52 nmol/L and 95 nmol/L (P = .02) and 189 µg/d and 340 µg/d (P = .02) in African Americans compared with non-African Americans, respectively.
Garcia Aparicio et al. ¹² 2006	M	30 asymptomatic HIV+, 16 healthy HIV-, adults	46% (HIV+)	86% (HIV+)	Mean 25(OH)D levels (Oct-June) were higher in HIV+ controls compared with HIV+ individuals (36 nmol/L vs 28 nmol/L, respectively; P = .04), but between HIV+ participants 25(OH)D did not significantly differ by treatment status (naïve compared with PI-based treatment). Mean PTH levels were higher in the HIV+ group (45 pg/mL) compared with healthy controls (24 pg/mL; P < .0001).
Dolan et al. ¹³ 2006	F	100 HIV+, 41 100 HIV- adults			Mean baseline 25(OH)D concentrations did not differ between HIV+ (37.0 nmol/L) and HIV- controls (41.8 nmol/L; P = .08).

Stephensen et al. ³⁴	M/F	359 adolescents (238 HIV+, 121 HIV- controls), drawn from families predominately of low socioeconomic status	87% (< 37.5) nmol/L	Mean 25(OH)D concentrations in HIV+ and HIV- participants were 20.3 and 19.3 nmol/L, respectively; and were not significantly different (P = .62). In multivariable analysis predictors of plasma 25(OH)D were (in rank order): latitude, total vitamin D intake, alcohol intake, black race, BMI, summer and fall seasons, and plasma neopterin (a marker of immune activation).
Seminari et al. ³²	M/F	68 HIV+ ART-experienced (> 5 years) adults	81.3% (< 45 nmol/L)	Elevated PTH in 26% of participants
Ramayo et al. ³³	M/F	74 HIV+ adults (50% IVD users; 30% with AIDS)		Median 25(OH)D levels in spring and autumn were 85 and 78 nmol/L (P = .03) and differed by treatment status with levels of 65 and 83 nmol/L in drug naïve and ART-treated patients, respectively (P = .04). Serum 25(OH)D was independently associated with the odds of receiving ART (OR 6.5, 95% CI 1.2-30, P = 0.04). Median 25(OH)D levels were not statistically different by gender. The Advantage 25(OH)D assay (Nichols Diagnostics) utilized in this study is known to overestimate 25(OH)D concentration.

Arpad et al. ³⁹	2004	M/F	40 HIV+ African American (58%) and Hispanic children and adolescents	7.1% (<30 nmol/L)	30%	Mean 25(OH)D concentration was 68 nmol/L.
Teichmann et al. ⁴⁰	2003	F	50 HIV+, 50 HIV- adults			Mean 25(OH)D concentrations for HIV+ and HIV- participants were 93 and 54 nmol/L, respectively (P < .01). Likewise, PTH was higher in HIV+ individuals (P < .05)
Teichmann et al. ⁴¹	2000	M/F	100 HIV+ (44 with heroin-dependency), 40 HIV-, adults			Mean 25(OH)D concentrations for HIV+ and HIV- participants were 99.0 nmol/L and 165 nmol/L. 25(OH)D levels differed by HIV status only in the male heroin-dependant subgroup (P < .05). Serum 25(OH)D was not associated with total CD4 count.
Kuehn et al. ⁴²	1999	M/F	828 HIV+, 549 HIV-, adults		50% (among 21 AIDS patients with hypocalcaemia)	Mean serum calcium (corrected for albumin) was lower in HIV+ compared to controls (P < .001). Study was designed to investigate the prevalence of hypocalcaemia; actual 25(OH)D levels were not directly reported.

Haug et al. ⁴⁶ 1998	M/F	54 HIV+, 20 healthy HIV- adults		Median 25(OH)D concentration of 70 nmol/L did not differ by HIV status and was not associated with serum 1,25(OH) ₂ D
Oxlo, Norway	64			
Haug et al. ⁴⁷ 1994	NR	53 HIV+ adults; 28 HIV- controls		Median 25(OH)D levels were not significantly different between HIV symptomatic participants (67 nmol/L), asymptomatic participants (132 nmol/L) and HIV- controls (126 nmol/L). Serum 25(OH)D was not associated with CD4 count, CD8 count, serum neopterin, clinical events, or 1,25(OH) ₂ D concentration.
Coodley et al. ⁴⁸ 1993	M/F	47 adults (15 with wasting syndrome, 26 with AIDS)	11% deficient (normal range defined as mean + 2 standard deviations)	
Portland, Oregon, U.S.	NR			

25(OH)D indicates 25-hydroxyvitamin D; HIV+, HIV-positive; HIV-, HIV-negative; RCT, randomized controlled trial; HR, hazard ratio; RR, relative risk; OR, odds ratio; NNRTI, non-nucleoside reverse transcriptase inhibitor; ART, antiretroviral therapy; PTH, parathyroid hormone; PI, protease inhibitor; BMI, body mass index; IVD, intravenous drug use; NR, not reported; 1,25(OH)₂D, 1,25-dihydroxyvitamin D.

⁴⁶ Mean, median, or range

⁴⁷ Outpatient setting in all cohorts except Teichmann et al.⁴⁴ which involved a small proportion of inpatient participants

2.1.1. Validity issues of the prevalence studies

Most reports were of small cross-sectional studies. Although, cross-sectional analyses may generate valid prevalence data, a number of important issues limit comparability between studies. First, there has been substantial and progressive improvement in the quality of HIV-care since the early days of HIV-therapy. In this review, study publication dates range 16 years from as early as 1993, in the pre-highly active ART era, to the most recent publication in 2009. Across this period varying features relevant to HIV-disease prognosis or to ART-adverse effects may limit comparability between studies. Furthermore, within a single population the prevalence of vitamin D insufficiency or deficiency has been shown to change over time.⁶⁴

Second, clinical, cultural and geographic differences between study populations limit comparability by modifying baseline risk for suboptimal vitamin D status.

Finally, 25(OH)D assays have been subject to inter-assay non-comparability. For instance, the Nichols Advantage 25(OH)D RIA assay (Nichols Institute Diagnostics) utilized in five studies^{30,45,46,51} consistently overestimated 25(OH)D₃ concentrations and was withdrawn from the marketplace in 2006.³¹ Additionally, the Advantage assay failed to accurately detect circulating 25(OH)D₂⁶⁵ further complicating interpretation of 25(OH)D status in countries where vitamin D₂ had been utilized therapeutically.

Since the establishment of the Vitamin D External Quality Assessment Scheme (DEQAS; Internet: www.deqas.org) in 1989, there has been a gradual improvement in the performance of 25(OH)D assays with respect to assay linearity, precision, and absolute values⁶⁵ Nevertheless, lack of comparability between assay methods, and between different versions of the same assay (the DiaSorin RIA kit, was reformulated between 1994 and 2000) represents an important source of between-study heterogeneity.

Most studies in this review are cross-sectional. Thus, findings are at high risk of bias. Causal inference from these data is speculative at best and provide only limited guidance for development and testing of my thesis hypotheses. Two studies prospectively investigated health effects of vitamin D supplementation. One of these was an uncontrolled pilot study¹³³, and likewise as with the cross-sectional investigations its findings are susceptible to bias. The other study was a Randomized Controlled Trial (RCT) of vitamin repletion in individuals initiating treatment for tuberculosis (TB) infection.⁶² Overall the study was at low-to-moderate risk of bias given the adequacy of sequence generation, allocation concealment, blinding, and intention to treat analysis (for mortality only, but not for primary outcome). Also, the report provides no evidence of selective reporting. However, missingness of primary outcome data was not addressed; and HIV-positive individuals represented only a subgroup of a larger TB-infected cohort.

2.2. Summary of the evidence regarding 25(OH)D status in HIV Infection

No investigation assessed vitamin D status within a Canadian HIV-positive population. Two studies, both conducted in the north-eastern United States, and another set in The Netherlands, are by geographic latitude reasonably comparable with settings that comprise the Canadian HIV Vascular Study cohort. In the north-eastern United States prevalence of 25(OH)D deficiency (≤ 50 nmol/L) in HIV-infected adults was 42%⁵⁹ and 47.3%.⁶⁰ The prevalence of severe deficiency (< 25 nmol/L) was 11.3%⁵⁹ and 10.5%.⁶⁰ Approximately 75% of patients in each study were categorized as having suboptimal vitamin D status (< 75 or < 80 nmol/L).

In The Netherlands, among 254 ambulatory HIV-positive individuals, the prevalence of vitamin D "deficiency" was 29% (defined as < 35.5 nmol/L in April-September or < 25 nmol/L in October-March).⁵⁸

The validity of generalization of these observational data to the Canadian HIV Vascular Study cohort is limited by population differences in extent of ethnic and gender diversity. All three studies were comprised proportionally of a greater number of individuals at increased risk of suboptimal vitamin D status (darker skinned individuals and women) than is found in the primarily white male population of the Canadian HIV Vascular Study. Based on these issues of clinical diversity, vitamin D deficiency or insufficiency within the Canadian cohort could presumably be less prevalent.

However, in a general population study, comprised mainly of healthy Caucasian men and woman residing in Calgary, Canada, prevalence rates were not substantially different than in the above-mentioned studies. In winter months, 39% had vitamin D deficiency (< 50 nmol/L), and 86% had levels less than optimal (< 80 nmol/L).¹² Prevalence of vitamin D deficiency and suboptimal status declined during the summer months to 14% and 68%, respectively. In consideration of this Calgary cohort, and of the three HIV-positive populations mentioned above, I expect that suboptimal vitamin D status in the Canadian HIV Vascular Study cohort will be prevalent particularly during the winter months. Whether the severity of deficiency will be greater than what was found in the Calgary HIV-negative cohort is not clear. In the section that follows I overview investigations that have compared vitamin D status between HIV-positive and HIV-negative adults.

2.2.1. 25-hydroxyvitamin D differences by HIV status

Nine studies assessed mean 25(OH)D levels in HIV-positive individuals compared with HIV-negative controls.^{34,44,45,48,49,53,54,57,62} In three studies,^{48,49,54} 25(OH)D levels differed by HIV status.

In an outpatient setting in Germany,⁴⁹ 25(OH)D levels were lower in a group of 50 HIV-positive woman than in 50 healthy HIV-negative age-matched controls ($P < .05$). Reported 25(OH)D means were 93 nmol/L and 154 nmol/L in HIV-positive and HIV-negative groups, respectively.

In a small study set in Sassari Spain,⁵⁴ mean serum 25(OH)D levels were lower in 30 asymptomatic HIV-positive individuals than in 16 healthy HIV-negative controls ($P = 0.04$). Among HIV-positive individuals, 86% were reported to be vitamin D "deficient"—the 25(OH)D threshold for deficiency was not stated. Forty-six percent of deficient cases were characterized as severe (< 25 nmol/L). Notably, the study period corresponded mainly to colder months of the year (October-June). While inclusion of warmer month 25(OH)D determinations would presumably have attenuated the high prevalence of vitamin D "deficiency" within the study, it is not clear whether attenuation would vary by HIV-status.

In an outpatient setting in Germany Teichmann et al.⁴⁸ found lower 25(OH)D levels among HIV-positive compared with HIV-negative individuals in a male-heroin dependent subgroup. In the larger study population 25(OH)D levels did not differ by HIV status.

Six additional studies^{34,44,45,53,57,62} did not detect significant differences by HIV status although effects suggestive of differences were reported in subgroups with advanced HIV disease.^{44,45} Owing to small sample size a number of these studies may have been underpowered to detect group differences by HIV-status.

In summary, although suboptimal vitamin D status is highly prevalent across a diverse range of HIV-positive populations, there is a lack of convincing evidence to conclude that persons with HIV infection are at increased risk of lower vitamin D status compared with HIV-negative controls.

2.2.2. Seasonal variation of 25(OH)D status

I found no evidence to suggest that seasonal 25(OH)D variation might differ by HIV status. In general, the effect of seasonal variation on vitamin D solar production in persons living with HIV infection appears congruent with variation noted in community populations. Suboptimal vitamin D status is most prominent in settings that experience substantial seasonal variation in UVB intensity,^{50,58-60} and particularly when 25(OH)D levels are determined during colder months of the year.⁵⁴ In Tanzania, serum 25(OH)D was positively associated with the harvest season, presumably due to increased duration of exposure to direct sunlight.⁵⁷

2.2.3. 25-hydroxyvitamin D differences by ART exposure

There is some evidence that ART may impact vitamin D status. In The Netherlands individuals exposed to non-nucleoside reverse transcriptase inhibitors (NNRTIs) had lower median 25(OH)D levels compared with individuals on protease inhibitor (PI)-based ART ($P = 0.017$).⁵⁸ Additionally, low vitamin D status may have been more prevalent in the NNRTI-exposed group (36.5% and 23.0%, for NNRTI-exposed and PI-exposed individuals, respectively; $P = 0.070$) and these participants had an 86% increased risk of

vitamin D "deficiency" compared to those not exposed to NNRTIs (odds ratio (OR) 1.86; 95% confidence interval (CI): 1.07-3.22). However, in multivariable logistic regression analysis treatment type did not remain a significant risk factor for vitamin D deficiency.

In a Boston study ⁶⁰ low vitamin D tended to be more common among patients receiving NNRTIs compared with other ART regimens (OR 3.03; 95% CI: 0.99-9.27, $P = 0.06$). Likewise, in another study set in the north-western United States, median vitamin D levels were lower among patients receiving NNRTIs compared with those on PI-based regimens; 25(OH)D levels were 42.4 nmol/L and 64.9 nmol/L ($P = 0.002$), respectively. ⁵⁹ In this study, NNRTI-usage was associated with a sixfold increased risk of vitamin D deficiency (OR 6.62; 95% CI: 1.91-22.89).

In a small study set in southern Spain, 25(OH)D levels differed by treatment status. ⁵¹ Median 25(OH)D levels were 65 nmol/L and 83 nmol/L for ART-naïve and ART-exposed patients, respectively. In contrast, several other studies did not detect 25(OH)D differences by treatment status. ^{54,56,58}

The findings of reduced 25(OH)D levels among patients exposed to NNRTI-therapy may represent a true clinical effect. Alternatively, it is possible that some factor related to the likelihood of having been prescribed a NNRTI therapy may account for these statistical associations. In the early era of highly active ART, NNRTIs represented a best option for patients (for instance, substance abusers) perceived to be at increased risk of nonadherence to the relatively laborious dosing strategies of the early-era PI-based regi-

mens. In a study of 1761 woman who started ART between the years 1996 to 2005, NNRTI-based ART as an initial regimen, was more common among participants who were more likely to have reported use of any injected drug or of non-injected crack, cocaine, or heroin ($P < .05$).⁶⁶ While the effect of substance abuse on the prevalence of sub-optimal vitamin D status is not clear, in a small study of HIV-positive adults, 25(OH)D levels were lower in males with, compared with those without, heroin dependancy.⁴⁸ Thus, it is plausible that the NNRTI-25(OH)D status association is best explained by the confounding effect of substance abuse, which presumably is a cause of NNRTI-exposure and a cause (or marker of a cause) of suboptimal vitamin D status.

In summary, there is consistent evidence from studies that have examined 25(OH)D differences by types of ART-exposure that NNRTI-use is associated with reduced 25(OH)D status. To my knowledge no study has examined this association prospectively and thus it is not clear whether NNRTI-exposure is a determinant of 25(OH)D deficiency. In addition, there is conflicting evidence about whether HIV-treatment in general affects vitamin D status.

2.2.4. 25-hydroxyvitamin D differences by immune status

Five studies provided comparison of 25(OH)D levels with various immune parameters.^{45,49,51,58,59} While there is evidence that calcitriol deficiency may be component of immunodeficiency (this is addressed in section 2.2.5.2), there is a lack of convincing evidence to suggest that 25(OH)D status differs by immunological status. In what might

be the exception Ramayo et al.⁵¹ found higher median 25(OH)D levels among patients exposed to ART compared with a more immunocompromised group of ART-naïve, potentially undernourished (many had low serum albumin), adults ($P = 0.04$). While it is tempting to speculate that some feature of immunodeficiency such as HIV-associated enteropathy or increased utilization might contribute to 25(OH)D malnutrition, this association has not been confirmed by evidence that markers of HIV-disease progression correspond to declining levels of circulating 25(OH)D. Of the 4 studies that reported this comparison none found independent statistical associations between circulating 25(OH)D and markers of immunodeficiency including current absolute CD4 count,^{45,49,58,59} CD8 count,^{45,58} serum neopterin,⁴⁵ history of wasting,⁵⁹ or clinical events.⁴⁵

2.2.5. HIV-associated disruptions along the vitamin D metabolic pathway

As I have previously described there is a high prevalence of suboptimal vitamin D status in HIV-positive populations but I have been unable to identify any compelling evidence to suggest that these high rates are unique to HIV-positive populations. In contrast, there may exist metabolic differences relevant to HIV status. Under certain conditions HIV-positive adults appear to be at greater risk of disrupted vitamin D metabolism, presenting as elevated or reduced PTH or low levels of circulating 1,25(OH)₂D. As follows, I overview evidence of disrupted metabolism in HIV-positive adults and present *in vitro* findings that might account for these disruptions in the scenario of HIV infection.

2.2.5.1: Evidence of parathyroid hormone disruption in HIV infection

Under normal circumstances 25(OH)D is inversely associated with serum PTH. The 25(OH)D threshold below which secondary hyperparathyroidism (and osteoporosis) occurs has varied across studies. According to a recent systematic review, most threshold estimates cluster between 40 and 50 nmol/L or 70 and 80 nmol/L.³³ Of note, the second cluster (70-80 nmol/L) has been utilized as rationale for defining the lower bound of the proposed *optimal* 25(OH)D status category as 75 or 80 nmol/L. Aloia et al.³³ have argued against use of a specific threshold for clinical purposes since a number of factors need to be considered:

The variability in the estimates for the 25(OH)D threshold may be explained by ethnic difference in calcium economy, the extent of vitamin D deficiency, different calcium intakes, inaccuracy of 25(OH)D assays, the age and health of the populations studied, and the mathematical analyses used.³³

In a multiple regression analysis involving thresholds derived from 18 studies serum 25(OH)D concentration and dietary calcium accounted for approximately 67% of the 25(OH)D threshold variance. Thus, this suggests that the 25(OH)D threshold for prevention of secondary hypoparathyroidism may be relative to dietary calcium intake, but also

may be influenced by other health related factors. Whether some feature particular to the scenario of HIV infection can influence the threshold has not to my knowledge been investigated.

Lower serum levels of PTH have been found in HIV-positive patients compared with healthy-controls, and this has occurred paradoxically under conditions of 1,25(OH)₂D deficiency^{44,49}—more typically, as is often seen in advanced renal disease, 1,25(OH)₂D deficiency triggers a corresponding hyperparathyroidism. Aukurst et al.⁴⁴ have proposed a mechanism whereby the inflammatory cytokine tumor necrosis factor-alpha (TNFα)—this often becomes elevated with advancing immunodeficiency—directly suppresses PTH secretion.

Whereas low PTH may be a component of advanced HIV infection, several studies have shown that patients on ART experience higher levels of PTH compared with ART-naïve individuals and with HIV-negative controls⁵⁴, and hyperparathyroidism is associated with concurrent antiretroviral use.^{58,60,67} In Rodriguez et al.,⁶⁰ high PTH was found in patients exposed to ART, but not in participants not receiving therapy. Likewise, Van Den Bout-Van Den Beukel et al.⁵⁸ found higher PTH among patients receiving treatment and this was most prominent among individuals exposed to NNRTIs compared to PI-containing regimens. Among vitamin D deficient participants, NNRTI-exposure compared with no NNRTI-exposure was associated with a nearly fourfold increased risk of

hyperparathyroidism (OR 3.93; 95% CI 1.65-9.37). As expected, serum PTH was inversely correlated with 25(OH)D levels ($r = -0.39$, $P = 0.001$) and elevated PTH was associated with low serum calcium ($r = 0.15$, $P = 0.03$).

2.2.5.2: Evidence of low 1,25-dihydroxyvitamin D status in HIV infection

I identified five studies that assessed circulating 1,25(OH)₂D levels in HIV-positive adults compared with HIV-negative controls (see Table 2). As I discuss below, 1,25(OH)₂D homeostasis may be disrupted under various conditions relevant to HIV infection.

A series of investigations conducted in Oslo Norway found lower 1,25(OH)₂D in HIV-positive individuals with advanced HIV infection.^{30,44-46} Lower 1,25(OH)₂D levels were found in individuals coinfecting with disseminated *Mycobacterium avium complex* (MAC) than in those without MAC infection ($P < 0.05$).³⁰ Nearly half of MAC-infected patients had 1,25(OH)₂D levels below the threshold of detection.

Table 2. Studies Reporting 1,25-dihydroxyvitamin D Concentrations in HIV-positive Individuals

Study/Year Region	Sex Age ^a	Cohort ^b	Mean or Median 1,25(OH) ₂ D Concentrations	Notable Findings/Comments
Ramayo et al. ⁵¹ 2005 Seville, Spain	M/F 37	74 HIV+ adults (50% IVD users; 30% with AIDS)	Lower in treatment-naïve compared with ART exposed participants ($P = .01$) and independently associated with the odds of receiving ART (OR 5.5, 95% CI 1.2–36, $P = .04$)	
Madeddu et al. ⁵² 2004 Sassari, Italy	M/F 20–61	172 HIV+, 64 HIV- adults	Lower in HIV+ participants compared with controls ($P < .04$) and levels further declined from baseline among 27 ART-exposed participants followed for 14 months ($P < .05$).	PTH concentrations did not significantly differ by treatment or HIV status.
Teichmann et al. ²⁹ 2003 Germany	F 37	50 HIV+, 50 HIV- adults	Lower in HIV+ participants compared with controls ($P < .01$), and correlated with CD4 count ($r = 0.45$, P $< .05$)	

Teichmann et al. ⁴⁷ 2000 Germany	M/F NR	100 HIV+ (44 with heroin- dependency), 40 HIV- adults	Lower in HIV+ male participants compared with male controls ($P < .05$); and associated with CD4 count ($r = 0.23$, $P < .05$)	Lower PTH in HIV+ male participants compared with male controls ($P < .05$). PTH positively associated with CD4 count ($r = 0.28$, $P < .05$)
Aukrust et al. ²² 1999 Oslo, Norway	M/F 38	73 HIV+, 25 sex and age- matched adult controls	Lower in HIV+ participants with AIDS ($n = 38$) compared with controls ($P < .001$)	Lower PTH in HIV+ participants compared with controls ($P < .01$)
Haug et al. ⁹ 1998 Oslo, Norway	M/F 16-64	54 HIV+, 20 HIV- adults	Lower in HIV+ participants compared with controls ($P < .01$); 54% of HIV+ individuals had 1,25(OH) ₂ D deficiency.	In HIV+ participants 1,25(OH) ₂ D was negatively correlated with TNF α ($r = -0.55$, $P < .001$), but was not associated with 25(OH)D concentration. Lowest 1,25(OH) ₂ D levels were characterized by advanced clinical HIV infection.
Haug et al. ⁹ 1996 Oslo, Norway	M/F NR	53 HIV+ adults with AIDS	Lower in participants with MAC infection ($n = 11$) compared with those without MAC infection ($P < .05$); 15% of AIDS patients had undetectable levels.	1,25(OH) ₂ D was not associated with 25(OH)D. All patients with detectable TNF α had low 1,25(OH) ₂ D. In MAC infection TNF α was inversely correlated with 1,25(OH) ₂ D ($r = -0.78$, $P < .05$)

Haug et al. ²⁵ 1994	NR	53 HIV+, 28 HIV- adults	Lower in HIV+ participants compared with controls (P < .01)	In HIV+ participants 1,25(OH) ₂ D was associated positively with CD4 (r = 0.35, P < .05) and CD8 counts (r = 0.33, P < .01), and negatively with serum neopterin (r = -0.36, P < .01); but was not associated with serum 25(OH)D. Severe 1,25(OH) ₂ D deficiency was associated with shorter survival (P < .01).
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1,25(OH)₂D indicates 1,25-dihydroxyvitamin D; HIV+, HIV-positive; HIV-, HIV-negative; IVD, intravenous drug; ART, antiretroviral therapy; OR, odds ratio; PTH, parathyroid hormone; NR, not reported; TNF α , tumor necrosis factor alpha; MAC, disseminated mycobacterium avium complex; 25(OH)D, 25-hydroxyvitamin D.

* Mean, median, or range

* Outpatient setting in all cohorts except Teichmann et al.⁴⁴ which involved a small proportion of inpatient participants

While low $1,25(\text{OH})_2\text{D}$ status has also been found in HIV-positive adults without advanced disease,^{49,67} circulating $1,25(\text{OH})_2\text{D}$ levels may correlate with extent of immunodeficiency. Positive correlations with absolute CD4 cell counts were found in Haug et al.⁴⁵ ($r = 0.45$, $P < 0.05$) and Teichmann et al.⁴⁹ ($r = 0.35$, $P < 0.05$), but not in Haug et al.,⁴⁶ and Madeddu et al.⁶⁷ However in Haug et al.⁴⁶ severe $1,25(\text{OH})_2\text{D}$ deficiency did corresponded to lower CD4+ counts ($P < 0.01$).

In addition, low circulating $1,25(\text{OH})_2\text{D}$ may be associated with other markers of HIV-disease progression. Haug et al. demonstrated inverse $1,25(\text{OH})_2\text{D}$ associations with the presence of MAC coinfection,³⁰ with the inflammatory cytokine $\text{TNF}\alpha$,⁴⁶ and with serum neopterin⁴⁵. Neopterin and $\text{TNF}\alpha$, both of which are markers of ongoing immune activation, have been associated with HIV-disease progression and AIDS-associated mortality.⁶⁸⁻⁷¹

Calcitriol is an important immune system regulator, but the clinical relevance of low circulating $1,25(\text{OH})_2\text{D}$ in the condition of advancing immunodeficiency is not clear. If deficiency occurs as a consequence of immune dysregulation, for instance secondary to elevated $\text{TNF}\alpha$,⁴⁴ presumably, ART-mediated immune reconstitution might restore, at least partially, circulating $1,25(\text{OH})_2\text{D}$ levels. Consistent with this proposition Ramayo et al. found higher serum $1,25(\text{OH})_2\text{D}$ levels in ART-exposed compared with a more immunocompromised ART-naïve group ($P = 0.01$).⁵¹ The circulating $1,25(\text{OH})_2\text{D}$ level was independently associated with the odds that a participant was receiving ART (adjusted

OR 5.5; 95% CI: 1.2-36.0). However, in a study where circulating $1,25(\text{OH})_2\text{D}$ differed by HIV status, levels did not differ by ART-exposure status.⁶⁷ Moreover, in longitudinal analysis of a subgroup of 14 participants receiving ART, $1,25(\text{OH})_2\text{D}$ levels declined over 14 weeks, from a mean of 118.6 pmol/L (45.6 pg/mL) at baseline to a mean of 89.4 pmol/L (34.4 pg/mL) at follow-up ($P < .05$). These findings deserve cautious interpretation particularly given that with a cohort of only 14 participants the presence of even a single outlier could have substantial influence on the estimated means. Hence, the influence of ART on circulating $1,25(\text{OH})_2\text{D}$ is not clear.

These findings suggest that HIV-positive adults may experience disrupted $1,25(\text{OH})_2\text{D}$ homeostasis. While low $1,25(\text{OH})_2\text{D}$ may in part be a consequence of reduced $25(\text{OH})\text{D}$ supply, concordant deficiency of both metabolites was found in only one study.⁴⁹ Circulating levels of these metabolites were found to be correlated in another study ($r = 0.6$, $P < 0.001$),⁵¹ but not in 3 others.^{30,45,46}

Under normal conditions $1,25(\text{OH})_2\text{D}$ and $25(\text{OH})\text{D}$ levels are only loosely correlated or are not correlated at all unless $25(\text{OH})\text{D}$ deficiency is severe. In moderate $25(\text{OH})\text{D}$ deficiency, calcitriol is stable or may be elevated as a compensatory response to the low $25(\text{OH})\text{D}$ supply. Thus, in the absence of severe $25(\text{OH})\text{D}$ deficiency, low $1,25(\text{OH})_2\text{D}$ presumably indicates disrupted calcitriol homeostasis via effects on its synthesis or degradation.

2.2.5.3: In vitro evidence of disrupted vitamin D metabolism

Levels of circulating and intracellular vitamin D metabolites are a consequence of differential rates of CYP450 mediated production and degradation. The exact CYP450 pathways have yet to be completely specified. However, in vitro investigations have provided evidence that medications commonly used in the management of HIV infection might impair vitamin D hydroxylation by modulating CYP450 activity.^{72,73}

Cozzolino et al.⁷² found that in vitro exposure to HIV-PIs impaired human hepatocyte 25(OH)D and macrophage 1,25(OH)₂D synthesis in a dose-dependent manner. In macrophages the PI inhibitory effect was greater for 1,25(OH)₂D synthesis (1 α -hydroxylase) than for its degradation (24-hydroxylase) resulting in a net 1,25(OH)₂D reduction. PI exposure at concentrations comparable to maximal plasma levels following drug administration, resulted in reduced conversions to 1,25(OH)₂D of 79.4%, 63.4% or 31.7%, for ritonavir, indinavir or nelfinavir, respectively. In contrast, all PIs demonstrated similar capacity to inhibit 1,25(OH)₂D degradation. Furthermore, the minimal inhibitory concentration of ritonavir differs for 1 α - and 24-hydroxylase enzymes in that 1,25(OH)₂D synthesis is inhibited at a lower concentration than what would be required to mount a compensatory inhibition of the catabolic activity of 24-hydroxylase. Thus, it appears that a PI-effect on vitamin D metabolism may be enzyme specific having greater impact on synthesis than on degradation.

Additionally, PI effects on vitamin D metabolism may differ by tissue type. In contrast to what has been reported in human hepatocytes and in macrophages, human myeloid leukemia cells exposed to ritonavir experienced a net increase in intracellular 1,25(OH)₂D levels presumably through downregulation of 24-hydroxylase (CYP24) expression.⁷³

The clinical relevance of PI-effects on vitamin D metabolism is not clear. A variety of CYP450 isoenzyme-mediated pathways provide some level of redundancy in metabolite synthesis and degradation. For instance, while the primary 25-hydroxylase is thought to be CYP2R1 other isoenzymes capable of 25(OH)D synthesis include CYP27A1, CYP3A4 and CYP2J3. Some of these alternate pathways may be substrate specific. For instance CYP3A4 has specific affinity for vitamin D₂, but does not appear to hydroxylate vitamin D₃.^{74,75} The clinical relevance of these specificities are poorly understood, but CYP3A4 expression is upregulated by calcitriol deficiency and could account for some of the 25(OH)D₂ and 1,25(OH)₂D in circulation. Despite these alternative pathways, renal CYP27B1 is considered the primary 1 α -hydroxylase and thus is the main contributor to serum 1,25(OH)₂D supply.

Given this evidence that certain CYP450 enzymes have differential affinity for vitamin D₂ and vitamin D₃ substrates, the magnitude of disruption caused by a PI—or any other drug that may affect CYP450 activity—might differ depending on an individual's source of vitamin D input. For instance, it is plausible based on this in vitro evidence that

vitamin D₃, but not D₂, may be the preferred source of fortification or supplementation in ART-exposed individuals. Whether this specificity is relevant in other tissues, or to degradation of 25(OH)D₂ or 1,25(OH)₂D₂ via 24-hydroxylase (CYP24A1), is not clear.

Additionally uncertain is the impact that a differential effect might have on 25(OH)D determination. Assays such as the Elecsys 25-hydroxyvitamin D method utilized in this current study cannot detect serum 25(OH)D₂.⁶⁵ Thus, they may fail to capture the effect of CYP3A4 perturbations on total circulating 25(OH)D levels. Fortunately, in Canada vitamin D₂ is not used clinically, and dietary D₂ input is expected to be minimal.

In addition to PIs, a number of other HIV-relevant medications may negatively impact vitamin D metabolism including: rifabutin,⁷⁶ rifampicin,⁷⁷⁻⁷⁹ isoniazid,^{77,78} tenofovir,⁸⁰ and efavirenz.^{81,82}

In summary, although suboptimal vitamin D status is highly prevalent across a diverse range of HIV-positive populations, there is a lack of convincing evidence to conclude that 25(OH)D levels differ by HIV status. Substantial heterogeneity limits the validity of between study comparisons. Methods of measuring 25(OH)D have not been uniform and assay ranges are not necessarily comparable. Other variables that may limit generalization include varied latitudes, season, lifestyle factors, oral intake and fortification of foods, skin colour, gender, morbidities, and historical trends in patient care.

2.3. Vitamin D-associated mortality in people living with HIV infection

In HIV-negative populations vitamin D deficiency may increase mortality risk^{83,84} and vitamin D supplementation, even at modest levels, may provide a mortality benefit.⁸⁵ In a meta-analysis of nine large RCTs involving 57,311 participants, vitamin D supplementation compared with placebo, resulted in a modest decrease in the risk of all-cause mortality (summary relative risk 0.93, 95% CI 0.87-0.99).⁸⁵

The impact of vitamin D status in people living with HIV infection has received only minimal attention. In a small longitudinal analysis including 7 asymptomatic and 10 symptomatic HIV-positive adults, those with low serum 1,25(OH)₂D (< 65 pmol/L) at baseline had shorter survival times at a median 9 months of follow-up.⁴⁵ In a Cox regression analysis only 1,25(OH)₂D remained predictive of survival. Inference from these data deserve cautious interpretation; in addition to the small sample size, few study details were reported.

In an RCT of vitamin D supplementation involving 365 TB-infected adults attending outpatient care in Guinea-Bissau, Africa, 100,000 IU of cholecalciferol at inclusion and at 5 and 8 months from baseline, had no overall affect on mortality (relative risk (RR) 1.19, 95% CI 0.58-1.95) at 12-month follow-up.⁶² In a subgroup of 95 HIV-positive participants vitamin D had no significant effect on mortality. Surprisingly, the hazard-ratio (HR) estimate was in the direction of harm (HR 1.8, 95% CI 0.8-4.1); whereas in HIV-negative participants the HZ was twofold less (HR 0.9, 95% CI 0.3-2.8). A test for

interaction was not significant ($P = 0.08$). These negative findings are in contrast to observational data and immunological benefits of vitamin D supplementation reported earlier,⁸⁶ and this study has been criticized on grounds of possible contamination bias: intervention and control groups experienced equal rises in 25(OH)D concentrations.⁸⁷

In summary, vitamin D supplementation may modestly reduce the risk of all cause mortality in the general population, but few studies have investigated a survival benefit in HIV infection. In patients coinfectd with TB infection vitamin D supplementation did not improve survival. Additionally, but perhaps more related to the condition of impaired vitamin D metabolism than to a consequence of decreased nutritional intake, the active vitamin D metabolite, 1,25(OH)₂D may be an important predictor of survival. However, evidence in support of this proposition is derived from prospective study of just 17 HIV-positive patients. In the sections that follow, I narrow the focus of this discussion to more specifically address possible cardiovascular implications of suboptimal vitamin D status.

2.4. Vitamin D and cardiovascular disease in people living with HIV infection

There is emerging evidence that vitamin D deficiency is associated with the development of CVD. In this section I provide an overview of evidence concerning an association between vitamin D status and CVD. This is followed by discussion of the state of

evidence as it applies to individuals living with HIV infection. I summarize by stating that there is a paucity of evidence about vitamin D status and cardiovascular disease in adults living with HIV infection.

2.4.1. Summary of observational evidence

The putative cardiovascular-associated activities of vitamin D are diverse and widespread.³ Observational studies have reported associations between low vitamin D levels and CVD mortality;^{32,84} incident CVD including heart failure, myocardial infarction (MI) and stroke;^{37,88-92} coronary artery calcification;⁹³ peripheral artery disease;⁹⁴ and prevalent CVD risk factors including hypertension, diabetes mellitus, and hypertriglycerides;^{1,5,95-97} Low vitamin D status was associated with increased carotid IMT in one study,³⁵ but not in two others;^{98,99} and in a fourth study vitamin D status was associated with the internal but not the common carotid IMT¹⁰⁰ (I expand upon the carotid IMT evidence in section 2.4.3).

In meta-analyses of observational data high versus low vitamin D status was associated with a 33% reduction in risk for prevalent cardiovascular disease (myocardial infarction, stroke, ischaemic heart disease, and peripheral vascular disease), a 55% reduction in risk for diabetes, and a 51% reduction in risk for metabolic syndrome.¹⁰¹ These data deserve cautious interpretation. First, the risk of bias associated with the source of these data (longitudinal cohort, case-control, and cross-sectional studies) limits inference regarding causal effects of vitamin D on cardiometabolic disorders. Second,

statistical heterogeneity was considerable. The percentage of variation in the risk estimates attributable to heterogeneity (I^2) was 76%. Extensive diversity across the pooled studies suggests that an average summary value may be misleading.¹⁰² However, the consistent direction of observed associations likely offsets the relevance of this high variability; in 85% of studies cardiometabolic risk was reduced in individuals with high compared with low levels of vitamin D. Furthermore, this pattern held across study design and cardiometabolic type.¹⁰¹ While these statistical associations are possibly a consequence of unmeasured (or poorly measured) confounding factors, these consistent findings provide a sound basis upon which to justify additional investigation, ideally in appropriately conducted RCTs, but also within more focused cohort studies where the nature of these associations may differ.

2.4.2. Summary of clinical trial evidence

Few clinical trials have been specifically designed to assess cardiovascular effects of vitamin D supplementation.

The largest RCT analysis to date, evaluated the effects of vitamin D₃ plus calcium supplementation on risk of cardiovascular events as a prespecified secondary outcome measure among 36,282 post-menopausal woman. This was a prespecified secondary outcome analysis of the Woman's Health Initiative (WHI) study.¹⁰³ Over a 7-year period, treatment with 400 IU/d vitamin D₃ plus 1000 mg/d calcium, compared with placebo, had no demonstrable impact on MI or coronary heart disease death (HR 1.04; 95% CI

0.92-1.18) or on incident cerebral-vascular disease (HR 0.95; 95% CI 0.82-1.10). The validity of these findings have been questioned on a number of grounds but mainly on the basis that the dose may have been too low to achieve a detectable clinical effect.^{5,104-106}

In a randomized placebo controlled fracture prevention trial comprised of 2686 older adults, the equivalent of 800 IU/d vitamin D₃ had no significant effect on incident CVD and CVD-mortality at five-years followup.¹⁰⁷ The relative risk estimates for incident CVD and CVD-mortality were 0.90 (95% CI 0.77-1.06, P = 0.22) and 0.84 (95% CI 0.65-1.10, P = 0.20), respectively.

2.4.3. Summary of evidence regarding atherogenic effects of suboptimal D status

Calcitriol possesses strong immunomodulating properties and may be a strong determinant of atherogenic inflammatory components including matrix metalloproteinase 9 (MMP-9), interleukin-6 (IL-6), TNF α , and markers of inflammation such as C-reactive protein (CRP).^{108,109} In a study of 139 men and woman with congestive heart failure (CHF), those randomized to receive daily 2000 IU of vitamin D₃ compared with placebo, demonstrated an improved inflammatory milieu characterized by increased levels of the anti-inflammatory cytokine interleukin-10 (IL-10) and decreased PTH.¹¹⁰ After 9 months of treatment TNF α had increased in the untreated group, but had stabilized in the group allocated to vitamin D supplementation.

In contrast, among 47 healthy post-menopausal woman, a 12-week daily course of 800 IU vitamin D₃ and 1000 mg calcium had no impact on circulating inflammatory markers that included TNF α , IL-6, and CRP.¹⁰⁹

There is conflicting evidence concerning the relationship between vitamin D status and vascular disease. In a study involving 390 individuals with type II diabetes, low vitamin D status was independently associated with carotid IMT ($P < 0.001$) after adjustment for multiple confounding factors including traditional risk factors, kidney function, and metabolic syndrome.³⁵ In contrast, carotid IMT was not associated with vitamin D status in a cohort of relatively healthy Old Order Amish adults.⁹⁸ Similarly, there was no association between 25(OH)D status and common carotid IMT in a group of 614 older adults from the Hoorn Study cohort.⁹⁹ In this same cohort low vitamin D status was significantly associated with risk of all-cause and CVD mortality.¹¹¹ Thus, it is conceivable that CVD clinical outcomes are not mediated by factors that produce detectable vascular disease and if this were so, carotid IMT would not be an appropriate measure of a vitamin D-CVD association.

In a study involving 654 older adults from the Rancho Bernardo Study cohort selected on the basis of a negative history of coronary heart disease, revascularization, or stroke, 25(OH)D was independently associated with internal carotid IMT ($P = 0.02$), but not with common carotid IMT ($P = 0.83$).¹⁰⁰ These findings are difficult to interpret. The clinical utility of isolated IMT assessment at the internal carotid artery has not been valid-

ated and for the purpose of CVD risk assessment, the common carotid artery is the preferred segment for measurement.¹¹² This in part is because common carotid IMT is more strongly associated with main CVD risk factors.¹¹³ While it is possible that segment specific differences in the associations between carotid IMT and CVD might translate into differences in clinical outcomes, this has not been confirmed by longitudinal investigation.¹¹³ However, the internal carotid IMT, compared with thickening at the common carotid segment, may be more sensitive to change, and Grunfeld et al.¹¹⁴ have proposed that in the atherosclerosis associated with HIV infection, IMT thickening may occur earliest at the internal carotid location.

Improved vascular function was reported in a small pilot study that randomized 48 vitamin D deficient (<50 nmol/L) individuals with type 2 diabetes mellitus to a single oral dose of 100,000 IU vitamin D₂, or placebo.¹¹⁵ After adjustment for a treatment associated reduction in systolic blood pressure, flow mediated vasodilation (FMD) of the brachial artery was 3.01% (95% CI 0.28-5.75, P = 0.03) higher in vitamin D-treated participants compared with participants randomized to placebo. These findings deserve cautious interpretation given that the analysis was restricted to 34 patients who completed the trial; an intention to treat analysis was not reported. Moreover, the validity of brachial FMD as a measure of CVD risk has recently been questioned.¹¹⁶

In summary, findings concerning possible vascular effects of suboptimal vitamin D status have not been consistent. Carotid IMT may not be the best measure of vitamin D

associated vascular disease. Similarly, a vitamin D-carotid IMT association may be more difficult to detect in the absence of substantial vascular inflammation or it may require a more sensitive measure of IMT progression such as prospective assessment at the internal carotid artery.

2.4.4. Vitamin D status, blood pressure, and hypertension

A number of studies have demonstrated statistical associations between arterial blood pressure and 25(OH)D levels. Piltz et al. provide a comprehensive systematic review on this topic.⁵ The majority of the larger population-based cross-sectional investigations have demonstrated inverse associations between blood pressure and 25(OH)D.

In a nested case-control study (within the Nurse's Health Study 2) women in the lowest compared with the highest quartile of plasma 25(OH)D levels had an adjusted odds ratio of incident hypertension of 1.66 (95% CI 1.10-1.97).¹¹⁷

In longitudinal analysis, Forman et al. utilized *estimated* 25(OH)D levels to investigate associated risks of incident hypertension among 77,531 women in the Nurse's Health Study and 38,388 men in the Health Professionals' Follow-Up Study.³⁶ Relative risks comparing lowest to highest estimated 25(OH)D deciles, with adjustment for age, body mass index (BMI), physical activity, race and (in women) menopausal status, were 1.57 (95% CI 1.44-1.72) in women and 2.31 (95% CI 2.03 to 2.63) in men, after 18 and 16 years follow-up, respectively. In analyses restricted to the 613 men and 1198 women with measured 25(OH)D levels, relative risks for participants whose levels were < 37

nmol/L compared to individuals with levels ≥ 75 nmol/L were 6.13 (95% CI 1.00-37.8) in men and 2.67 (95% CI 1.05-6.79), during 4 years follow-up. Pooled analysis of the men and woman with measured 25(OH)D yielded a relative risk of 3.18 (95% CI 1.39-7.29)

In contrast to these positive findings, in a study involving 524 men and woman randomly selected from the longitudinal Ely study, baseline 25(OH)D levels correlated with baseline blood pressure, but not with changes in blood pressure over 10 years followup.¹¹⁸

It has been suggested that the observed geographic variation in the prevalence of hypertension may in part be explained by differences in ambient UVB-exposure.¹¹⁹ This hypothesis was tested in a small RCT involving 18 individuals with mild hypertension.¹²⁰ Six weeks of thrice-weekly UVB exposure lowered systolic and diastolic blood pressure by -6 mm Hg (-14 to -1, $P < .001$) and 6 mm Hg (-12 to -2, $P < .001$), respectively. In contrast, UVA exposure, as a control, had no effect on blood pressure.

Vitamin D supplementation reduced blood pressure in some but not all of the RCTs reviewed by Piltz et al.⁵ Supplementation over an 8 week period with 800 IU/d of vitamin D₃ plus 1200 mg calcium, compared with calcium alone, reduced systolic blood pressure by 9.3% ($P = 0.02$) in a study population of 148 vitamin D deficient elderly woman.¹²¹ Likewise in a small study of involving 34 vitamin D deficient individuals ran-

domized to receive a single dose of 100,000 IU vitamin D₃ or placebo, follow-up systolic blood pressure at 8 weeks was 14 mmHg lower in the group allocated to supplementation (P = 0.001).¹¹⁵

In an RCT involving 189 elderly men and woman a single dose of 100,000 vitamin D₃ had no effect on blood pressure at 5 weeks follow-up.¹²² Likewise, in the Woman's Health Initiative study low dose vitamin D plus calcium had no impact on blood pressure and did not reduce the risk of incident hypertension over 7 years follow-up.¹²³ As described by Pilz et al.,⁵ these and the majority of other trials that failed to demonstrate antihypertensive effects of vitamin D supplementation were characterized by normotensive cohorts without prevalent vitamin D deficiency. Most were not designed with the primary intent of assessing the efficacy of vitamin D supplementation in blood-pressure lowering and may have been underpowered to detect effect differences.

A number of overlapping mechanisms may be at play in an antihypertensive effect of vitamin D. These would include direct effects of 1,25(OH)₂D on vascular smooth muscle cell proliferation,¹²⁴ renoprotective effects,¹²⁵ correction of elevated PTH,¹²⁶ modulation of systolic calcium concentrations,² suppression of the renin-angiotensin system (RAS),^{127,128} and improved insulin sensitivity.¹²⁹

Calcitriol and its analogues have been shown to modulate the RAS through suppression of renin biosynthesis, and 1,25(OH)₂D may serve as a counter-balance to prevent over-activation of the RAS.^{125,128} The main effector of the RAS, angiotensin II, is a potent

vasoactive peptide and induces increased blood pressure, but also contributes to vascular remodelling and endothelial dysfunction.¹³⁰ Notably, angiotensin II has a range of pro-inflammatory properties and is able to induce vascular injury independent of its vasoactive effects.¹³⁰ In this respect the RAS, under modulation by 1,25(OH)₂D, would serve as a mediating factor along a causal pathway between 1,25(OH)₂D status and vascular inflammation. Furthermore, some of these effects would circumvent renin effects attributable to increased blood pressure. By extension, not all vitamin D-renin modulated vascular effects would be attributable to corresponding increases in blood pressure.

2.4.5. Does hypertension modify the 25-hydroxyvitamin D-CVD association?

There is some evidence to suggest that hypertension may modify the vitamin D-CVD association.

In a prospective observational study of 1739 Framingham Offspring participants, low 25(OH)D status (< 37 nmol/L) was associated with a greater than twofold increased risk for incident cardiovascular events (HR 2.13, 95% CI 1.30-3.48) in participants with hypertension but not in those without hypertension (HR 1.04, 95% CI 0.55-1.96).⁸⁹ A formal test for interaction was of borderline significance (P = 0.08) suggesting that hypertension may modify the effect of 25(OH)D deficiency on incident cardiovascular disease. Validation of this interaction would require a larger sample size as studies are often underpowered to detect interaction.¹³¹

In the Rancho Bernardo Study cohort (described previously in section 2.2.3), 1,25(OH)₂D was only associated with carotid IMT in a subgroup analysis restricted to participants with hypertension.¹⁰⁰ A formal test for interaction was statistically significant ($P = 0.036$). It is not clear how comparable this interaction is to findings from the Framingham Offspring cohort since the interaction was found for 1,25(OH)₂D but not for 25(OH)D, and under normal conditions these measures are only weakly correlated. Additionally, as numerous subgroup analyses were performed the finding may have occurred due to chance¹⁰⁰

It is conceivable that hypertension magnifies the adverse effects of suboptimal vitamin D status. This would be consistent with a general trend in the literature whereby a vitamin D-CVD association is more often confirmed in cohorts with advanced compared with less advanced CVD risk. Likewise in a study comprised of adults with elevated CVD risk, serum 1,25(OH)₂D was more strongly associated with vascular calcification in participants at very high risk for coronary heart disease ($r = -0.57$) compared with adults with elevated but less severe risk ($r = -0.18$).⁹³

Similarly, the severity of vitamin D deficiency might also impact the strength of found associations. For instance, while the majority of large observational studies have demonstrated inverse associations between 25(OH)D levels and blood pressure, two not-

able exceptions, the Rancho Bernardo Study and the Longitudinal Aging Study Amsterdam, were comprised of cohorts with notably high baseline 25(OH)D levels.^{5,100,132} In the Rancho Bernardo Study 86% of participants had 25(OH)D levels exceeding 75 nmol/L.¹⁰⁰

2.4.6. Vitamin D status and cardiovascular disease in HIV-positive adults

As I have previously described suboptimal vitamin D status is highly prevalent in HIV-positive populations, and while there is only limited evidence to suggest that prevalence of 25(OH)D differs by HIV-status, HIV-positive individuals may be at increased risk of disrupted vitamin D metabolism. Correction of suboptimal vitamin D status in this population may be clinically important for prevention of CVD. However, causality has yet to be confirmed in adequately designed RCTs. Furthermore, I identified just one study that assessed cardiovascular implications of vitamin D in an HIV-positive cohort. In this uncontrolled pilot study, 20 vitamin D deficient HIV-infected adults were provided 2000 IU/d vitamin D₃ for 14 weeks at which point the daily dose was halved for an additional 34 weeks supplementation.¹³³ Compared with baseline measures, triglycerides, cholesterol, adiponectin, leptin, IL-6 and TNF α were not significantly different after 24 or 48 weeks of supplementation. Surprisingly, HOMA index (an estimate of insulin sensitivity) and fasting glucose levels were significantly higher at 24 weeks of supplementation, but these returned to baseline values by 48 weeks. Vitamin D₃ exhibited dose-response relationships with 1,25(OH)₂D and PTH levels in that measures were improved with 2000 IU, but not with 1000 IU, of daily vitamin D₃.

In summary, the evidence regarding health effects of vitamin D status and vitamin D repletion in HIV-positive adults is primarily derived from small exploratory studies and cross-sectional analyses at high risk of bias. Some of this evidence is consistent with the proposition that an HIV-positive status may increase an individual's susceptibility to disrupted vitamin D metabolism. Based on research conducted in HIV-negative cohorts, there is sufficient evidence upon which to justify the development of large RCTs and prospective observational studies to evaluate CVD implications of suboptimal vitamin D status. These study protocols might be improved by selecting a study population on the basis of elevated CVD-risk. Furthermore, the power to detect group differences may be improved when cohorts have representation along the spectrum of vitamin D status. Participants in the Canadian HIV Vascular Study should meet both of these criteria. Their HIV-status is associated with increased CVD risk, and as Canadians they may be at high risk for suboptimal vitamin D status.

The data concerning vascular effects of suboptimal vitamin D status, including its impact on carotid IMT, have been inconsistent; in HIV-positive adults the evidence is sparse. This thesis will attempt to contribute to a better understanding of vitamin D status and about its relationship with the extent of vascular disease in Canadian HIV-positive adults.

Chapter 3

Testing the hypotheses: methodological considerations and the study protocol

3.1. Study hypotheses and objectives

I hypothesize that there will be greater extent of vascular disease, determined by baseline carotid IMT, among HIV-positive adults with lower compared with higher plasma levels of 25(OH)D. Furthermore, I hypothesize that a primary mechanism underlying this association is metabolic perturbation of the renin-angiotensin system (RAS) secondary to reduced 25(OH)D supply. Consequent activation of the RAS leads to increased carotid IMT via direct and indirect effects involving inflammatory mediated vascular injury and remodelling. Part of this effect will be mediated through arterial blood pressure effects on the arterial wall, and as such I hypothesize that adjustment on systolic blood pressure (as a mediator) in the analysis will attenuate the true carotid IMT-25(OH)D association.

Given the seasonal variability of vitamin D supply, I hypothesize that suboptimal vitamin D status will be particularly prevalent through the ‘winter-half’ of the year (November through April) when solar induced synthesis is limited.

Additionally, vitamin D quartiles will differ by age, ethnicity, BMI, systolic blood pressure, leisure activity (sweating), insulin resistance (HOMA-IR), NNRTI use, and

plasma PTH. Finally, I hypothesize that the carotid IMT-25(OH)D association will be curvilinear and that flexion points from linearity will resemble cutpoints commonly utilized to categorize vitamin D status.

My primary research objective is to test whether there is an association between baseline extent of carotid IMT and vitamin D status in a large cohort of HIV-positive adults living at a latitude conducive to prevalent suboptimal vitamin D status.

Secondary objectives are as follows:

1. To characterize the shape of the relationship between plasma 25(OH)D and carotid IMT in this Canadian HIV-positive population, and
2. To examine the effect of 25(OH)D status categorization (as per the 25(OH)D cutpoints in common use) on the strength of a carotid IMT-25(OH)D relationship, and
3. To determine the prevalence of vitamin D insufficiency and deficiency, and to describe cohort specific characteristics of vitamin D status, within the Canadian HIV Vascular Study.

3.2. Rationale for the study

The prevalence of suboptimal vitamin D status in Canada is high. There is abundant, but somewhat conflicting, epidemiological evidence that implicates vitamin D as an associated feature across a wide range of conditions including osteoporosis, cancer, infection, diabetes mellitus, multiple sclerosis, arterial hypertension, and CVD. There is evidence to suggest that vitamin D supplementation may benefit cancer prevention,¹³⁴ bone

density,^{135,136} risk of falls,^{137,138} risk of fractures,¹³⁹⁻¹⁴² tuberculosis infection,⁸⁶ hypertension,^{5,121} and diabetes.¹ Additionally, a meta-analysis of 18 randomized trials found reduced mortality among participants randomized to vitamin D supplementation.⁸⁵

As described previously in Chapter Two a number of factors related to HIV infection may disrupt vitamin D metabolism. These include: immunodeficiency and the use of certain medications. HIV infection may predispose individuals to disrupted calcium homeostasis presenting as reduced or elevated PTH, reduced 1,25(OH)₂D, or hypocalcaemia. Likewise, HIV-positive individuals may be at risk of decreased bone mineral density, osteoporosis, and fracture.^{56,143,144} In a meta-analysis of 12 cross-sectional studies 67% of 884 HIV-positive individuals had reduced bone mineral density (BMD), of whom 15% were osteoporotic.¹⁴³ Compared with HIV-negative controls, HIV-positive individuals were sixfold and threefold more likely to experience reduced bone density and osteoporosis, respectively. While these data derived from cross-sectional analyses need to be interpreted with caution, an HIV infection-bone pathology association even if non-causal – for instance, instead explained by increased baseline risk within a population more likely to develop HIV infection – presumably elevates the importance of vitamin D status in persons living with HIV infection.

Likewise, HIV-positive adults appear to be at increased risk of CVD.¹⁻⁵ A number of factors are likely at play.⁴² HIV-positive groups have elevated levels of cardiovascular risk factors some of which may be unrelated to HIV infection or its management (e.g., a

higher baseline prevalence of smoking or saturated fat intake). Inflammatory effects of ongoing HIV-1 viremia may accelerate atherogenic mechanisms and may in part be responsible for the low high-density lipoprotein (HDL) cholesterol levels often seen in HIV infection. Finally, ART exposure can elevate traditional CVD risk factors including low-density lipoprotein (LDL) cholesterol and triglycerides. Given putative biological and epidemiological evidence it is plausible that any one of these CVD-relevant features of an HIV-infected population may be influenced by vitamin D status and hence, vitamin D status may be a modifiable CVD risk factor in HIV-positive adults.

While, vitamin D repletion among HIV-negative persons is considered feasible, safe, and attainable,¹³ efficacy related to the prevention of CVD has not been established in an HIV-positive population (nor, for that matter, has its efficacy been established for any other health outcome in HIV-infection). While correction of vitamin D deficiency makes intuitive sense given the vitamin's putative pleiotrophic role, a number of important questions remain unanswered. These would include questions about appropriate 25(OH)D repletion targets, vitamin D dosage, potential harms, and populations most likely to benefit. Furthermore, health effects may differ in clinically important ways across HIV-positive and HIV-negative populations. For instance, while HIV-positive populations appear to be at increased risk for a number of conditions with some relevance to vitamin D status, unique features of HIV infection, its management, and the population at risk, introduce additional complexity into the causal milieu in which vitamin D nutrition may rep-

resent just one causal component of a multifactorial disease process. Thus, questions concerning the clinical relevance of vitamin D status in HIV-positive adults, may require investigation directly within HIV-positive cohorts. In other words, the specificities of an HIV-infected cohort may limit the validity of inferences drawn from HIV-negative study populations. The specifics of how vitamin D repletion might best be utilized in HIV-positive adults to reduce risk of vitamin D related disease can only be determined through the development of well-conducted clinical trials. The main rationale for this current investigation is the need to generate within an HIV-positive cohort prerequisite background data for refinement of hypotheses concerning vitamin D status and cardiovascular disease that can in future be subjected to longitudinal study.

3.3. Methodological considerations

In this section I discuss important epidemiological concepts as they pertain to development of the current investigation. The intent of this section is to provide a rationale for the cross-sectional design, to fully explicate important limitations of the proposed study, and to present strategies intended to minimize risk for erred inference from the study findings.

As a primary end most epidemiological investigations seek to identify and accurately measure causal associations between health-relevant phenomena as they actually exist in the world. To be sure, associations are at best approximated; individual associations

exist within a complex causal network that is never completely understood and measurement usually involves some level of error or deviation from what would otherwise have been the true value for each parameter of interest.

In designing the current study, my over-riding aim was to minimize, within the practical context of the study environment, those sources of measurement error that can have undue influence on the validity of inferences from study observations. Two types of error need to be addressed. These are, *random error*, for instance when measurements lack precision; and *systematic error*, as when some non-random influence affects the capacity for valid inference. In the sections that follow, these various measurement issues are addressed in the context of the current study. First, I discuss strengths and weaknesses of the cross-sectional design architecture. This is followed by discussion of threats to validity which are categorized under the broad headings of *selection bias*, *information bias*, and *confounding bias*.

3.3.1. Strengths and weaknesses of a cross-sectional design

The relative *strength* of a particular study design, and often its cost, corresponds to its capacity to address measurement error, and in most situations this corresponds to its capacity to yield strong evidence for inference of causality. In contrast, the relative *merit* of a study design corresponds to how the particular study question can best be answered in the context of various cost-to-benefit considerations. For instance, *ideally* etiological

research should always be longitudinal.¹⁴⁵ However, in lieu of preliminary data to justify the time and expense of deriving more costly prospective observational data the cost-benefit assessment often favours efficiency over absolute costs to validity.

In the paragraphs that follow, I discuss major strengths and weaknesses of the current cross-sectional investigation.

A cross-sectional design has several major strengths. First and foremost, it is efficient and feasible. In the context of the Canadian HIV Vascular Study it provides a cost-effective means of rapidly testing preliminary hypotheses. Study questions can then be sorted and refined as necessary so that only the most promising hypotheses are extended for testing within the much stronger, but less efficient, longitudinal component of the Canadian HIV Vascular Study.

Second, in chronic conditions, as in atherosclerosis, that develop only after many years of exposure, a cross-sectional analysis may provide a more accurate measure of association compared to a longitudinal study where follow-up might be insufficient for detection of disease progression. In this respect the cross-sectional design shares some similarity with a case-control study since ascertainment of those participants with the outcome (the cases), and controls (the remainder of the population studied), is not dependant on the development of disease over a prospective period of time.¹⁴⁵

Third, the cross-sectional design is not typically dependant on the ascertainment of patient follow-up data. This is a relative strength compared to longitudinal investiga-

tions where some level of loss-to-follow can be expected. Loss-to-follow-up can present as a special form of selection bias should the loss occur differentially by exposure status.

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Fourth, the cross-sectional design is well suited for addressing descriptive questions about prevalence.¹⁴⁷ The current study will provide rapid and efficient data regarding characteristics of vitamin D insufficiency/deficiency in the Canadian HIV Vascular Study cohort.

The cross-sectional study design has a number of major weaknesses. First, because all variables are measured simultaneously, observed associations lack intuitive directionality; thus, causal inference cannot be corroborated by a temporality of events. In this respect, valid inference from the observed associations should allow for *at least* two contrasting interpretations: as hypothesized, vitamin D insufficiency/deficiency may be a component cause of vascular disease; alternatively, the presence of vascular disease may predate development of suboptimal vitamin D status. This latter interpretation would be consistent with the scenario of reduced sun exposure secondary to CVD-associated illness. Lack of temporality also limits the strength of assumptions upon which study analyses are based. Without a firm reference point upon which to hang directionality, important assumptions required for adequate statistical adjustment become ever the more

complex and much less certain. As I discuss in section 3.3.4, erred assumptions about the causal relations between study variables could lead to the introduction of bias during the analysis stage of the study.

Second, the cross-sectional design is not ideal for analysis of exposures that change over time.¹⁴⁵ In the current study, I utilize plasma 25(OH)D as a proxy for the historical 25(OH)D mean. My explicit assumption is that baseline 25(OH)D will reflect a historical exposure period of sufficient duration to capture a meaningful disease induction period – where the causal mechanism is complete and thus sufficient to cause disease¹⁴⁸ – plus the time required for vitamin D related vascular change to become detectable. This supposition has support in that 25(OH)D has been shown to longitudinally predict incident cardiovascular disease.^{32,89,92} However, some factors deserve consideration as possible threats to the validity of this assumption. These would include the influence of historical changes in prevailing attitudes about sun exposure or about the presumed importance of vitamin D nutrition. Additionally, the effect of vitamin D status on CVD risk may not entirely follow a typical dose-response relationship. For instance, as proposed by Vieth (2009),¹⁴⁹ risk associated with vitamin D status may in part be explained by the extent of 25(OH)D fluctuation across seasons. Ascertainment of this component of risk would require serial 25(OH)D assessment across seasons.

Given these important limitations, valid inference from this cross-sectional study about a causal association between vitamin D status and vascular disease must incorporate a large degree of uncertainty. Stronger inference would require longitudinal assessment of vitamin D status prior to carotid IMT assessment.

In summary, the cross sectional design is particularly suited for the proposed descriptive prevalence component of the study, it is efficient and cost effective, and is not prone to bias associated with loss-to-follow-up. There are a number of notable validity trade-offs inherent in the cross-sectional structure. Foremost, the design lacks a temporal structure upon which to assess the directionality of effects. Additionally, cross-sectional investigations are highly vulnerable to a number of validity threats including the three main forms of study bias: selection bias, information bias, and confounding bias. These are discussed in the sections that follow.

3.3.2. Selection bias

Selection bias can occur whenever the study population differs in some systematic (non-random) way from the defined population of interest.¹⁵⁰ All study types, but particularly observational investigations, are susceptible to this bias. In this current study selection bias can be partially explicated and minimized; however, some uncertainty regarding the magnitude of its effect on study observations will inevitably remain. Thus, selection bias represents a built-in validity threat; and an important limit on inference.

The target population in the Canadian HIV Vascular study includes all HIV-positive adults, aged 35 years and older, receiving care at one of five participating university associated HIV-clinics in Canada. In the ideal scenario, all eligible candidates would have had an equal opportunity for participation. The selected cohort would thus represent a random selection from the eligible study population. Unfortunately, bias at this recruitment stage is difficult to avoid; selection entirely at random precludes the fact that some factors related to the study variables – this is an essential criteria for selection bias – are also likely relevant to decisions that influence participation. As a consequence there may be decreased comparability between the sample available for analysis and the population that the study is designed to assess.

In the paragraphs that follow, I identify five main factors that may bias selection of participants or the availability of their data for this cross-sectional analysis.

First, patients may differ according to their altruistic belief in the value of participation (volunteer bias). If these beliefs are associated with behaviours that affect study variables this may bias a vitamin D-carotid IMT association. An important example of this particular problem is the finding that the propensity for participation may differ by smoking status. A selection bias will arise if those smokers who participate differ in some important way from those smokers who do not participate.

Second, the severity of an eligible patient's illness may affect the capacity for completion of questionnaires or of other baseline measures including scheduled ultra-

sonography. For instance, the presence of severe pain, chronic diarrhoea, or severe nausea, might reduce the capacity to comply with scheduled visits and thus decrease the probability of participation (healthy-volunteer bias). Likewise, it is foreseeable that study personnel might be more likely to approach healthy, seemingly more agreeable, patients than those displaying physical discomfort or a negative affect. In contrast, patients requiring close supervision, and accordingly more frequent clinical visits, would have increased opportunity for participation. Thus, unless accrual is entirely sequential the opportunity for participation may differ by the severity or presentation of illness.

Third, demographic factors may bias participation. For instance, the recent immigrant status of a disproportionate amount of woman in the target population may be associated with a number of foreseeable barriers to participation including those involving transportation, child care, or translation services for provision of informed consent. Given this scenario, the accrual of woman would be biased towards the selection of woman in a non-immigrant strata or may result in an over-representation of a characteristic associated with an immigrant woman's decision to participate.

Fourth, selection bias can occur at all stages of study design and implementation including as a consequence of an incomplete dataset. For instance, in the current study the dataset available for analysis may not accurately represent the dataset that otherwise would have been available if all important parameters were completely captured from questionnaires, chart reviews, specimen assays and imaging studies. Selection bias occurs

if the data loss is systematic as a consequence of some factor associated with vitamin D status and carotid IMT. For instance, missing data may differ by the thoroughness of a practitioner's charting style, the complexity of the patient's case, or by the capacity of the patient to comprehend instructions and thus provide a complete medical history. A particular advantage of the cross-sectional design compared with a longitudinal study is that cross-sectional analyses are less prone to bias that occurs as a consequence of differential loss to follow up. However, a similar missingness issue exists in this current study given that a full baseline dataset is dependent on completion of a necessary sequence of steps, any one of which represents a barrier to complete measurement. For instance, study personnel have limited control over potential barriers to blood collection (e.g., a busy phlebotomy office) or to carotid ultrasound imaging (e.g., scheduling conflicts and attendance). Bias is introduced if factors associated with any of these barriers and with vitamin D status and carotid IMT affect in some non-random way what participant data enters the final dataset.

Finally, statistical conditioning on a variable to control for confounding can introduce a collider stratification bias which, as Hernán et al. have argued, shares a structural commonality with other selection biases.¹⁴⁶ When the variable undergoing adjustment is a common effect of exposure and outcome, or is an effect of a cause of exposure or outcome, statistical adjustment or stratification can result in a spurious statistical association

occurring within strata of the adjusted variable. Collider-stratification bias is represented structurally in Figure 1; and again in Figure 2 where in this situation the collider is a common effect of exposure and outcome.

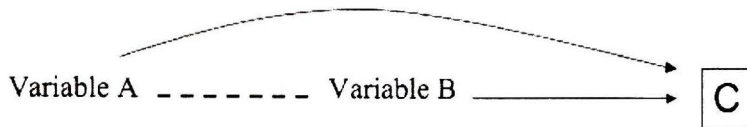


Figure 1. In this graphical representation of *collider stratification bias* adjustment on a collider variable (C) results in a conditional association, represented by the dashed line (---), between two of its causes (variables A and B).

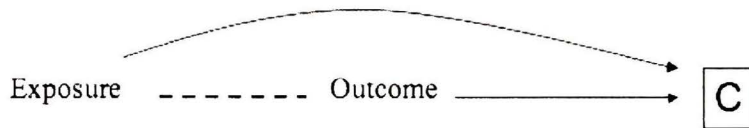


Figure 2. Conditioning on a common effect C generates a conditional association (represented by the dashed line) between exposure and outcome within strata of C.

Here the square around the covariate, represented by C, signifies conditioning on a particular value of C. In this example, even if vitamin D and carotid IMT are not truly associated, a conditional association will occur within strata of the common effect C. A more complex model of selection bias (Figure 3) demonstrates that the common effect can be of variables statistically associated with vitamin D or carotid IMT.

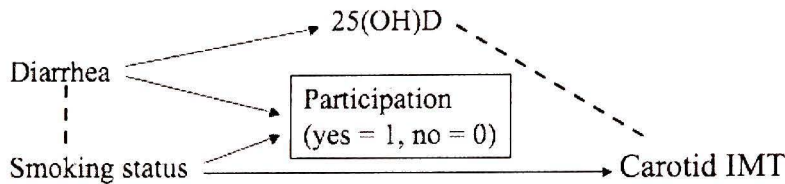


Figure 3. A structural illustration of selection bias

In the illustrated scenario, a conditional association between 25(OH)D and carotid IMT is generated because participant selection is biased by factors associated with exposure and outcome and thus, the cohort is not a random sample of the eligible population. Instead the presence of severe diarrhea (a plausible barrier to participation) and the occurrence of smoking (smokers are typically underrepresented in study cohorts) affect participation. If an eligible participant with severe diarrhea (and therefore at a greater risk of non-participation) did in fact not participate because of the diarrhea, then it is less likely that the second cause of non-participation (smoking status) was present. Thus, diarrhea and smoking status are conditionally associated within strata of participation. Likewise, a statistical association between 25(OH)D and carotid IMT can be detected given the backdoor path from 25(OH)D through diarrhea and smoking status, to carotid IMT.

In this scenario the effect of conditioning on the collider *participation* generates a spurious statistical association between vitamin D status and carotid IMT. Alternatively, the collider could be a covariate under adjustment for confounding. In effect, confounding is addressed at the expense of introducing a "back-door"¹⁵¹ association between vitamin D status and carotid IMT. This is described in greater detail in section 3.3.5 where I use directed acyclic graphs (DAGs) to present a structural representation of the presumed causal associations between the study variables. The presented DAGs are then utilized with the intent of facilitating identification of a best set of covariates that will efficiently address confounding and the negative consequences of conditioning on a collider variable.

3.3.3. Information bias

The validity of findings in a study is dependent on accurate assessment of measured parameters. Information bias occurs when measurement error is non-random. It is particularly problematic if the extent of error is sufficient to produce misclassification of those study variables that have important influence in the analysis. Several strengths in this current study should help minimize its influence.

First, due to the cross-sectional design neither vitamin D status nor vascular disease are likely to influence the manner in which data is obtained by study personnel.

Second, measurement techniques and procedures for the determination of exposure and outcome have been well standardized and validated and are performed under competent supervision. I expect instrumentation to be well-calibrated. Carotid ultrasound has been measured and analyzed according to methods validated and used in a number of large investigations including SECURE,¹⁵² SHARE,¹⁵³ HART,¹⁵⁴ and STARR.¹⁵⁵ Carotid artery high-resolution ultrasound to measure carotid IMT is a well-validated method for assessing the extent of vascular disease. Although measurement of 25(OH)D involves some imprecision,¹⁵⁶ the error is expected to be mainly non-differential:¹⁵⁷ the direction of error is not expected to differ in some systematic way between patients with or without greater extent of carotid IMT. Measurement of 25(OH)D has been performed in a research-based laboratory that conforms to the Vitamin D External Quality Assessment Scheme (DEQAS).

One potential source of information bias is that the Elecsys 25(OH)D assay measures 25(OH)D₃, but not 25(OH)D₂. A high dietary intake of vitamin D₂ could lead to misclassification of 25(OH)D status. However, in Canada vitamin D₂ supplements are not commonly available. Likewise, food sources are mainly limited to fortified milk-alternatives such as soy or rice milk. In the unlikely event of substantial vitamin D₂ intake, misclassification would likely bias a vitamin D-carotid IMT association towards the null. The most problematic sources of information bias in this current study are discussed below in the context of bias associated with mismeasurement of confounders.

3.3.4. Confounding bias

The greatest validity threat in non-experimental research is confounding bias. In this current study, as in all observational investigations, statistical control of confounding will to some extent be limited by measurement error, and it is not possible to completely account for all known and unknown confounding factors. Additionally, uncertainty about causal associations between study variables can lead to errors in confounder identification and statistical control. For example, a variable identified for adjustment might instead mediate rather than confound a 25(OH)D-carotid IMT association. Another problem, as I have described in section 3.3.5, would be the introduction of a collider stratification bias by conditioning on a common effect of exposure and outcome.

The problem of confounding bias in this current study is discussed in two parts. First, I attempt to explicate the process of covariate selection by means of graphical rep-

resentation of the analysis model in the form of directed acyclic graphs (DAGs). Second, I describe main sources of measurement error that may limit the capacity for statistical control in the analysis.

3.3.5. Graphical representation of the causal network

Often, a variable is labelled as a confounder on the basis of some form of statistical criteria. Use of this approach without reference to subject matter may lead to invalid conclusions.¹⁵⁸ A number of authors have argued that covariate selection should be based on a more explicit and conscious variable screening process in order to prevent unintended consequences of inappropriate statistical adjustment.^{151,158,159 160,161} Towards this end, I present direct acyclic graphs (DAGs) that explicate the assumptions upon which my analysis is based, and apply a systematic method presented by Greenland et al.¹⁵¹ and others,¹⁶¹ for evaluating the potential negative implications of statistical control.

As has been described in detail by Judea Pearl, DAGs provide a basis for "identifying and explicating the assumptions needed for substantiating causal claims".¹⁶² The framework described by Pearl allows problems to be framed in the way that the investigator perceives nature to work,¹⁶³ and in a format whereby necessary causal assumptions can be explicated, manipulated, and presented graphically. Moreover, in application to epidemiology DAGS provide a framework for describing the structural basis of confounding and other key epidemiological concepts.

A few comments on DAGS are in order. DAGS link variables (nodes) with directed lines (edges) whereby the line represents a direct causal effect, and the direction, represented by an arrow, encodes a temporal sequence between the first variable (the parent) and the second variable (the child). This temporal feature specifies that the graphs are "acyclic" since a variable cannot cycle back in time and cause itself. The absence of a directed line between any two variables indicates that the investigator is able to assume the absence of a direct effect of one variable on the other.

Causal DAGs can frame true causal associations but they also encode the causal determinants of statistical associations, and thus reflect the component contributions to the association that, A) are a consequence of the causal effect of exposure on outcome or, B) are the consequence of a summation of spurious, non-causal, "back-door" paths between exposure and outcome. In other words, they provide information relevant to the detection of spurious and causal associations. Causal DAGs provide information about the effect of variable adjustment on other variables within the causal model. For instance, the DAG encodes potential negative validity consequences of conditioning on a particular variable, and elucidates optional variable subsets for sufficient or best-case adjustment.

The primary hypothesis of the current study can be represented by the partial DAG illustrated in Figure 4.

Suboptimal 25(OH)D Status —————→ Carotid IMT

Figure 4. Partial DAG representing the primary hypothesis

The above DAG satisfies the three necessary criteria of causation as defined by Pearl.¹⁶⁴ First, the cause (suboptimal vitamin D status) and effect (increased carotid IMT) are correlated. Second, the cause proceeds the effect. Third, the association is non-spurious, that is, it is not explained by any third variable. By definition the DAG represented in Figure 4 is incomplete given that a causal DAG must also reflect all common causes of any pair of variables.¹⁴⁶ A more complete scenario is represented in Figure 5, whereby U is a common cause of both suboptimal vitamin D status and increased carotid IMT.

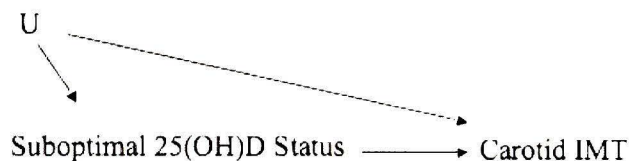


Figure 5. Partial DAG representing the primary hypothesis. U represents common causes of exposure and outcome

My qualitative a priori assumptions about the underlying causal structure of the current study are encoded in the causal DAG given in Figure 6.

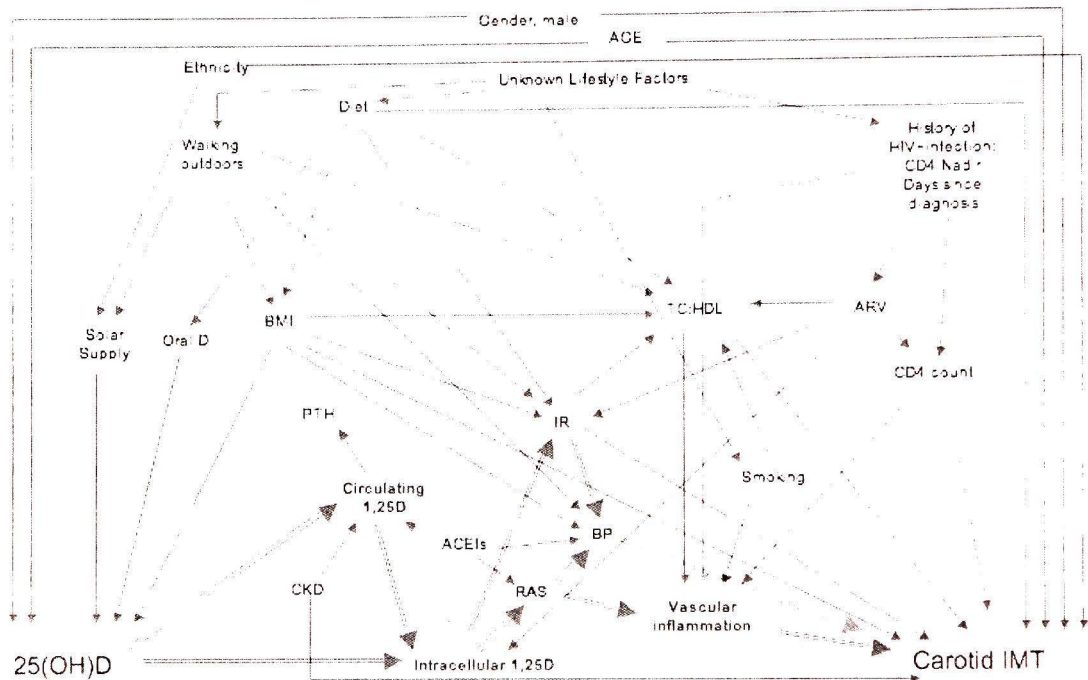


Figure 6. Causal assumptions of the analysis model.

The causal pathway between 25(OH)D and carotid IMT is represented by the double lines ($\Rightarrow$). Mediating variables are bolded. Note, insulin resistance (IR) and blood pressure (BP) are assumed to both mediate and confound the 25(OH)D-carotid IMT association. Also, the causal effects of age, ethnicity, and gender have been minimized in the interest of avoiding an unwieldy DAG. 1,25D indicates 1,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; CKD, chronic kidney disease; ACEIs, angiotensin-converting enzyme inhibitors; ARV, antiretroviral drug therapy regimen; BP, blood pressure; BMI, body mass index; IR, insulin resistance; PTH, parathyroid hormone; RAS, renin-angiotensin system; TC:HDL, total cholesterol to HDL cholesterol ratio.

An important criterium of a confounder is that it is not on a causal pathway between exposure and outcome (note that here I am referring specifically to the confounding component of a variable since it is possible for the same variable to both mediate and confound an association). Unless the causal network has been completely and accurately defined – a rare situation in epidemiological research – it can be difficult to know with certainty that a covariate does not mediate a causal association. As described

in Figure 6 I expect, based on inference from clinical trial data in HIV-negative populations, that insulin resistance (IR) and elevated blood pressure may each mediate some component of the causal path between plasma 25(OH)D concentration and carotid IMT. If my assumptions are correct, adjustment on these variables would attenuate or eliminate the hypothesized association.

This problem, an issue of overadjustment,¹⁶⁵ could lead to an erroneous conclusion that vitamin D status is not causally important. Alternatively, should either IR or blood pressure have both confounding and mediating roles, failure to account for confounding in the analysis would bias the effect measure presumably towards overestimation of the true causal association between plasma 25(OH)D and carotid IMT.

In acknowledgement of this potential problem and based on the causal assumptions described by the DAG in Figure 6, I conduct the primary analysis without adjustment on either of these two variables, but instead adjust for BMI, total cholesterol to HDL cholesterol ratio (TC:HDL), age (causal relation not represented in Figure 6), and ART, in an attempt to close backdoor pathways mediated by insulin resistance or blood pressure. Unfortunately, some important variables remain unmeasured. The potential influence of dietary factors that affect insulin sensitivity and blood pressure may to some extent remain and confound the 25(OH)D-carotid IMT association.

The impact of this decision to not adjust for insulin resistance and blood pressure on the basis of their presumed mediating roles will be assessed by way of sensitivity ana-

lysis whereby they will individually be inserted into the analysis for evaluation of influence on the effect measure. Specific methods to account for overlap between a variable's mediating and confounding effects have been proposed¹⁶⁵, but are beyond the scope of this current investigation.

As I have previously discussed, statistical methods for adjustment of confounding are fallible. Covariate selection on the basis of statistical criteria – for instance, where variable selection is automatic according to stepwise selection – can lead to erroneous conclusions. For the purpose of generating qualitative guidance with respect to the covariate selection process I utilize a graphical algorithm presented by Greenland et al.¹⁵¹ and others.¹⁶¹ The procedure provides a mechanism for examining the sufficiency of a given set of variables for closure of pathways that confound the true causal association between exposure and outcome. A particular advantage of the procedure is that it provides graphical representation of pathways generated by the analysis itself. That is, it illustrates the problem of collider-stratification bias whereby adjustment of a *collider* variable (*the child*), creates a conditional association between two or more of its causes (*the parents*). The algorithm provides a means of assessing whether these induced associations may require inclusion of additional covariates in the analysis.

As previously stated, the causal assumptions upon which my primary analysis is based are illustrated by the causal DAG in Figure 6.

Figure 7 represents the first step in the procedure: all exposure effects are removed, thus producing a DAG representing the null-hypothesis of no exposure effects. The remaining lines (regardless of the direction of arrows) represent *backdoor paths* mediated by nondescendants of 25(OH)D. Each path represents the underlying structure of a possible spurious, non-casual statistical-association between exposure and outcome. The goal of statistical control is closure of all paths by conditioning on a minimally sufficient subset of covariates.

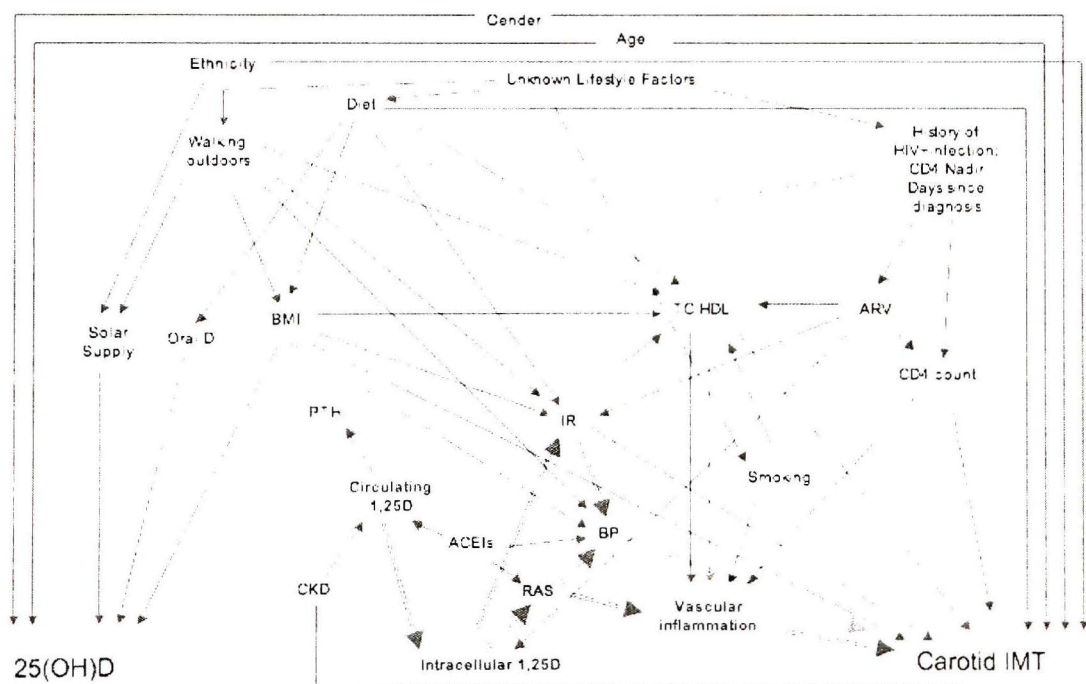


Figure 7. Step one completed; thus, providing a DAG representing the null hypothesis of no exposure effects.

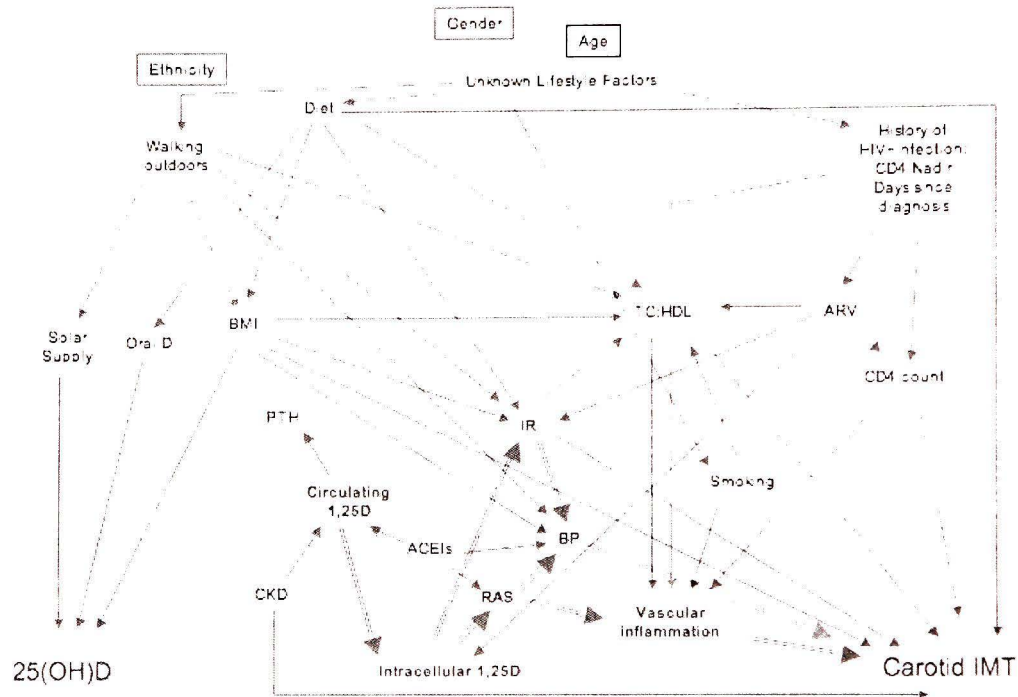


Figure 8. Conditioning on the variable subset containing ethnicity, gender and age

Figure 8 illustrates conditioning on the variable subset containing ethnicity, gender, and age (indicated by the square around each adjusted variable). The next step in the procedure is illustrated in Figures 9 through 11. Here we connect every pair of variables that share a common *child* that is itself contained within the subset of adjusted covariates or has a descendant within the subset. The resulting DAG illustrates the problem of conditioning on a collider and the risk of generating additional backdoor paths that may require additional adjustment in the analysis. For instance, a diet high in saturated fat

can elevate TC:HDL, but so too can PI use. If we know that the TC:HDL is elevated in an individual on a low fat diet the probability of the presence of another cause of elevated lipids such as PI-use is increased.

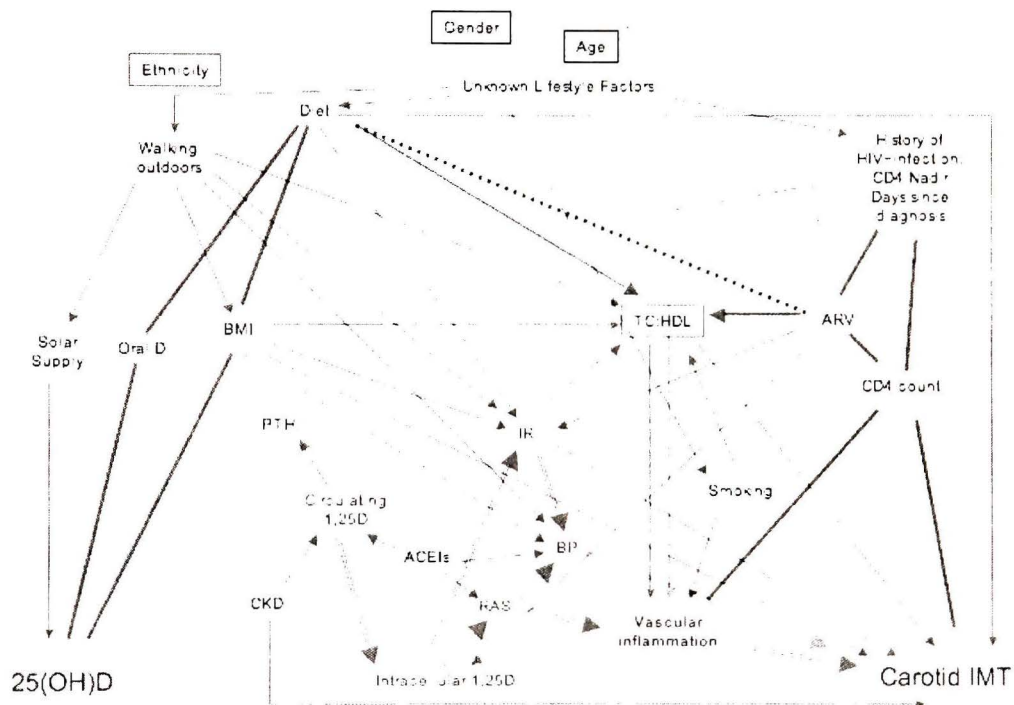


Figure 9. Conditioning on the child (TC:HDL) creates a conditional association, represented by the dashed line (---), between two of its parents (ARV and diet). For illustrative purposes, only one pair (ARV and diet) of many possible parent combinations is presented. The complete representation is illustrated in Figure 11.

In this scenario, diet and ART become conditionally associated within strata of their common effect elevated TC:HDL. The effect of adjusting on the collider TC:HDL (Figure 9) is represented by the undirected line generated between diet and ART. Note the emergence of a backdoor path from 25(OH)D through BMI (or oral D), diet, ART, history of HIV infection (or smoking or intracellular 1,25(OH)₂D or insulin resistance) through to

carotid IMT. A more complete illustration of the effect of conditioning on TC:HDL (i.e. it includes the conditional association between each pair of its parent variables) is presented.

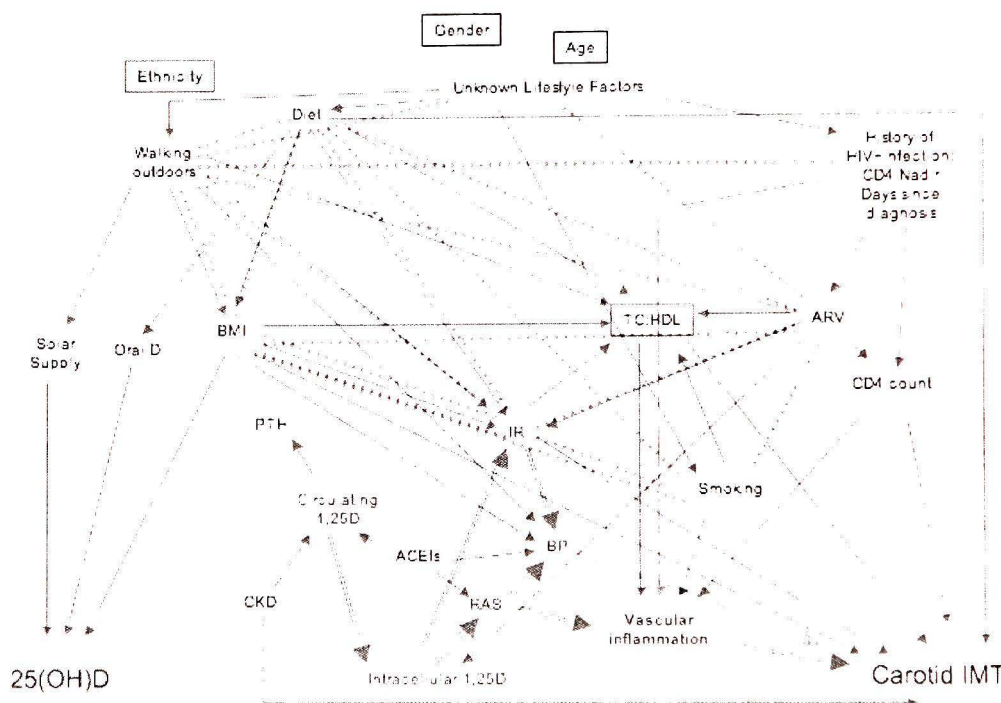


Figure 10. Complete graphical representation of conditional associations generated by control of TC:HDL. Conditional associations are represented by the dashed line (---).

The final step in the algorithm, is to visually assess whether a covariate set is sufficient for closure of all backdoor paths between exposure and outcome. This is facilitated by removal of all lines passing through the conditioned variable set. Figures 11, 12, and 13 illustrate a progressive reduction of paths with each DAG corresponding to the step-wise addition of a variable *chunk*.

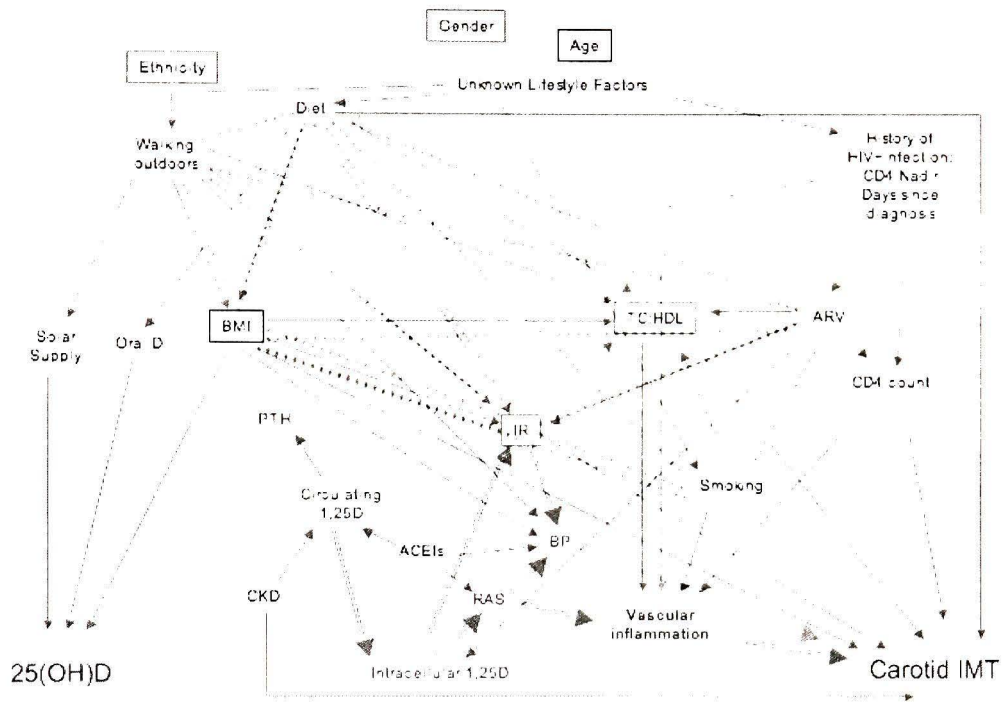


Figure 11. Conditional associations generated by control of the colliders, TC:HDL, BMI, and IR (IIOMA-IR).

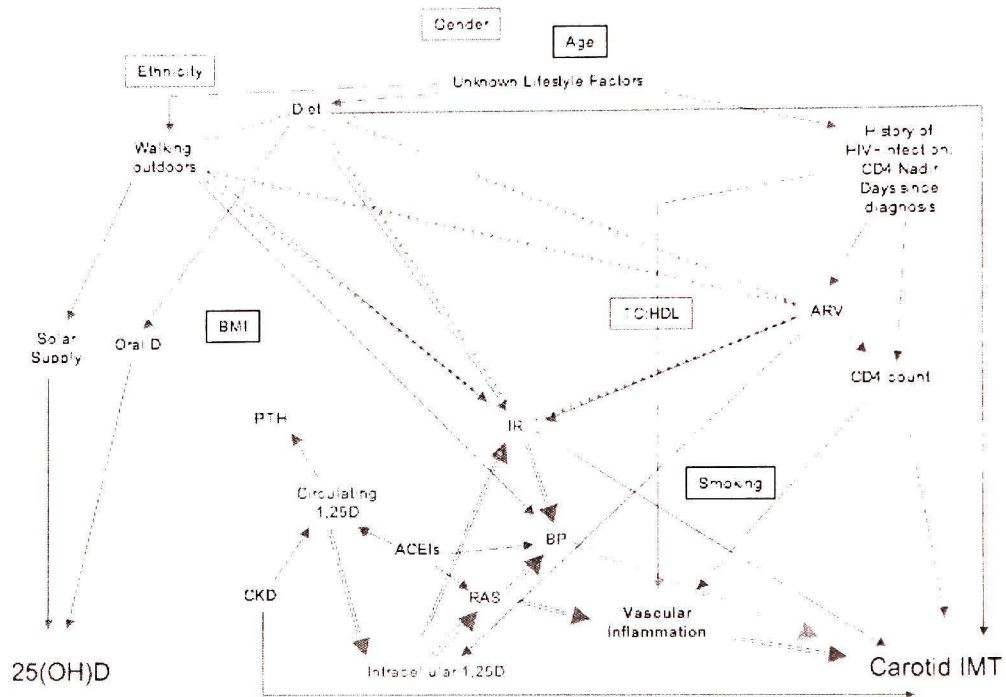


Figure 12. The effect of conditioning on a covariate subset of ethnicity, gender, age, smoking, BMI and TC:HDL.

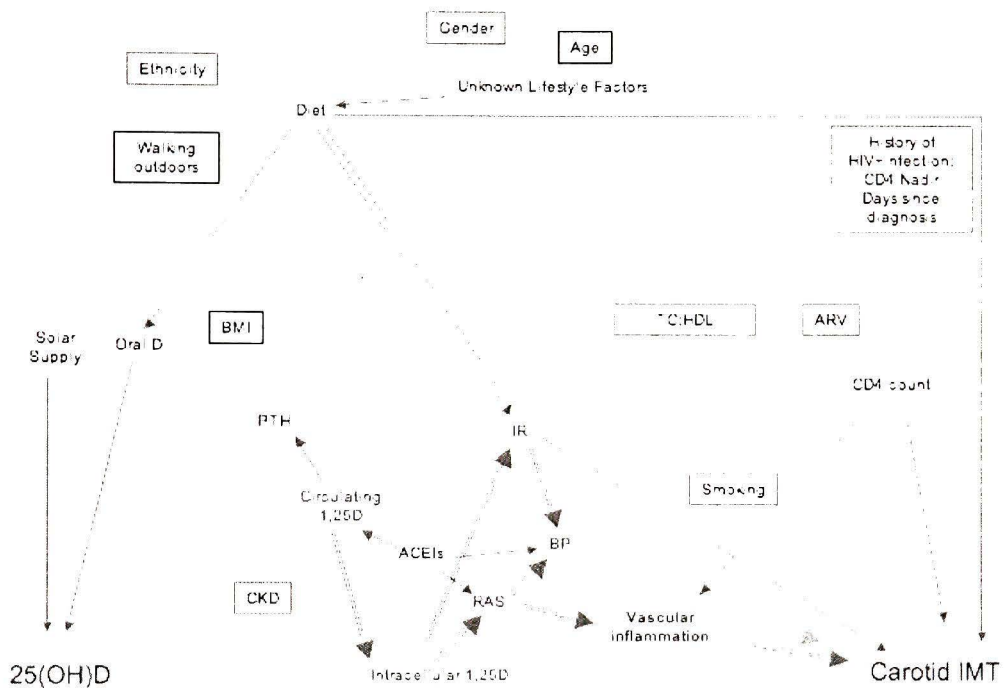


Figure 13. The effect of conditioning on a covariate subset of ethnicity, gender, age, smoking, BMI, TC:HDL, ARV, CD4 nadir, Days since diagnosis, walking outdoors, and CKD. Note, conditioning on CD4 count and CKD may be unnecessary.

Figure 13 illustrates the consequence of conditioning on the covariate set that includes ethnicity, male gender, age, walking outdoors, BMI, TC:HDL, smoking, CD4 nadir, days since diagnosis, ART, CD4 count and CKD. Note all backdoor paths are eliminated except for the path mediated by diet. Unfortunately, because this variable remains unmeasured its contribution to confounding will not be directly addressed in the analysis. The DAGs in Figures 14 and 15 represent the effects of conditioning on IR or BP. The remaining paths between circulating $1,25(\text{OH})_2\text{D}$ and carotid IMT indicate that, while ad-

justment on these variables would attenuate the 25(OH)D-carotid IMT association, some component casual effect would remain. The clinical relevance of this casual association is not known; and the association, due to a smaller effect size, might not be detectable.

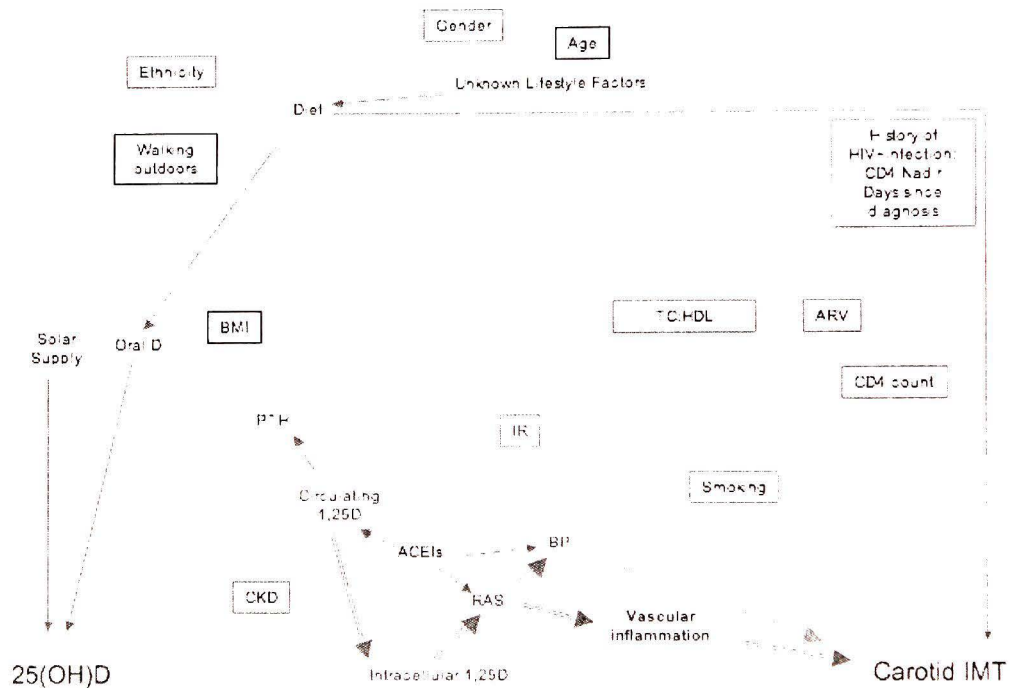


Figure 14. The effect of conditioning on a covariate subset of ethnicity, gender, age, smoking, BMI, TC:HDL, ARV, CD4 nadir, days since diagnosis, CD4 count, walking outdoors, CKD, and IR.

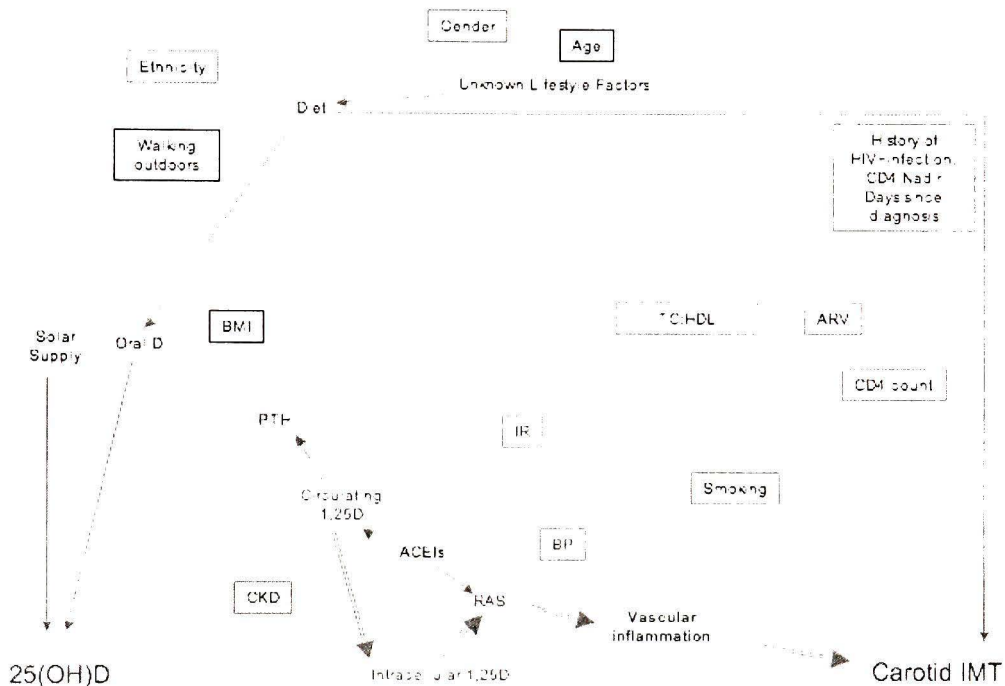


Figure 15. The effect of conditioning on a covariate subset of ethnicity, gender, age, smoking, BMI, TC:HDL, ARV, CD4 nadir, days since diagnosis, CD4 count, walking outdoors, CKD, IR, and blood pressure.

3.3.6. Mismeasurement of confounders

Residual confounding, a consequence of error in measurement of confounders, can lead to incomplete statistical control and substantial bias.¹⁶⁶⁻¹⁶⁹ The effect of this error is complex. Residual confounding can attenuate the statistical association between confounder and exposure and thus obscure the confounder's actual influence.¹⁶⁷ In a simulation study, Fewell et al. found that the direction of bias in the effect estimate, as a consequence of measurement error in confounders, is dependent on such factors as whether the confounders are correlated, the number of unmeasured confounders, and the

correlation of the confounders with exposure.¹⁶⁹ Of note, they reported that unmeasured confounding produced the most serious bias and that small or moderate bias in the measurement of individual variables could in summation substantially bias the outcome. Other factors that have been shown to influence the overall bias include the magnitude and correlation of measurement errors, and the format of the data (dichotomous or continuous).^{165,169,170}

A main limitation in this current study is that the Canadian HIV Vascular Study was not designed specifically with the aim of measuring vitamin D associated risk of vascular disease. Consequently, some characteristics relevant to statistical control for confounding in this current study are unmeasured. Notably absent are reasonable estimates of participants' lifestyle habits relevant to dietary and solar sources of vitamin D.

As I have previously described, the main source of vitamin D supply for most individuals is solar induced synthesis in the dermal layer of the skin. Thus, circulating 25(OH)D is to a large extent affected by the duration and intensity of sun exposure and by those factors that modify vitamin D synthesis such as skin pigmentation, clothing, sunscreen use, and age. It is plausible that any one of these factors might confound an association between circulating 25(OH)D and vascular disease; and ideally, in the interest of statistical efficiency, I would adjust directly on an accurately measured parameter of solar induced vitamin D synthesis. This would close a number of back-door pathways between 25(OH)D and carotid IMT.

Unfortunately, direct measurement of solar induced vitamin D production is not practical in epidemiological research and indirect estimates are imprecise.¹⁷¹ Exposure items on the recently administered questionnaire can provide crude approximations, but data are available for only 75 of 283 participants. Furthermore, these data are subject to historical bias given that current attitudes towards sun exposure may not reflect attitudes that were prevalent during the period of baseline specimen collection. These data are also subject to recall bias since a participant's recollection of previous behaviour may be influenced by factors related to vitamin D status and cardiovascular risk.

In lieu of direct information, I intend to utilize the variables *ethnicity* and *walking outside* as proxies for skin pigmentation and exposure to sunlight, respectively. I expect some degree of residual confounding since these estimates are crude. For instance, there is substantial variation in skin pigmentation across self-reported ethnicity; and walking outside is only one of many ways by which a participant might be exposed to sunlight, and it does not account for biological factors that modify vitamin D synthesis.

An important limitation in this study is the lack of adequate information pertaining to dietary and supplemental vitamin D intake. As described previously, relevant data derived from the recently administered study questionnaire are incomplete and susceptible to historical and recall biases. This lack of information is problematic because a statistical association between dietary or supplemental vitamin D intake and baseline ca-

rotid IMT may be due to shared lifestyle or socioeconomic characteristics that remain unmeasured. Statistical adjustment on vitamin D intake would presumably provide an efficient means of controlling for a number of these unmeasured confounders.

Similarly, adjustment on some proxy for a healthy diet, such as the extent of fruit and vegetable intake, would provide a means of reducing confounding effects mediated by unmeasured healthy lifestyle factors. Unfortunately, while the smaller CIHR component of the dataset includes a measure of fruit and vegetable intake, for the majority of participants dietary intake has not been assessed.

The problem of unmeasured confounding represents a major limitation of observational based nutritional epidemiology. Failure to understand complex differences between participants with low versus high status of a studied nutrient may explain why observational findings often differ from those derived from randomized trials.¹⁷²

Unmeasured confounding may be most problematic when confounders are uncorrelated. Therefore adjustment on lifestyle factors strongly associated with vitamin D but also presumably associated with a range of characteristics associated with a healthy lifestyle may mitigate the influence of these unmeasured characteristics. For instance, accurate assessment of vitamin D input, and of fruit and vegetable consumption, would have provided a means of addressing unknown confounding factors associated with a healthy lifestyle. In lieu of this control, unmeasured confounding may lead to substantial bias in the estimated 25(OH)D-carotid IMT association.

In the next few paragraphs I discuss the issue of residual confounding that may occur as a consequence of misclassification of CVD risk factors.

In the Canadian HIV Vascular Study smoking status is strongly associated with carotid IMT extent and progression.¹¹⁶ Presumably, it may also be statistically associated with vitamin D status and thus, it may confound the 25(OH)D-carotid IMT relationship. As presented graphically in Figure 6, a pathway between 25(OH)D and smoking status may be mediated by TC:HDL and BMI, or by some unknown lifestyle factor that affects vitamin D status through dietary effects on BMI or vitamin D intake. Accurate assessment of smoking status is problematic since categorization based on data derived from self-reported tobacco use tends to underestimate smoking prevalence.¹⁷³ Additionally, measurement of the CVD risk attributable to smoking is challenged by mediating factors such as smoking frequency, depth and duration of inhalation, and the amount of rebreathing of expelled smoke.¹⁶⁵

Measurement of blood pressure and the ascertainment of hypertension can be problematic in epidemiological research. Error can occur as a consequence of practitioner level variation in the way blood pressure is assessed and managed. For example, health providers may vary in their propensity to round blood pressure measures downward. This rounding error would be further complicated if the propensity to round differed by patient characteristics such as other components of the patient's CVD risk profile. Another measurement problem would occur if physicians differed in their adherence to diagnostic

thresholds for classification of hypertension. For instance, there may be inter-practitioner variation in duration of the assessment period that an individual physician provides for discrimination of true hypertension from "white-coat syndrome", and as follows, the threshold for initiating antihypertensive treatment might also differ. Thus, assessment of the true confounding effect of hypertension on the basis of a participant's exposure to antihypertensive therapy may be biased by practitioner level imprecision in how hypertension becomes classified.

In summary, the validity of the current study is threatened by residual confounding issues that occur as a consequence of incomplete measurement or misclassification of important variables. The direction and magnitude of bias on the study effect estimates are dependent on a number of inter-related factors including: the magnitude of measurement error; the strength of associations between mismeasured or unmeasured confounders and 25(OH)D status, and their individual effects on exposure-outcome risk; and whether confounders or the error in their measurement are correlated.^{165,169,170}

3.4. Study population and setting

The Canadian HIV Vascular Study is a prospective cohort investigation designed to evaluate the association between atherosclerotic progression; anti-retroviral drug regimen; immune reconstitution; and standard and nonstandard cardiovascular risk factors. HIV positive persons, aged 35 years or older, attending university affiliated clinics in five

Canadian Centres (Hamilton, Toronto, Calgary, Quebec City and Vancouver) were recruited between October 2002 and June 2009. Participants were enrolled irrespective of cardiovascular risk factors or past cardiac history.

For this current investigation a cross-sectional dataset was created consisting of 283 participants with baseline carotid IMT.

3.5. Clinical characteristics

Demographic and clinical characteristics were collected at each centre by questionnaires administered by research staff and by chart review. Blood pressure is measured twice and results averaged.

To capture data related to vitamin D oral intake and solar production a single page questionnaire (see appendix C) was administered to a subset of the Canadian HIV Vascular Study cohort during the months of July through October in 2009. These data are not representative of the entire cohort and the questionnaire has not yet been validated. Furthermore, as described in section 3.3.6, owing to their more recent time-frame of collection they may not reflect the condition of vitamin D input at the time of specimen collection. In consideration of these limitations, information gleaned from the vitamin D questionnaire will be used descriptively, but will not be utilized for the purpose of statistical adjustment in primary or secondary analyses.

3.6. Laboratory analyses

Participants had been instructed to complete an overnight fast prior to venous blood collection for measurement of plasma glucose, total cholesterol, HDL cholesterol, and triglycerides. LDL cholesterol was calculated by the Friedewald equation. CD4-T-lymphocyte counts were obtained by FACS analysis and plasma HIV-1 viral load was measured by Chiron bDNA assay at the Central Public Health Laboratory in Toronto, Ontario. All other laboratory assays were conducted using stored EDTA plasma samples that were frozen at -70°C.

Plasma 25(OH)D₃ concentrations and intact PTH were determined by electro-chemiluminescence immunoassay (Roche Elecsys vitamin D₃ assay and the Elycys PTH assay, respectively; Roche Diagnostics, Indianapolis, IN), analyzed on the Roche Modular Analytics E170 at the Clinical Research and Clinical Trials Laboratory at Hamilton General Hospital. The Clinical Research and Clinical Trials Laboratory participates in the Vitamin D External Quality Assessment Scheme (DEQAS; internet: www.deqas.org).

Intra- and inter-assay coefficients of variation (CV) for 25(OH)D₃ measures were well within acceptable limits. Intra-assay CVs were 5.1%, 2.3% and 2.2% for control values of 57, 78.5, and 215 nmol/L, respectively, while inter assay CVs were 6.0%, 3.2%, and 3.3% for control values of 54, 77, and 210 nmol/L.

The Elecsys vitamin D assay cannot detect 25(OH)D₂ and thus it can underestimate total 25(OH)D concentrations. However, in this Canadian cohort the occurrence of vitamin D₂ is expected to be minimal.

3.7. Carotid ultrasound methods

Measurement of carotid IMT by high resolution B-mode ultrasound is a non-invasive, sensitive, and reproducible technique for quantifying subclinical vascular disease. A number of factors are known to contribute to the intima-media thickness. These include effects associated with aging, blood pressure, and atherogenesis.¹⁷⁴ Carotid IMT is associated with risk for myocardial infarction, stroke, death from coronary heart disease, and with the extent of angiographic coronary artery disease.^{112,175}

At baseline and annually participants in the Canadian HIV Vascular Study undergo high-resolution B-mode ultrasound at participating ultrasound laboratories according to a standardized protocol. The scanning and measurement protocol, developed and validated by Lonn et al.,¹⁵² measures carotid IMT as the mean of 12 carotid IMT segments, consisting of near and far wall measures located 1 and 2 cm above the carotid bifurcation (Figure 16). This technique, involving an initial transverse scan, followed by a longitudinal circumferential scan, aims to image the thickest component of the arterial wall at each prespecified arterial segment.

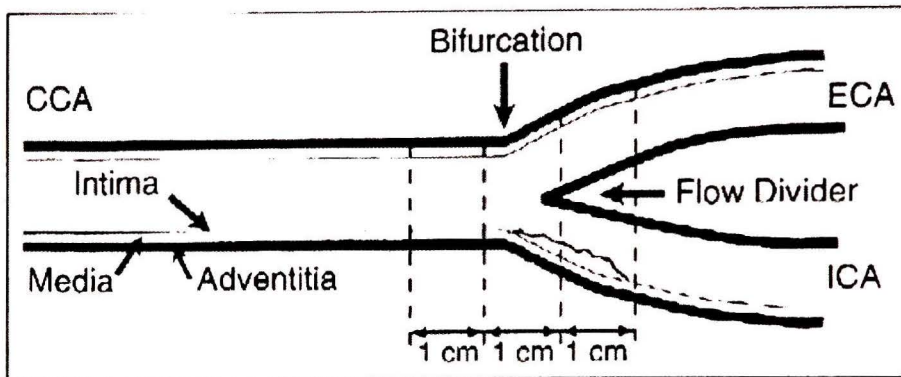


Figure 16. Diagrammatic representation of right carotid artery scan. The tip of the flow divider is shown representing the anatomic landmark used to define the carotid artery segments analyzed. A plaque is seen in the far wall of the bifurcation and internal carotid artery (ICA) segments. CCA – common carotid artery; ECA – external carotid artery. (Lonn et al. 1996)

Videotaped images were read at the Core Ultrasound Laboratory (Hamilton, Ontario) by a blinded reader under the supervision of Dr. Lonn. Calculation of carotid IMT was facilitated by use of computer-assisted software (Image-Pro V4.5.1; Glen Burnie, Maryland). A mean maximum IMT was computed by averaging maximum IMT measurements from each of the twelve preselected segments. This provides a summary parameter pertaining to the left and right common carotid artery, bulb, and internal carotid arteries.

Prior validation of 12-segment carotid IMT at the Core Ultrasound Laboratory demonstrated good reproducibility with interclass correlation ≥ 0.90 and coefficient of variation $< 5\%$ for repeat examinations.

3.8. Power analysis

On average, carotid IMT in the Canadian HIV Vascular Study Cohort progresses at a rate of 0.02 (SD = 0.13) mm/year.¹¹⁶ A difference of 0.05 mm representing 2.5 years of aging was judged by the author to be clinically important. Based on a mean (SD) baseline carotid IMT of 0.81 mm (0.25) and an existing sample size of 286, power of the study to detect an effect size of 0.20 (0.05/SD) is 0.92, two-tailed $\alpha = 0.05$, comparing a 0.05 mm difference between the carotid IMT mean above (H0) with the carotid IMT mean below (H1) the third 25(OH)D quartile. Power analysis was performed using G*Power 3.0.10. See appendix D.

3.9. Analysis

Descriptive statistics are presented as mean with standard deviation, or median with interquartile range, for continuous data, and as number and percent for categorical variables. Prior to analyses, data was represented graphically. Outliers were assessed for plausibility and influence.

For parametric tests, carotid IMT was logarithmically transformed. All statistical tests were performed using two-sided tests at the 0.05 level of significance. P values were reported to three decimal places with values less than 0.001 reported as < 0.001 . Missing values were handled using imputation with the mean (median for categorical variables) and regression-based imputation.

3.9.1. Seasonal variation of 25(OH)D

As vitamin D status varies by season, 25(OH)D₃ was analyzed as seasonally adjusted z-scores determined by date of sample collection and its correspondence to one of 4 seasonal periods. In a simulation study evaluating misclassification bias related to 25(OH)D variation, failure to account for date of sample collection resulted in attenuation of risk estimates whereas in models utilizing 25(OH)D categories that accounted for date of sample collection (i.e., season-specific or month-specific quartiles) bias associated with temporal variation was reduced. In this latter scenario effect estimates closely approximated *truth* (long-term average 25(OH)D levels).¹⁷⁶

One advantage of z-score adjustment is its preservation of vitamin D as a continuous variable. A particular limitation of seasonal standardization is that summer status may not necessarily correspond to the severity of winter season deficiency (personal correspondence with R. Vieth, unpublished data, 2009). In a subset of 16 participants selected on the basis of available data, the intraclass correlation coefficient (ICC, case-type "3"¹⁷⁷) was estimated to assess agreement between samples collected during "colder" (November through April) or "warmer" months (May through October).

3.9.2. Characteristics of vitamin D status

Prevalence data regarding vitamin D status are presented as the proportion of participants within each vitamin D status category (i.e. deficiency, insufficiency, and sufficiency). Vitamin D status is also presented across ethnic categories.

I describe vitamin D status by presenting relevant clinical characteristics stratified by season-standardized 25(OH)D₃ quartiles. Across group differences were compared using analysis of variance (ANOVA) for continuous variables and using the χ^2 test for categorical variables. The following characteristics were compared: age (years), male sex (%), BMI, estimated skin colour (based on ethnicity categories where *white* was classified as *light skin*; *South Asian*, *Chinese*, *First Nation* and *other* were classified as *medium skin*, and *Black* was classified as *dark skin*), physical intensity of leisure activity (low, moderate, and high, estimated from self-reported frequency of sweating during leisure activity), current smoker (%), absolute CD4 cell count (> 500, 200-499, and < 200 cells/mm³), CD4 nadir (< 200 cells/mm³), current ART (no treatment, PI-based regimen, NNRTI-based regimen, triple NRTI-based regimen, other regimen), blood lipids (TC:HDL, HDL, and lipid lowering medication use), fasting plasma glucose, systolic blood pressure, diastolic blood pressure, history of hypertension (systolic $\geq$ 140 mm Hg, diastolic $\geq$ 90, or receiving anti-hypertensive medication;%), history of cardiovascular disease (%), PTH, and carotid IMT.

Selected data extracted from the vitamin D questionnaire is utilized descriptively to characterize vitamin D input. Vitamin D supplemental intake was defined as *none*; *moderate*, estimated to provide 200 to 400 IU vitamin D₃ daily (participants who indicated use of a multivitamins or calcium with vitamin D or cod liver oil only); or *high*, estimated to provide greater than 400 IU vitamin D₃ daily (participants who indicated use of more than one source or use of a specific vitamin D₃ supplement). Daily intake from fortified food sources was calculated based on an estimated 100 IU vitamin D per glass of milk or milk substitute. Estimated vitamin D₃ intake from dietary fish was not calculated since the fish intake item on the questionnaire pertains to combined salmon and tuna intake; as food sources their vitamin D content differs substantially.

3.9.3. Regression analyses

As the primary analysis I estimated linear regression models to explore the relationship between season-standardized 25(OH)D₃ and carotid IMT (as the dependant variable). As the relationship was hypothesized to not be linear, for purposes of the primary analysis, 25(OH)D₃ was modelled by quartile, as a nominal categorical variable.

The analysis approach was that models should include all scientifically relevant variables as determined on the basis of experimental evidence, biological plausibility, and the presumed sufficiency of the subset to adjust for confounding (described in section 3.3.4). If a full-model were to appear unstable (or *over-fit*) variable selection was to be determined by *chunkwise* selection. Covariate subsets, in order of prespecified importance,

were *chunk* one (age, gender, smoking status including current and former, TC:HDL); *chunk* two (ethnicity, BMI); *chunk* three (ART-type) , *chunk* four (CD4 nadir, current lipid lowering medication use), and *chunk* five (physical activity, duration of HIV infection (years since diagnosis), CD4 count, and history of chronic kidney disease (CKD)). The causal assumptions that underlie conditioning on these covariate sets have been presented graphically in Figures 8 through 15. A variable for study centre and a categorical variable for season were introduced within each model. The decision to avoid conditioning on measures of insulin resistance and hypertension, on the basis that these may mediate the 25(OH)D-carotid IMT association, was tested by inclusion of relevant variables for each in regression models. The included variables were systolic blood pressure or hypertension and fasting plasma glucose (a more precise measure of insulin resistance i.e., HOMA-IR was not available).

To assess statistical interaction, multiplicative terms were computed between 25(OH)D and the variables, age, hypertension, and BMI. The threshold for inclusion within main effects models was a significant likelihood ratio tests at the P level of 0.05.

For continuous data, abnormalities of scale were investigated through univariable restricted cubic spline analyses with three knots, located at the 5th, 50th and 95th percentiles. For regression analyses BMI was classified into three categories: under or normal weight ($\text{BMI} < 25 \text{ kg/m}^2$), overweight ($\text{BMI } 25.0\text{--}29.9 \text{ kg/m}^2$) and obesity ($\text{BMI} > 30.0$

kg/m²). Ethnicity was categorized as White, Black, First Nation, South Asian, Chinese, and other. Absolute CD4 T-cell count and CD4 nadir were analysed as nominal categorical variables as per definitions previously described.

To enhance our understanding of the shape of the relationship between 25(OH)D status and carotid IMT, in addition to the quartile categorization as per the primary analysis, 25(OH)D₃ was modelled using commonly used thresholds of status (deficiency, ≤ 37.5 nmol/L; insufficiency, > 37.5 to 74.9 nmol/L; and optimal, ≥ 75 nmol/L (referent)) and as a continuous variable.

Multivariable restricted cubic spline regressions were conducted to describe the relationship between vitamin D status and carotid IMT. A 25(OH)D₃ spline function was generated based on four knot (using the default setting) and three knot (selected on the basis of clinically relevant 25(OH)D₃ levels) settings.

On the basis that hypertension may magnify the adverse vascular effects of vitamin D deficiency⁸⁹—hypertension and presumably vitamin D deficiency can influence vascular remodelling— subgroup analyses were performed to assess for the presence of quantitative heterogeneity ("effect modification") across patients stratified on the basis of hypertension status.

To assess for model instability and numerical problems (complete separation and collinearity) coefficients and their standard errors were assessed for magnitude aberrations. Collinearity was further investigated by calculating variance inflation factor (VIF)

and measures of tolerance. A tolerance of 0.1 or less or a VIF of 10 or greater was considered evidence of high collinearity. Linear regression results are expressed as R^2 (where appropriate), regression coefficients, standard errors, corresponding two-sided 95% confidence intervals, and associated p-values. P-values are reported to four decimal places with values less than 0.0001 reported as <0.0001 . Goodness-of-fit was estimated by comparing R^2 values, and by examining the residuals for the model assumptions. Analyses were performed using STATA version 10.1 (StataCorp, College Station, TX).

Chapter 4

Results and Discussion

4.1. Study results

A total of 283 participants from the Canadian HIV Vascular study were included in the analysis on the basis of available carotid IMT and 25(OH)D₃ measurements.

Baseline participant characteristics are presented in Table 3. Of the participants, 89% were men, the mean age (SD) was 51.8 (8.3) years, 85 % were white, and 36% were current smokers. Less than 8% of participants had a history of intravenous drug use.

Carotid IMT ranged from a min of 0.47 mm to a max 2.24 mm, with a mean (SD) of 0.81 (0.25). Almost one third of participants were classified (as per the study definition) as having a history of hypertension. Lipid lowering medication was used by 15% of participants; for the majority of these, medication type (statin or fibrate) was not differentiated.

The mean (SD) 25(OH)D₃ concentration was 85.6 (33.0) nmol/L. The overall prevalence of 25(OH)D *deficiency* (≤ 37.5 nmol/L) was 2.1%. No participant had a 25(OH)D₃ level characterized as *severe* (≤ 25 nmol/L). The prevalence of *insufficiency* (> 37.5 to 74.9 nmol/L) was 38.9% whereas 59% of participants could be classified as vitamin D *sufficient* (or *optimal status* , ≥ 75 nmol/L).

Table 3 Baseline characteristics of the study population (n = 283)	
25(OH)D ₃ , nmol/L*	85.6 (33.0)
Age, years*	51.8 (8.3)
Male sex, number (%)	250 (89)
Body mass index, kg/m ² *	25.1 (4.3)
Current smoker, number (%)	101 (36)
Ethnicity, number (%)	
White	238 (85)
Black	14 (5)
First Nation	5 (2)
South Asian	2 (< 1)
Chinese	2 (< 1)
Other	21 (7)
Estimated skin colour, number (%)†	
Light skin	238 (84)
Medium skin	30 (11)
Dark skin	14 (5)
Physical activity, number (%)‡	
Low	102 (36)
Moderate	115 (41)
High	66 (23)
Absolute CD4 T-cell count, number (%)	
> 500 cells/mm ³	111 (40)
200-499 cells/mm ³	123 (44)
< 200 cells/mm ³	45 (16)
CD4 T-cell nadir < 200 cells/mm ³ , number (%)	161 (58)

Current ART, number (%)	
PI-based regimen	80 (29)
NNRTI-based regimen	108 (39)
NRTI-only regimen	23 (8)
Other regimen	24 (9)
No current therapy	45 (16)
Total cholesterol:HDL ratio*	5.0 (2.3)
HDL cholesterol mmol/L*	1.08 (0.4)
Lipid lowering medication use, number (%)	42 (15)
Plasma glucose nmol/L*	5.5 (1.3)
Systolic blood pressure mm Hg*	121 (16)
Diastolic blood pressure mm Hg*	77 (11)
Hypertension, number (%)§	80 (29)
History of cardiovascular disease, number (%)	19 (7)
Parathyroid hormone pmol/L*	3.07 (1.31)
Carotid IMT*	0.81 (0.25)

25(OH)D₃ indicates 25-hydroxyvitamin D₃; ART, antiretroviral therapy; PI, protease inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside/nucleotide reverse transcriptase inhibitor.

*Mean(SD)

†Based on ethnicity categories where *white* was classified as *light skin*; *South Asian*, *Chinese*, *First Nation* and *other* were classified as *medium skin*, and *Black* was classified as *dark skin*.

‡Estimated from self-reported frequency of sweating during leisure activity

§Systolic ≥ 140 mm Hg, diastolic ≥ 90 , or receiving anti-hypertensive medication.

By ethnic categories, 61% (146/238) of whites would be classified as vitamin D sufficient, whereas only 40% (4/10) of blacks had 25(OH)D₃ levels reflecting sufficiency. In the remaining ethnic categories combined, inclusive of the South Asian, Chinese, and *other* category—presumably these represent participants with skin colour intermediate between whites and blacks—60% (18/30) had 25(OH)D₃ concentrations exceeding the sufficiency threshold.

Clinical and demographic characteristics of study participants according to seasonally adjusted 25(OH)D₃ quartiles are shown in Table 4. Quartile groups were not statistically different by age, TC:HDL ratio, systolic blood pressure, diastolic blood pressure, smoking status, plasma glucose, immune status parameters, estimated skin-colour

groupings or by carotid IMT. Quartiles differed significantly by mean BMI ($P = 0.0242$), history of hypertension ($P = 0.04$), and mean PTH concentration ($P = 0.0002$). In the highest 25(OH)D₃ quartile, representation by participants exposed to PI-based ART was 45% compared to the next highest, the NNRTI-based group which constituted 26% of the quartile. The proportion of PI-based ART exposure differed significantly across quartiles ($P = 0.005$). Similarly, the proportion of participants exposed to lipid lowering medication was also significantly different across quartiles ($P = 0.036$).

Table 4. Unadjusted baseline characteristics of the study participants by quartiles (Q) of seasonally adjusted plasma 25(OH)D₃ levels

Characteristic	Q1 n = 71	Q2 n = 71	Q3 n = 71	Q4 n = 70	p Value†
25(OH)D ₃ , nmol/L*	50.7 (9.5)	73.0 (5.5)	90.2 (7.8)	129.0 (31.3)	
Age, years*	52.2 (8.1)	51.6 (7.7)	52.2 (9.3)	51.3 (7.9)	0.8976
Male sex, number (%)	59 (86)	65 (92)	62 (86)	64 (93)	0.403
Body mass index, kg/m ² *	26 (4)	26 (5)	25 (4)	24 (4)	0.0242
Current smoker, number (%)	20 (29)	24 (34)	28 (39)	29 (41)	0.403
Estimated skin colour, number (%)††					
Light skin	57 (81)	57 (81)	60 (83)	64 (91)	0.305
Medium skin	8 (11)	7 (10)	10 (14)	5 (7)	0.620

Dark skin	5 (7)	6 (9)	2 (3)	1 (1)	0.156
Physical activity, number (%)‡					
Low	25 (36)	25 (35)	24 (33)	28 (40)	0.866
Moderate	30 (43)	27 (38)	32 (44)	26 (37)	0.770
High	15 (21)	19 (27)	16 (22)	16 (23)	0.881
Absolute CD4 T-cell count					
> 500 cells/mm ³	42.7	35.2	38.9	42.7	0.775
200-499 cells/mm ³	39.7	45.1	45.8	45.6	0.871
< 200 cells/mm ³	12 (18)	14 (20)	11 (15)	8 (12)	0.618
CD4 T-cell nadir < 200 cells/mm ³	42 (62)	37 (52)	39 (54)	43 (62)	0.506
ART-type (current), number (%)					
PI-based regimen	19 (28)	15 (21)	15 (21)	31 (45)	0.005
NNRTI-based regimen	29 (42)	32 (46)	29 (40)	18 (26)	0.09
NRTI-only regimen	8 (12)	4 (6)	7 (10)	4 (6)	0.498
Other regimen	4 (6)	7 (10)	7 (10)	6 (9)	0.805
No current therapy	9 (13)	12 (17)	14 (19)	10 (14)	0.738
total cholesterol:HDL ratio*	4.9 (1.6)	5.1 (1.7)	5.0 (1.7)	4.8 (1.4)	0.7551
HDL cholesterol*	1.0 (0.3)	1.1 (0.3)	1.1 (0.4)	1.1 (0.4)	0.2381
Lipid lowering medication use, number (%)	6 (9)	7 (10)	4 (6)	14 (20)	0.036
Plasma glucose nmol/L*	5.7 (2.3)	5.5 (0.8)	5.4 (0.7)	5.3 (0.5)	0.1731

Systolic blood pressure, mm Hg*	123 (19)	122 (14)	123 (17)	117 (15)	0.1554
Diastolic blood pressure mm Hg*	79 (13.0)	78 (10.6)	78 (9.6)	74 (11.6)	0.1404
History of hypertension, number (%)§	24 (35)	23 (32)	23 (32)	10 (15)	0.04
History of cardiovascular disease, number (%)	7 (10)	3 (4)	5 (7)	4 (6)	0.567
Parathyroid hormone*	3.5 (1.6)	3.1 (1.1)	3.1 (1.2)	2.6 (1.1)	0.0002
Log carotid IMT*	-0.28 (0.27)	-0.22 (0.29)	-0.23 (0.26)	-0.27 (0.26)	0.4493

25(OH)D₃ indicates 25-hydroxyvitamin D₃; ART, antiretroviral therapy; PI, protease inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside/nucleotide reverse transcriptase inhibitor.

†P values for quartile differences were calculated using ANOVA for continuous variables and the χ^2 test for categorical variables.

*Mean(SD)

††Based on ethnicity categories where *white* was classified as *light skin*; *South Asian*, *Chinese*, *First Nation* and *other* were classified as *medium skin*, and *Black* was classified as *dark skin*.

‡Estimated from self-reported frequency of sweating during leisure activity

§Systolic ≥ 140 mm Hg, diastolic ≥ 90 , or receiving anti-hypertensive medication.

Table 5 summarizes estimated vitamin D consumption for the 74 participants who provided vitamin D questionnaire data. An estimated intake from nutritional supplements or milk products of at least 400 iu per day was attained by 28% and 8% of participants, respectively. Of the seven participants to have reported soy or rice milk intake—the main source of vitamin D₂—only one of these would be classified as having a daily vitamin D₂ intake of at least 400 IU.

Table 5. Supplemental and dietary vitamin D intake	
Estimated supplemental intake, number (%) [*]	
None	25(34)
Moderate	28(38)
High	21(28)
Participants with a daily intake from all milk sources [†] of at least 400 IU, number (%)	6(8)
Participants consuming a milk substitute (soy or rice milk), number (%)	7 (9)
[*] Moderate supplemental intake estimated to provide 200 to 400 IU vitamin D ₃ daily (participants who indicated use of only one of multivitamins, calcium with vitamin D ₃ , or cod liver oil), high supplemental intake estimated to provide greater than 400 IU vitamin D ₃ daily (participants who indicated use of more than one source or use of a specific vitamin D ₃ supplement). [†] Cow's, goat, rice, or soy. Assuming fortification of 100 IU vitamin D per glass of milk or milk substitute.	

In unspecified (by protocol) analyses, plasma 25(OH)D₃ levels were not significantly different by month ($P = 0.0874$, ANOVA) or by seasonal category (November through January, February through April, May through July, August through October; ANOVA, $P = 0.2079$). Plasma 25(OH)D₃ levels were statistically different across *colder* and *warmer* months (November through April; May through October, $P = 0.0373$); however, the seasonal variation was minimal; *colder* and *warmer* month means (SD) were 82.2 (29.3) and 90.5 (37.4), respectively. Plasma 25(OH)D₃ by seasonal category of specimen collection date is illustrated in Figure 17.

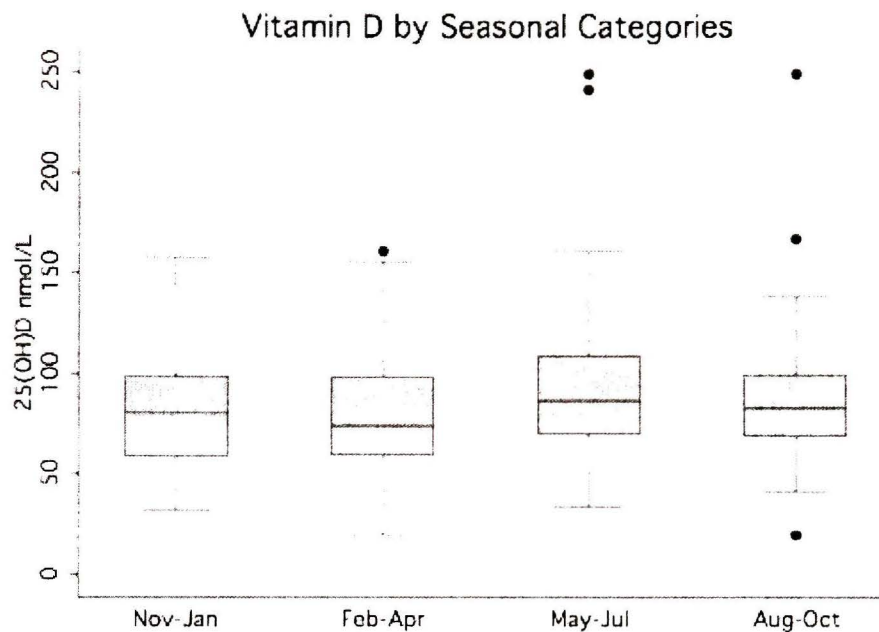


Figure 17. Vitamin D concentration within each seasonal category according to date of specimen collection.

Repeat 25(OH)D₃ measures across two seasonal periods (*colder* months vs. *warm-er* months) were available for 16 participants. Intra-participant agreement between 25(OH)D₃ measures collected during one of each season was high (ICC = 0.84) suggesting that seasonal variation had minimal influence on determination of 25(OH)D₃ status in the study population. Figure 18 illustrates the relationship between data-driven z-scores and unadjusted 25(OH)D₃ values.

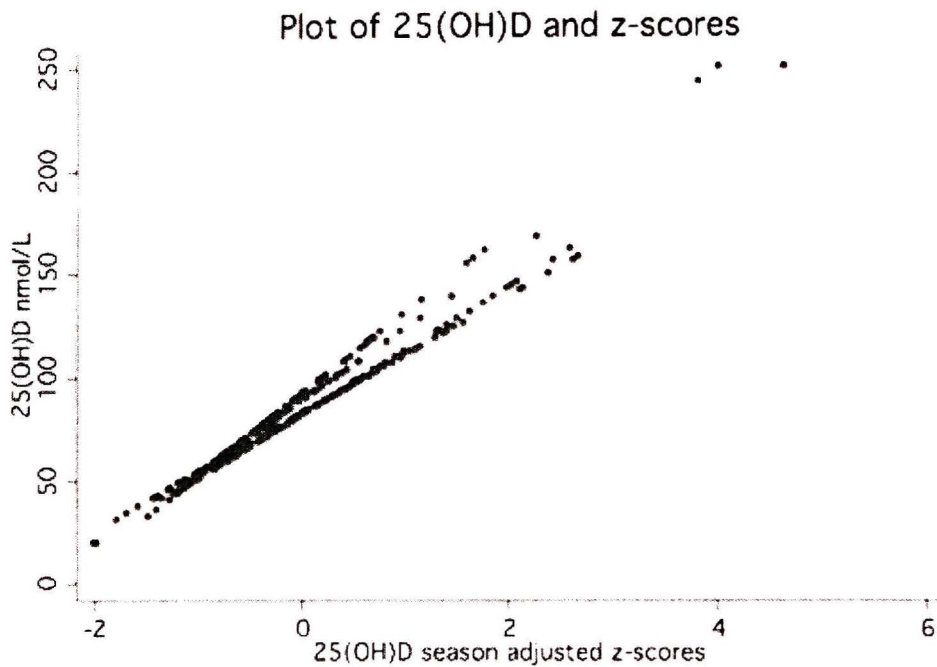


Figure 18. Plot of 25(OH)D values on season adjusted z-scores illustrating a reasonably strong linear relationship

Results of univariable and multivariable linear regression models are shown in Table 6. In univariable linear regression analysis season adjusted 25(OH)D by quartile was not statistically associated with carotid IMT ($P = 0.4493$, $R^2 = 0.01$). Adjustment for known risk factors and other variables hypothesized to potentially confound a 25(OH)D-carotid IMT association had no appreciable effect on the probability of an association.

Likewise, evidence of an association was not affected by the inclusion of the prespecified multiplicative terms, or by performing analyses stratified by hypertension status (data not presented).

Table 6. Results of univariable and multivariable linear regression analyses: season adjusted 25(OH)D ₃ estimates for carotid IMT.				
	25(OH)D ₃ Estimate	Standard error	95% CI	P Value
Univariable (n=283)*				
Quartile 1	referent			
Quartile 2	0.062	0.05	-0.028 - 0.152	0.174
Quartile 3	0.05	0.05	-0.039 - 0.14	0.267
Quartile 4	0.01	0.05	-0.08 - 0.1	0.828
Full Model (n=266)*†				
Quartile 1	referent			
Quartile 2	0.057	0.04	-0.02 - 0.134	0.149
Quartile 3	0.033	0.04	-0.043 - 0.109	0.393
Quartile 4	-0.003	0.04	-0.081 - 0.075	0.941
Continuous‡†	-0.005	0.01	-0.032 - 0.023	0.733

*Estimates based on season adjusted 25(OH)D₃ z-scores by quartile

†Adjusted for age, gender, smoking status (including current and former smoking),

TC:HDL, ethnicity, BMI category, ART-type, CD4 nadir category, CD4 category, lipid lowering medication use, physical activity, years since HIV diagnosis, history of kidney disease, and season.

‡25(OH)D₃ season-adjusted z-scores modelled as a continuous variable.

Regression diagnostics were unremarkable except for the following two issues. First, a few observations were highlighted and assessed for their potential to exert undue influence on model estimates. Deletion of identified observations had minimal impact on regression estimates; observations were not removed from presented analyses. Second, the variable for clinic site contributed a model specification error (*link test* for model specification); consequently, the variable was excluded from regression analyses.

As prespecified secondary analyses, 25(OH)D₃ was modelled according to commonly used thresholds of status (deficiency, ≤ 37.5 nmol/L; insufficiency, > 37.5 to 74.9 nmol/L; and optimal, ≥ 75 nmol/L (referent) and as a continuous variable. Again, 25(OH)D₃ status failed to demonstrate a significant association with carotid IMT (data for status categories not presented).

Because the shape of the relationship between 25(OH)D₃ and carotid IMT was hypothesized to be curvilinear, I conducted restricted cubic spine linear regression analyses

(4 knots) in order that 25(OH)D₃ could be modelled as a continuous variable. As the influence of season on 25(OH)D₃ variability was shown to be minimal (and for purposes of presentation) the 25(OH)D₃ spline function was determined using unadjusted plasma 25(OH)D₃ values. By the default setting for a 4 knot placement (mkspline2, Stata IC 10.1), knots were established at 25(OH)D₃ values of 44.08, 72.84, 92.26, and 144 nmol/L. In univariable analysis (see Table 7) there is a statistically significant curvilinear relationship between 25(OH)D₃ and carotid IMT characterized by spline variable coefficients (these represent the slope in each respective region between knots) that are positive, negative, and positive, respectively. Formally, there is a significant departure from linearity (Wald test, P = 0.0181). After adjustment for age, gender, smoking status, TC:HDL, ethnicity, BMI status, ART-type, CD4 nadir, lipid lowering therapy use, physical activity, years since HIV diagnosis, history of kidney disease, and season, only the first variable coefficient (or slope) remained statistically significant (P = 0.040).

Table 7. Carotid IMT against a restricted cubic spline function of 25(OH)D ₃ with 4 knots.*				
	25(OH)D ₃ Estimate†	Standard Error	95% CI	P Value
Univariable model				
Variable 1	0.006	0.002	0.002 -0.011	0.007
Variable 2	-0.023	0.01	-0.042 - -0.003	0.021
Variable 3	0.063	0.03	0.004 - 0.122	0.038

Full Model§				
Variable 1	0.004	0.002	0.0001 - 0.008	0.043
Variable 2	-0.014	0.009	-0.031 - 0.003	0.101
Variable 3	0.037	0.026	-0.015 - 0.088	0.166
*25(OH)D₃ spline variable coefficients based on 4 knots corresponding to unadjusted 25(OH)D₃ values of 44.1, 72.8, 92.3, and 144 nmol/L. †Variable coefficients are estimates of slope, each variable corresponding to an interval between the knots. §Adjusted for age, gender, smoking status (including current and former smoking), TC:HDL, ethnicity, BMI category, ART-type, CD4 nadir category, CD4 category, lipid lowering medication use, physical activity, years since HIV diagnosis, history of kidney disease, and season.				

Figure 19 (obtained using POSTRCSPLINE¹⁷⁸ in Stata/IC 10. 1) illustrates a curvilinear relationship between 25(OH)D₃ and predicted log transformed carotid IMT (based on the full model with each variable set at its mean value).

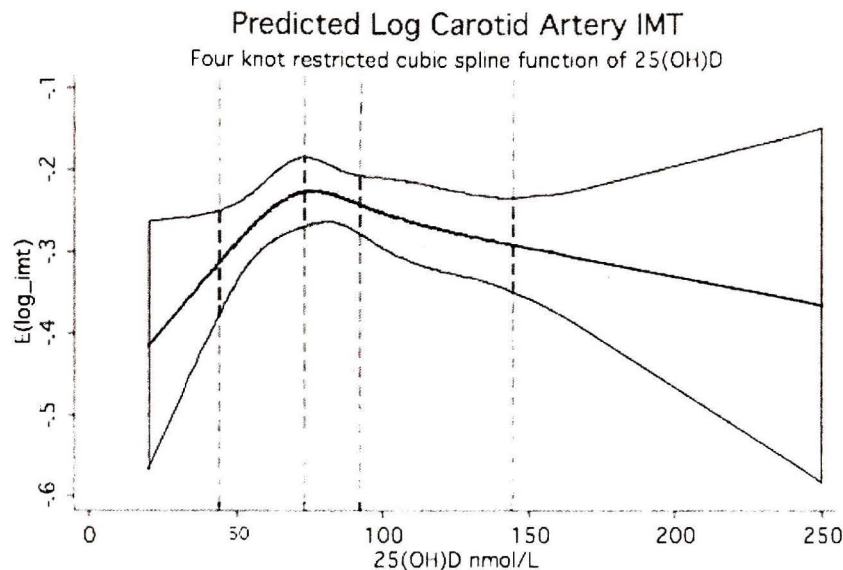


Figure 19. Predicted log carotid IMT based on a restricted cubic spline function of 25(OH)D with knots set at 44.1, 72.8, 92.3, and 144 nmol/L. Analysis was adjusted for age, gender, smoking status (including current and former smoking), TC:HDL, ethnicity, BMI category, ART-type, CD4 nadir category, CD4 category, lipid lowering medication use, physical activity, years since HIV diagnosis, history of kidney disease, and season with each variable set at its mean value. Graph constructed with POSTRCSPLINE¹⁷⁸ in Stata/IC 10.1.

Analyses were also performed utilizing knot placement determined primarily on the basis of clinical relevance: knots were set at 25(OH)D₃ values of 37.5, 80, and 120 nmol/L. In univariable analysis (see Table 8) the spline variable coefficients demonstrated a curvilinear relationship between 25(OH)D₃ and carotid IMT characterized by an association that is positive immediately below, and negative immediately above, the 80 nmol/L level. After adjustment for age, gender, smoking status, TC:HDL, ethnicity, BMI status, ART-type, CD4 nadir, CD4 category, lipid lowering medication use, physical activity, years since HIV diagnosis, history of kidney disease, and season, the first and

second 25(OH)D₃ spline variables remained statistically significant in the model (P = 0.044 and 0.026, respectively). Figure 20 illustrates a curvilinear relationship between 25(OH)D₃ and predicted log transformed carotid IMT (based on the multivariable 3 knot model with each variable set at its mean value).

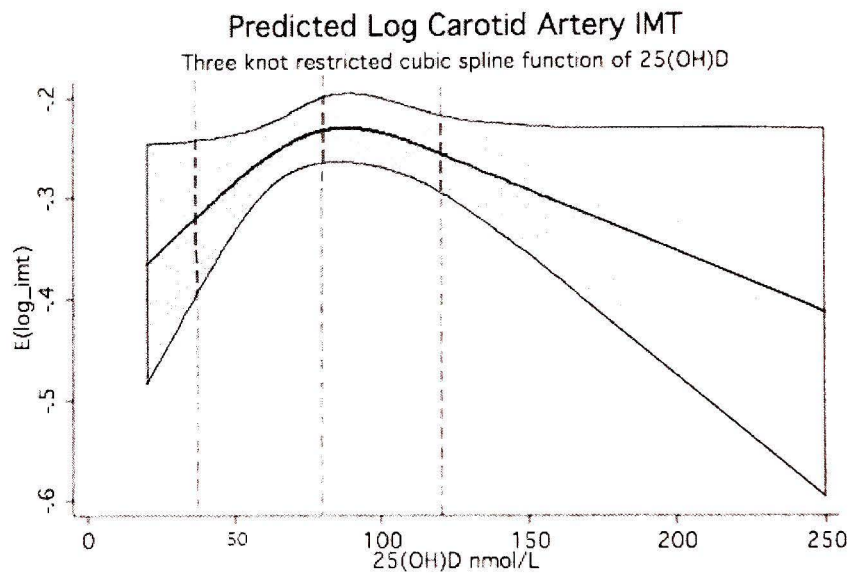


Figure 20. Predicted log carotid IMT based on a restricted cubic spline function of 25(OH)D with knots set at 37.5, 80, and 120 nmol/L. Analysis was adjusted for age, gender, smoking status (including current and former smoking), TC:HDL, ethnicity, BMI category, ART-type, CD4 nadir category, CD4 category, lipid lowering medication use, physical activity, years since HIV diagnosis, history of kidney disease, and season with each variable set at its mean value. Graph constructed with POSTRCSPLINE¹⁷⁸ in Stata/IC 10.1.

Figures 19 and 20 provide evidence of a possible threshold effect occurring within a 25(OH)D₃ interval spanning upper and lower regions of insufficiency and sufficiency status, respectively.

I performed a number of post hoc analyses in exploration of primary and secondary findings. These are described below.

A test for linear trend conducted by modelling a continuous 25(OH)D₃ variable comprised of the four within-quartile medians against carotid IMT was not statistically significant (P for trend = .762).

In addition to the prespecified subgroup analyses that were stratified by history of hypertension, analyses were conducted by smoking status, BMI categories, ART regimen, lipid lowering medication use, activity levels and CD4 nadir less than 200 cells/mm³. As with the previous exploratory subgroup analyses based on the primary model, subgroup stratification did not provide evidence of an association.

Table 8. Carotid IMT against a restricted cubic spline function of 25(OH)D ₃ with 3 knots chosen at clinically relevant values.*				
	25(OH)D ₃ Estimate †	Standard Error	95% CI	P value
Univariable model (n=283)				
Variable 1	0.003	0.002	0.0005 - 0.007	0.025
Variable 2	-0.003	0.001	-0.006 - -0.0005	0.021
Full Model§ (n = 266)				
Variable 1	0.003	0.001	0.0001 - 0.005	0.042
Variable 2	-0.003	0.001	-0.005 - -0.0004	0.019
*Three knots corresponding to unadjusted 25(OH)D ₃ values of 37.5, 80, and 120 nmol/L.				
†Variable coefficients are estimates of slope, each variable corresponding to an interval between the knots.				
§Adjusted for age, gender, smoking status (including current and former smoking), TC:HDL, ethnicity, BMI category, ART-type, CD4 nadir category, CD4 category, lipid lowering medication use, physical activity, years since HIV diagnosis, history of kidney disease, and season.				

Additional restricted cubic spline regression analyses were conducted to further explore the apparent curvilinear relationship between 25(OH)D₃ and carotid IMT. It should be noted that the generated 25(OH)D₃ spline variables are functions of both

25(OH)D₃ concentration and of the specified knots—they are not affected by the response variable carotid IMT. In other words, specification of the knots, by number or by specifying explicit values for knot placement, will influence the relative shape of the 25(OH)D₃-carotid IMT relationship. However, as illustrated in figures 19 through 21 (Figure 21 presents default 3 and 5 knot variations of the relationship) a notable flexion-point from linearity occurs in each case approximate to the upper and lower values of insufficiency and sufficiency, respectively.

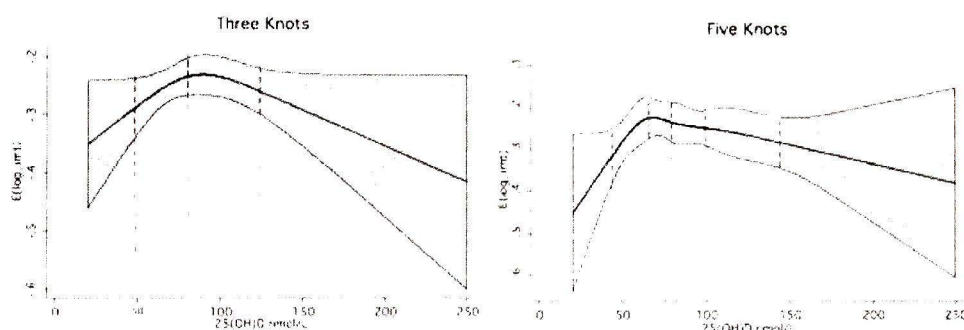


Figure 21. Predicted log carotid IMT based on restricted cubic spline functions of 25(OH)D with three knots (set at 49.1, 79.7 and 123.6 nmol/L) and five knots (set at 44.1, 64.9, 79.9, 98.6, and 144). Analyses were adjusted for age, gender, smoking status (including current and former smoking), TC:HDL, ethnicity, BMI category, ART-type, CD4 nadir category, CD4 category, lipid lowering medication use, physical activity, years since HIV diagnosis, history of kidney disease, and season with each variable set at its mean value. Graph constructed with POSTRCSPLINE¹⁷⁸ in Stata/IC 10.1.

Accordingly, the general shape appears reasonably robust to the influence of knot placement; and in each version there is an apparent positive 25(OH)D₃-carotid IMT association among participants with 25(OH)D₃ values in the suboptimal range (< 75 or < 80 nmol/L).

In order to explore the nature of this finding in greater detail I conducted separate linear regression analyses within each of two populations distinguished by the 25(OH)D₃

cutpoint 80 nmol/L. Note that in addition to its purported clinical relevance, 80 nmol/L also happens to fall on the 25(OH)D₃ median. Notable findings from analyses restricted to participants with less than sufficient 25(OH)D₃ levels are presented below.

None of the analyses restricted to participants with optimal status (in this case defined as a 25(OH)D₃ value $\geq$ 80 nmol/L) produced statistically significant results.

Main results of these exploratory analyses are presented in Table 9. After adjustment for all covariates as per the primary analysis, lower 25(OH)D₃ concentration was statistically significantly associated with higher carotid IMT ($P = 0.043$). Inclusion of 25(OH)D₃, compared with the base covariate model without 25(OH)D₃, demonstrated an improved fit (LR $\chi^2(1)=5.07$, $P = 0.0244$). The model explained approximately 55% of the variation in carotid IMT ($R^2 = .55$), an improvement of approximately 2% over the base covariate model. Evidence of an association was not affected by the inclusion of the prespecified multiplicative terms. Regression diagnostics revealed a potential problem of statistical influence. Removal of one observation in particular had a substantial effect on the statistical significance of 25(OH)D₃ in the model ($P = 0.104$) but had only slight impact on the 25(OH)D₃ coefficient.

Table 9. Estimates from multiple regression models carotid IMT restricted to participants with suboptimal 25(OH)D ₃ status*				
	25(OH)D ₃ * Estimate	Standard error	95% CI	p-value
Univariable (n =142)				
	0.005	0.002	0.001 - 0.008	0.008
Multivariable (n = 135)				
	0.003	0.001	0.0001 - 0.0058	0.043
Stratified by CD4 nadir				
CD4 nadir < 200 cells/mm ³				
Univariable (n =81)	0.006	0.002	0.001 - 0.01	0.019
Multivariable (n = 77)	0.006	0.002	0.001 - 0.01	0.013
CD4 nadir ≥ 200 cells/mm ³				
Univariable (n =59)	0.003	0.002	-0.002 - 0.008	0.224
Multivariable (n = 58)	0.001	0.002	-0.004 - 0.005	0.818

*Defined as $25(\text{OH})\text{D}_3 < 80 \text{ nmol/L}$.

§Adjusted for age, gender, smoking status (including current and former smoking), TC:HDL, ethnicity, BMI category, ART-type, CD4 nadir category, CD4 category, lipid lowering medication use, physical activity, years since HIV diagnosis, history of kidney disease, and season.

As a consequence of the reduced sample size most subgroup analyses within the suboptimal status population were not informative. In the subgroup defined by a CD4 nadir of less than 200 cells/mm^3 the effect of $25(\text{OH})\text{D}_3$ was statistically significant in univariable analysis ($P = 0.019$). It remained statistically significant after inclusion of all covariates as per the primary analysis ($P = 0.013$). Inclusion of $25(\text{OH})\text{D}_3$, compared with the base covariate model without $25(\text{OH})\text{D}_3$, demonstrated an improved fit ($\text{LR } \chi^2(1) = 9.00$, $P = 0.0027$). The model explained approximately 59% of the variation in carotid IMT ($R^2 = 0.59$, $P = 0.0002$), an improvement of approximately 5% over the base covariate model. The statistical significance of $25(\text{OH})\text{D}_3$ in the model was robust to deletion of the previously identified influential observation. In these models the effect of $25(\text{OH})\text{D}_3$ was not significant in analyses restricted to the subgroup of participants without a CD4 nadir of less than 200 cells/mm^3 .

4.2. Discussion

4.2.1. Summary of key results

I hypothesized that there would be greater extent of vascular disease, determined by baseline carotid IMT, among HIV-positive adults with lower compared with higher plasma levels of 25(OH)D. My hypothesis was not confirmed: carotid IMT was not associated with plasma 25(OH)D₃ status in primary analyses.

Surprisingly, 25(OH)D₃ levels exhibited minimal seasonal variation and levels did not substantially correspond to a hypothesized winter-season trough. Another unexpected finding was the high prevalence of vitamin D sufficiency and the low prevalence of vitamin D deficiency in this Canadian HIV-positive population.

In restricted cubic spline linear regression analyses—these were utilized to allow for a hypothesized 25(OH)D₃ curvilinear effect on carotid IMT—spline variables in the 3 knot *clinically relevant* model were statistically significant. The demonstrated curvilinear effect suggests that the 25(OH)D₃-carotid IMT relationship is positive below, and inverse above, the 80 nmol/L cutpoint. Surprisingly, the expected U-shaped relationship is inverted. Consistent with this finding, exploratory regression models restricted to participants comprising the suboptimal range of 25(OH)D₃ status demonstrated an unexpected *positive* linear association between plasma 25(OH)D₃ and carotid IMT.

4.2.2. Strengths and limitations

A main limitation of this investigation is that the Canadian HIV Vascular Study was not designed specifically with the aim of measuring vitamin D associated risk of vascular disease. Consequently, some factors relevant to statistical control are unmeasured or have been measured with perhaps some important level of imprecision. A notable problem was the absence of a precise summary measure for outdoor physical activity. Physical activity is associated with CVD risk, and outdoor activity (particularly during warmer months) is associated with cutaneous vitamin D input. The variable *walking outdoors* would have provided a reasonable measure of control; however, it was only partially captured in the dataset. The workaround was to infer activity level based on patient reported frequency of sweating during physical activity. The effect of this potential residual confounding as a consequence of the inherent imprecision in estimation of vitamin D associated physical activity is not clear.

I was unable to directly adjust for dietary vitamin D input and other dietary factors associated with a healthy lifestyle (e.g. fruit and vegetable intake). However, confounding attributable to diet is presumably controlled by adjustment of mediating factors such as BMI, blood lipids, and dyslipidemia. My original intent of assessing insulin resistance via a HOMA-IR calculation was modified to assessment of blood glucose control (via fasting plasma glucose). This is because HOMA-IR could not be calculated from baseline specimens. A viable alternative to HOMA, waist circumference, was available

for only a subset of the the study population. It is likely that plasma glucose would control for much of the CVD effect associated with insulin resistance; but presumably, residual effects could occur in a small minority of patients with combined hyperinsulinemia and normoglycemia. The direction of residual effect in this case would depend on whether insulin resistance mediates or confounds a 25(OH)D₃-carotid IMT association—plausibly it may do both.

A particular strength of the study was that procedures for the determination of plasma 25(OH)D₃ concentration and carotid artery IMT have been well standardized and validated and were performed under competent supervision. In this regard, findings from the primary univariable analyses (i.e. of no association) should be considered reasonably robust to the influence of measurement error. The problem of residual confounding applies mainly to interpretation of secondary and exploratory analyses where statistical adjustment was indeed necessary for further assessment of positive univariable findings.

This study is limited by its cross-sectional design. The positive association between 25(OH)D₃ and carotid IMT identified in analyses restricted to participants without vitamin D sufficiency cannot be considered causal without further confirmation from prospective investigation. Furthermore, a substantial number of exploratory analyses were performed increasing the probability of having experienced a type I error.

4.2.3. Interpretation

The primary finding that low vitamin D status is not associated with increased carotid IMT is consistent with two previous studies of vitamin D effects on common carotid IMT. Michos et al. cross-sectionally studied a population of 650 Old Order Amish adults living in Pennsylvania (U.S.) and were unable to detect a statistically significant association between 25(OH)D and measures of subclinical vascular disease including coronary artery calcification and carotid IMT.⁹⁸ In a cross-sectional analysis of 614 older adults in the Hoorn study (The Netherlands) low 25(OH)D status was not associated with common carotid artery IMT.⁹⁹ This occurred despite findings from a prior prospective study within the Hoorn cohort that found an association between low vitamin D levels and an increased risk of CVD and all-cause mortality.⁹⁹ This apparent discrepancy highlights some concern about the sensitivity of common carotid IMT (more specifically, as a measure of vascular disease extent) for detection of vitamin D-associated CVD. Hypothetically, the effect of low vitamin D levels on development of CVD might be mediated by factors, for instance by blood pressure or myocardial structural defects, that exert minimal early stage influence on the common carotid IMT.⁹⁹ Or alternatively, as found in a third vitamin D-carotid IMT outcome study, detection of vitamin D associated vascular disease might be partially dependant on the site of carotid IMT measurement. In an investigation involving 654 older adults from the Rancho Bernardo Study (California, U.S.), selected on the basis of a negative history of coronary heart disease, revascularization, or stroke, 25(OH)D was

independently associated with internal carotid IMT ($P = 0.02$), but not with common carotid IMT ($P = 0.83$).¹⁰⁰ As discussed in section 2.4.3, the internal carotid IMT might provide a more sensitive measure of vascular disease change and this might be particularly relevant in the assessment of HIV-associated atherosclerosis.¹¹⁴

The present finding of a non-linear 25(OH)D₃-carotid IMT relationship has not to my knowledge been previously reported. Michos et al. formally tested for a parabolic relationship between 25(OH)D and carotid IMT by the inclusion of a polynomial 25(OH)D term in regression models, however, the effect of the term was not statistically significant.⁹⁸ Wang et al. reported a U-shaped curvilinear relationship between 25(OH)D status and incident CVD risk in 1739 Framingham Offspring Study participants that was characterized by higher CVD risk in participants with lower levels of 25(OH)D.³⁸ In contrast, in the present study the U-shaped relationship between 25(OH)D₃ and CVD risk appears inverted. While the apparent shape confirms a study hypothesis regarding curvilinearity, the inverted orientation was unexpected. Furthermore, while I had hypothesized that flexion points from linearity would resemble flexion cutpoints utilized to categorize vitamin D status—the finding of a threshold effect approximate to 80 nmol/L is certainly in line with this proposition—the inverted shape substantially complicates interpretation. According to the restricted cubic spline analyses, carotid IMT may attain its highest values approximate to the cutpoint differentiating optimal from insufficiency status.

The positive 25(OH)D₃-carotid IMT association noted in participants with suboptimal vitamin D status was confirmed in regression analyses restricted to participants below the 25(OH)D₃ median. This finding might not be generalizable given issues related to the presence of influential data in the analysis. However, irrespective of whether the identified influential observation is included or excluded from analyses, there is on the whole some consistency in support of a positive 25(OH)D₃-carotid IMT association within the suboptimal interval of vitamin D status.

While the biological basis of this finding is not clear, it is consistent with findings from a recent (2010) study that detected a positive association between serum 25(OH)D concentrations and subclinical vascular disease in 340 African American adults with type 2 diabetes.¹⁷⁹ In this relatively vitamin D deplete population living in North Carolina (U.S.), 25(OH)D concentration, in cross-sectional analysis adjusted for age, gender, BMI, glycosylated hemoglobin, and glomerular filtration rate, was positively associated with carotid artery and aortic calcified atherosclerotic plaque ($P = .013$ and $.014$, respectively). This seemingly novel finding is in contrast with the bulk of observational evidence (discussed in section 2.4), that on the whole is consistent with an inverse association between vitamin D levels and CVD risk. Consequently, the study authors, Freedman et al, proposed that their findings might be unique to an African American population. An alternative explanation, in line with findings from the present study, is that the detected positive 25(OH)D-vascular disease association, is unique not by race, but by the popula-

tion's rather homogenous risk level for vitamin D insufficiency. Accordingly, Freedman et al, by selecting a study population on the basis of race, and by implication, by skin colour, may have unintentionally constructed a population with enough similarity to the present study's below median analyses so as to avoid attenuating features inherent in modelling a curvilinear relationship based on incorrect linear assumptions. In other words, Freedman et al, selected a population devoid of the inverse (or perhaps non-linear), component of the inverted-U shape. Hypothetically, their analysis is akin to examining the vitamin D-CVD relationship within one strata of the vitamin D status categorization. Vitamin D status in the African-American Diabetes Heart Study with a mean (SD) of 50.4 (30.5) tended to be somewhat lower than vitamin D status within the present study's suboptimal status category (mean 61.9 (13.4), for participants with $25(\text{OH})\text{D} < 80 \text{ nmol/L}$). Accordingly, the positive association between vitamin D concentration and detectable vascular disease might only be important among groups with an elevated risk for suboptimal vitamin D status.

The cross-sectional design of this study limits interpretation about the direction of effect. One plausible explanation for the the positive association is that low vitamin D levels, below the sufficient threshold, may simply indicate the presence of another factor associated with non-atherosclerotic vascular thickening. For instance physical activity can contribute positively to carotid IMT and low vitamin D status may be a marker of low

physical activity. However, in consideration of a worst case scenario, the positive association could imply that partial but incomplete restoration of vitamin D stores might confer an increased CVD risk.

There are several other unexpected findings. First, in this Canadian population I had hypothesized that 25(OH)D levels should adhere to seasonal variation characterized by an important winter season trough. Surprisingly, my hypothesis was not confirmed.

Second, the prevalence of vitamin D sufficiency was notably higher than expected, and the upper quartile (min max, 100.7 and 250 nmol/L) contributed substantially to detectable differences in across quartile comparisons of BMI, history of hypertension, ongoing exposure to PI-based ART, PTH, and to lipid lowering medication use. With regard to history of hypertension and PTH concentration, this finding is unexpected given that substantial contrast should mainly be noted at the 25(OH)D₃ median (80 nmol/L) as it is at this level where having attained optimal status incremental increases in 25(OH)D₃ levels should in theory no longer correspond to clinical improvement. For the characteristics BMI, ongoing exposure to PI-based ART, and lipid lowering medication use, their disproportionate representation in the upper quartile would be consistent with the proposition that these lead to elevation, or are associated with factors with the potential to elevate, 25(OH)D₃ concentration.

Third, I had hypothesized that NNRTI use would vary across quartiles, and I provided evidence (see section 2.2.5) that PI-use could impair vitamin D metabolism. In

contrast, the largest proportion of the highest quartile was comprised of participants exposed to PI-based ART. NNRTI exposure did not statistically significantly differ across quartiles; however, proportionally, NNRTI-exposed participants are distributed mainly in the lower 3 quartiles.

It is possible that these across quartile drug differences may be clinically important because the synthesis and degradation of 25(OH)D, 1,25(OH)D, and of a number of important drugs utilized in the management in HIV infection share common metabolic pathways. For instance, in a study of 63 participants prescribed atorvastatin following an acute MI, statistically significant improvements in lipid parameters (TC, LDL and triglycerides) were noted only among participants with 25(OH)D levels of at least 30 nmol/L.¹⁸⁰ It has been proposed that 25(OH)D and atorvastatin interact at the level of CYP3A4 metabolism. While few patients within the present study had a 25(OH)D₃ level lower than 30 nmol/L, combined interactions at the level of CYP3A4 metabolism might theoretically alter the 30 nmol/L threshold. To add to this scenario, lipid lowering therapy use in the upper quartile of 25(OH)D₃ might be a biomarker of statin efficacy as statins have also been shown to improve vitamin D status.¹⁸¹ By this proposition statin failure among patients with suboptimal vitamin D status could presumably correspond to increased carotid IMT up to a particular threshold (say 80 nmol/L) at which point statin efficacy, once attained, would attenuate further vascular thickening. Exploratory analysis to test this proposition were not informative, in part perhaps due to low sample size in subgroups.

4.3. Unanswered questions and future research

The primary aim of this study was mainly intended to address the following question: is suboptimal vitamin D status associated with the baseline extent of vascular disease? As per the primary analyses, I was unable to demonstrate an association. The related hypothesis that low vitamin D status would be associated with increased cardiovascular disease risk as evidenced by increased carotid IMT extent, was not confirmed in any of the primary, secondary, or exploratory analyses.

The apparent confirmatory nature of findings related to questions about the presence or absence of an association, and about linearity, shape, and the congruence of this cohort with others, should not be construed to imply that any one study hypothesis has been sufficiently confirmed. Most findings, including the curvilinear inverted-U-shape relationship—it was hypothesized (implicitly) to be upright—were unexpected. Appropriately, in light of this, the strength of inference from these study findings should be commensurate with the high risk of bias associated with a hypothesis generating investigation. With this in mind I address three main areas where study findings might inform future research.

First, one of the more unexpected findings was the high level of vitamin D sufficiency in this Canadian HIV-positive population. Likewise vitamin D deficiency ($25(\text{OH})\text{D}_3 < 37.5 \text{ nmol/L}$) was present in only 2.1% of the study population. While the present study was well designed to assess vitamin D status in the Canadian HIV Vascular

Study cohort, and in consequence these prevalence findings are relatively robust, they are generalizable mainly to HIV-positive white, middle-aged, males. Study findings suggest that suboptimal vitamin D status may be particularly prevalent in blacks: only three of ten black participants were vitamin D sufficient. Future studies should be constructed to assess prevalence data in underrepresented subgroups including females, older persons, dark skinned individuals, intravenous drug users, and persons living with AIDS. Furthermore, the determinants of vitamin D status in this cohort are not clear. Future research could address questions about the the nature of vitamin D input in a Canadian HIV-positive population. In particular, in the Canadian HIV Vascular Study development of hypotheses towards prospective investigation would be enhanced by determining correlates of vitamin D status.

Second, the study found evidence of an inverse-U shape relationship between 25(OH)D and carotid IMT that may peak approximate to upper and lower interval of insufficiency and sufficiency status, respectively. This finding may be somewhat unique to a Canadian HIV-positive cohort. Alternatively, it may represent a novel finding generalizable to an HIV-positive population, or beyond. In either of these two latter scenarios the most important finding may regard assumptions of linearity. The validity of findings derived from analytical methods such as linear and logistic regression analysis are to a certain extent based on assumptions of linearity. A strength of the present study was the use of restricted cubic spline regressions that provided a provision for detecting an associa-

tion that may be too non-linear to have been meaningfully summarized by a linear relationship. If not for these spline analyses the potential association would otherwise have been overlooked. Future investigations into the relationship between vitamin D status and vascular disease should consider the problem of non-linearity as a feature of primary analyses.

Third, emphasis should be placed on investigation of a possible positive association between 25(OH)D₃ and carotid IMT in participants characterized as having suboptimal vitamin D₃ status. This is the second reporting of a positive association between 25(OH)D concentration and vascular disease in otherwise vitamin D insufficient populations. These cross-sectional-based findings would eventually need to be confirmed by prospective investigation, however, at this preliminary stage an efficient cross-sectional investigation may be the most appropriate means of confirming these results in another population.

Fourth, it is unclear to what extent certain features particularly relevant to an HIV-positive population might alter vitamin D metabolism or perhaps even the clinical utility of applying common 25(OH)D status categories in this context. The present study highlights possible interactions (all-informal) between vitamin D and CD4 nadir, ART-type, and lipid lowering therapy use. Future research should evaluate the possibility that medications commonly used in the management of HIV infection might alter vitamin D metabolism, or conversely that vitamin D status might modify the effect of these drugs. In

particular, future investigations in this regard should be well powered to detect 25(OH)D differences across drug exposure categories. Ideally these studies would include bio-markers relevant to vitamin D physiology including PTH, 1,25(OH)₂D, and ionized calcium.

Fifth, analyses of these biomarkers may provide some insight into the validity of evidence regarding unexpected vascular effects in this study. In particular, potential vascular effects of secondary hyperparathyroidism could be explored in the context of the expected inverse PTH-25(OH)D relationship. Exploration of the 25(OH)D threshold, below which hyperparathyroidism occurs, may inform comparability between this HIV-positive population and previously studied cohorts, and may provide a reference point upon which to interpret the clinical relevance of potentially unique study findings.

4.4. Summary of the thesis

In this thesis I presented background evidence suggesting that while suboptimal vitamin D status is highly prevalent across a diverse range of HIV-positive populations, there is no strong basis for proposing that 25(OH)D levels differ by HIV status. Prevalence results of the current study were unexpected: the prevalence of vitamin D₃ deficiency was notably low whereas the majority of participants were vitamin D₃ sufficient. As in earlier investigations, current findings suggest that HIV-positive individuals may be at increased risk of altered vitamin D metabolism: I presented evidence suggesting that PI-exposure and lipid lowering medication use are associated with higher vitamin D₃

status (the upper 25(OH)D₃ quartile). The main finding of this thesis relates to the somewhat conflicting evidence regarding the relationship between vitamin D status and CVD. In primary analyses, as in some earlier studies that have assessed the relationship between 25(OH)D₃ and carotid IMT, plasma 25(OH)D₃ by quartile was not associated with carotid IMT extent. However, in restricted cubic spline regression analyses—these were utilized to accommodate assumptions of non-linearity—there was evidence of an inverted U-shaped 25(OH)D₃-carotid IMT relationship. Furthermore, in exploratory regression models restricted to participants comprising the suboptimal range of vitamin D status, lower 25(OH)D₃ concentration was statistically significantly associated with higher carotid IMT after adjustment for known CVD risk factors and other variables hypothesized to potentially confound a 25(OH)D₃-carotid IMT association. While inference from these exploratory findings requires cautious interpretation, future investigations into the relationship between vitamin D status and vascular disease should consider the problem of non-linearity as a feature of primary analyses; otherwise, such studies might fail to detect a true association.

References

1. Pittas AG, Lau J, Hu FB, Dawson-Hughes B. The role of vitamin D and calcium in type 2 diabetes. A systematic review and meta-analysis. *J Clin Endocrinol Metab* 2007, Jun;92(6):2017-29.
2. Zittermann A, Koerfer R. Vitamin D in the prevention and treatment of coronary heart disease. *Curr Opin Clin Nutr Metab Care* 2008, Nov;11(6):752-7.
3. Wallis DE, Penckofer S, Sizemore GW. The "sunshine deficit" and cardiovascular disease. *Circulation* 2008, Sep 30;118(14):1476-85.
4. Pérez-López FR. Vitamin D metabolism and cardiovascular risk factors in postmenopausal women. *Maturitas* 2009, Mar 20;62(3):248-62.
5. Pilz S, Tomaschitz A, Ritz E, Pieber TR, Medscape. Vitamin D status and arterial hypertension: A systematic review. *Nat Rev Cardiol* 2009, Aug 18.
6. Triant VA, Lee H, Hadigan C, Grinspoon SK. Increased acute myocardial infarction rates and cardiovascular risk factors among patients with human immunodeficiency virus disease. *J Clin Endocrinol Metab* 2007, Jul;92(7):2506-12.
7. D:A:D Study Group, Sabin CA, Worm SW, Weber R, Reiss P, El-Sadr W, et al. Use of nucleoside reverse transcriptase inhibitors and risk of myocardial infarction in hiv-infected patients enrolled in the D:A:D study: A multi-cohort collaboration. *Lancet* 2008, Apr 26;371(9622):1417-26.
8. Strategies for Management of Antiretroviral Therapy (SMART) Study Group, El-Sadr WM, Lundgren JD, Neaton JD, Gordin F, Abrams D, et al. CD4+ count-guided interruption of antiretroviral treatment. *N Engl J Med* 2006, Nov 30;355(22):2283-96.
9. Friis-Møller N, Weber R, Reiss P, Thiébaud R, Kirk O, d'Arminio Monforte A, et al. Cardiovascular disease risk factors in HIV patients--association with antiretroviral therapy. Results from the DAD study. *AIDS* 2003, May 23;17(8):1179-93.
10. Gozdzik A, Barta JL, Wu H, Wagner D, Cole DE, Vieth R, et al. Low wintertime vitamin D levels in a sample of healthy young adults of diverse ancestry living in the Toronto area: Associations with vitamin D intake and skin pigmentation. *BMC Public Health* 2008;8:336.

11. Vieth R, Cole DE, Hawker GA, Trang HM, Rubin LA. Wintertime vitamin D insufficiency is common in young Canadian women, and their vitamin D intake does not prevent it. *Eur J Clin Nutr* 2001, Dec;55(12):1091-7.
12. Rucker D, Allan JA, Fick GH, Hanley DA. Vitamin D insufficiency in a population of healthy western Canadians. *CMAJ* 2002, Jun 11;166(12):1517-24.
13. Holick MF. Vitamin D deficiency. *N Engl J Med* 2007, Jul 19;357(3):266-81.
14. Bikle D. Nonclassic actions of vitamin D. *J Clin Endocrinol Metab* 2009, Jan;94(1):26-34.
15. Hollis BW. Assessment of vitamin D status and definition of a normal circulating range of 25-hydroxyvitamin D. *Curr Opin Endocrinol Diabetes Obes* 2008, Dec;15(6):489-94.
16. Lu Z, Chen TC, Zhang A, Persons KS, Kohn N, Berkowitz R, et al. An evaluation of the vitamin D₃ content in fish: Is the vitamin D content adequate to satisfy the dietary requirement for vitamin D? *J Steroid Biochem Mol Biol* 2007, Mar;103(3-5):642-4.
17. Bikle DD. Vitamin D insufficiency/deficiency in gastrointestinal disorders. *J Bone Miner Res* 2007, Dec;22 Suppl 2:V50-4.
18. Chen TC, Chimeh F, Lu Z, Mathieu J, Person KS, Zhang A, et al. Factors that influence the cutaneous synthesis and dietary sources of vitamin D. *Arch Biochem Biophys* 2007, Apr 15;460(2):213-7.
19. Holick MF, MacLaughlin JA, Doppelt SH. Regulation of cutaneous previtamin D₃ photosynthesis in man: Skin pigment is not an essential regulator. *Science (Wash. DC)* 1981;211:590-3.
20. Hollis BW. Circulating 25-hydroxyvitamin D levels indicative of vitamin D sufficiency: Implications for establishing a new effective dietary intake recommendation for vitamin D. *J Nutr* 2005, Feb;135(2):317-22.
21. Lo CW, Paris PW, Holick MF. Indian and pakistani immigrants have the same capacity as caucasians to produce vitamin D in response to ultraviolet irradiation. *Am J Clin Nutr* 1986, Nov;44(5):683-5.
22. Holick MF. Vitamin D: A millenium perspective. *J Cell Biochem* 2003, Feb 1;88(2):296-307.
23. Mithal A, Wahl DA, Bonjour JP, Burckhardt P, Dawson-Hughes B, Eisman JA, et al. Global vitamin D status and determinants of hypovitaminosis D. *Osteoporos Int* 2009,

- Nov;20(11):1807-20.
24. Lips P. Vitamin D physiology. *Prog Biophys Mol Biol* 2006, Sep;92(1):4-8.
 25. Zerwekh JE. Blood biomarkers of vitamin D status. *Am J Clin Nutr* 2008, Apr;87(4):1087S-91S.
 26. Seamans KM, Cashman KD. Existing and potentially novel functional markers of vitamin D status: A systematic review. *Am J Clin Nutr* 2009, Jun;89(6):1997S-2008S.
 27. Bolland MJ, Grey AB, Ames RW, Horne AM, Mason BH, Wattie DJ, et al. Age-, gender-, and weight-related effects on levels of 25-hydroxyvitamin D are not mediated by vitamin D binding protein. *Clin Endocrinol (Oxf)* 2007, Aug;67(2):259-64.
 28. Prentice A, Goldberg GR, Schoenmakers I. Vitamin D across the lifecycle: Physiology and biomarkers. *Am J Clin Nutr* 2008, Aug;88(2):500S-6S.
 29. Brannon PM, Yetley EA, Bailey RL, Picciano MF. Summary of roundtable discussion on vitamin D research needs. *Am J Clin Nutr* 2008, Aug;88(2):587S-92S.
 30. Haug CJ, Aukrust P, Lien E, Müller F, Espevik T, Frøland SS. Disseminated mycobacterium avium complex infection in AIDS: Immunopathogenic significance of an activated tumor necrosis factor system and depressed serum levels of 1,25 dihydroxyvitamin D. *J Infect Dis* 1996, Jan;173(1):259-62.
 31. Jones G, Horst R, Carter G, Makin HLJ. Contemporary diagnosis and treatment of vitamin D-related disorders. *J Bone Miner Res* 2007, Dec;22 Suppl 2:V11-5.
 32. Dobnig H, Pilz S, Scharnagl H, Renner W, Seelhorst U, Wellnitz B, et al. Independent association of low serum 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels with all-cause and cardiovascular mortality. *Arch Intern Med* 2008, Jun 23;168(12):1340-9.
 33. Aloia JF, Talwar SA, Pollack S, Feuerman M, Yeh JK. Optimal vitamin D status and serum parathyroid hormone concentrations in African American women. *Am J Clin Nutr* 2006, Sep;84(3):602-9.
 34. Stephensen CB, Marquis GS, Kruzich LA, Douglas SD, Aldrovandi GM, Wilson CM. Vitamin D status in adolescents and young adults with HIV infection. *Am J Clin Nutr* 2006, May;83(5):1135-41.
 35. Targher G, Bertolini L, Padovani R, Zenari L, Scala L, Cigolini M, Arcaro G. Serum 25-

- hydroxyvitamin D₃ concentrations and carotid artery intima-media thickness among type 2 diabetic patients. *Clin Endocrinol (Oxf)* 2006, Nov;65(5):593-7.
36. Forman JP, Giovannucci E, Holmes MD, Bischoff-Ferrari HA, Tworoger SS, Willett WC, Curhan GC. Plasma 25-hydroxyvitamin D levels and risk of incident hypertension. *Hypertension* 2007, May;49(5):1063-9.
37. Giovannucci E, Liu Y, Hollis BW, Rimm EB. 25-Hydroxyvitamin D and risk of myocardial infarction in men: A prospective study. *Arch Intern Med* 2008, Jun 9;168(11):1174-80.
38. Wang TJ, Pencina MJ, Booth SL, Jacques PF, Ingelsson E, Lanier K, et al. Vitamin D deficiency and risk of cardiovascular disease. *Circulation* 2008, Jan 29;117(4):503-11.
39. van Etten E, Mathieu C. Immunoregulation by 1,25-dihydroxyvitamin D₃: Basic concepts. *J Steroid Biochem Mol Biol* 2005, Oct;97(1-2):93-101.
40. van Etten E, Stoffels K, Gysemans C, Mathieu C, Overbergh L. Regulation of vitamin D homeostasis: Implications for the immune system. *Nutr Rev* 2008, Oct;66(10 Suppl 2):S125-34.
41. Henry K. Internal medicine/primary care reminder: What are the standards of care for HIV-positive patients aged 50 years and older? *Curr HIV/AIDS Rep* 2009, Aug;6(3):153-61.
42. Currier JS, Lundgren JD, Carr A, Klein D, Sabin CA, Sax PE, et al. Epidemiological evidence for cardiovascular disease in HIV-infected patients and relationship to highly active antiretroviral therapy. *Circulation* 2008, Jul 8;118(2):e29-35.
43. Coodley GO, Coodley MK, Nelson HD, Loveless MO. Micronutrient concentrations in the HIV wasting syndrome. *AIDS* 1993, Dec;7(12): 1595-600.
44. Aukrust P, Haug CJ, Ueland T, Lien E, Müller F, Espevik T, et al. Decreased bone formative and enhanced resorptive markers in human immunodeficiency virus infection: Indication of normalization of the bone-remodeling process during highly active antiretroviral therapy. *J Clin Endocrinol Metab* 1999, Jan;84(1):145-50.
45. Haug C, Müller F, Aukrust P, Frøland SS. Subnormal serum concentration of 1,25-vitamin D in human immunodeficiency virus infection: Correlation with degree of immune deficiency and survival. *J Infect Dis* 1994, Apr;169(4):889-93.
46. Haug CJ, Aukrust P, Haug E, Mørkrid L, Müller F, Frøland SS. Severe deficiency of 1,25-

- dihydroxyvitamin D₃ in human immunodeficiency virus infection: Association with immunological hyperactivity and only minor changes in calcium homeostasis. *J Clin Endocrinol Metab* 1998, Nov;83(11):3832-8.
47. Kuehn EW, Anders HJ, Bogner JR, Obermaier J, Goebel FD, Schlöndorff D. Hypocalcaemia in HIV infection and AIDS. *J Intern Med* 1999, Jan;245(1):69-73.
48. Teichmann J, Stephan E, Discher T, Lange U, Federlin K, Stracke H, et al. Changes in calciotropic hormones and biochemical markers of bone metabolism in patients with human immunodeficiency virus infection. *Metabolism* 2000, Sep;49(9):1134-9.
49. Teichmann J, Stephan E, Lange U, Discher T, Fries G, Lohmeyer J, et al. Osteopenia in HIV-infected women prior to highly active antiretroviral therapy. *J Infect* 2003, May;46(4):221-7.
50. Arpadi S, Horlick M, MacMahon DJ, Bakshi S, Bamji M, Abrams E, Engelson E, Shane E. Vitamin D status and bone mineral density in perinatally HIV-infected children and adolescents living in New York City. Poster presented at: 15th International AIDS Conference; 2004 Jul 11 Bangkok, Thailand.
51. Ramayo E, González-Moreno MP, Macías J, Cruz-Ruiz M, Mira JA, Villar-Rueda AM, et al. Relationship between osteopenia, free testosterone, and vitamin D metabolite levels in HIV-infected patients with and without highly active antiretroviral therapy. *AIDS Res Hum Retroviruses* 2005, Nov;21(11):915-21.
52. Seminari E, Castagna A, Soldarini A, Galli L, Fusetti G, Dorigatti F, et al. Osteoprotegerin and bone turnover markers in heavily pretreated HIV-infected patients. *HIV Med* 2005, May;6(3):145-50.
53. Dolan SE, Kanter JR, Grinspoon S. Longitudinal analysis of bone density in human immunodeficiency virus-infected women. *J Clin Endocrinol Metab* 2006, Aug;91(8):2938-45.
54. García Aparicio AM, Muñoz Fernández S, González J, Arribas JR, Peña JM, Vázquez JJ, et al. Abnormalities in the bone mineral metabolism in HIV-infected patients. *Clin Rheumatol* 2006, Jul;25(4):537-9.
55. Curtis JR, Smith B, Weaver M, Landers K, Lopez-Ben R, Raper JL, et al. Ethnic variations in

the prevalence of metabolic bone disease among HIV-positive patients with lipodystrophy. *AIDS Res Hum Retroviruses* 2006, Feb;22(2):125-31.

56. Tomažič J, Ul K, Volčanšek G, Gorenšek S, Pfeifer M, Karner P, et al. Prevalence and risk factors for osteopenia/osteoporosis in an HIV-infected male population. *Wien Klin Wochenschr* 2007, Nov;119(21-22):639-46.
57. Friis H, Range N, Pedersen ML, Mølgaard C, Changalucha J, Krarup H, et al. Hypovitaminosis D is common among pulmonary tuberculosis patients in Tanzania but is not explained by the acute phase response. *J Nutr* 2008, Dec;138(12):2474-80.
58. Van Den Bout-Van Den Beukel CJ, Fievez L, Michels M, Sweep FC, Hermus AR, Bosch ME, et al. Vitamin D deficiency among HIV type 1-infected individuals in The Netherlands: Effects of antiretroviral therapy. *AIDS Res Hum Retroviruses* 2008, Nov;24(11):1375-82.
59. Rubin D, Wasserman P. Highly prevalent vitamin D deficiency and insufficiency among an urban cohort of human immunodeficiency virus (HIV)-infected men under care. Poster Presented At: 5Th International AIDS Society Conference on HIV Pathogenesis, Treatment and Prevention; 2009 Jul 19-22 Capetown, South Africa.
60. Rodríguez M, Daniels B, Gunawardene S, Robbins GK. High frequency of vitamin D deficiency in ambulatory HIV-positive patients. *AIDS Res Hum Retroviruses* 2009, Jan;25(1):9-14.
61. Arpadi SM, McMahon D, Abrams EJ, Bamji M, Purswani M, Engelson ES, et al. Effect of bimonthly supplementation with oral cholecalciferol on serum 25-hydroxyvitamin D concentrations in HIV-infected children and adolescents. *Pediatrics* 2009, Jan;123(1):e121-6.
62. Wejse C, Gomes VF, Rabna P, Gustafson P, Aaby P, Lisse IM, et al. Vitamin D as supplementary treatment for tuberculosis: A double-blind, randomized, placebo-controlled trial. *Am J Respir Crit Care Med* 2009, May 1;179(9):843-50.
63. Mehta S, Hunter DJ, Mugusi FM, Spiegelman D, Manji KP, Giovannucci EL, et al. Perinatal outcomes, including mother-to-child transmission of HIV, and child mortality and their association with maternal vitamin D status in Tanzania. *J Infect Dis* 2009, Oct 1;200(7):1022-30.
64. Ginde AA, Liu MC, Camargo CA. Demographic differences and trends of vitamin D

- insufficiency in the US population, 1988-2004. *Arch Intern Med* 2009, Mar 23;169(6):626-32.
65. Hollis BW. Measuring 25-hydroxyvitamin D in a clinical environment: Challenges and needs. *Am J Clin Nutr* 2008, Aug;88(2):507S-10S.
66. Golub ET, Benning L, Sharma A, Gandhi M, Cohen MH, Young M, Gange SJ. Patterns, predictors, and consequences of initial regimen type among HIV-infected women receiving highly active antiretroviral therapy. *Clin Infect Dis* 2008, Jan 15;46(2):305-12.
67. Madeddu G, Spanu A, Solinas P, Calia GM, Lovigu C, Chessa F, et al. Bone mass loss and vitamin D metabolism impairment in HIV patients receiving highly active antiretroviral therapy. *Q J Nucl Med Mol Imaging* 2004, Mar;48(1):39-48.
68. Fuchs D, Jäger H, Popescu M, Reibnegger G, Werner ER, Dierich MP, et al. Immune activation markers to predict AIDS and survival in HIV-1 seropositives. *Immunol Lett* 1990, Oct;26(1):75-9.
69. Fuchs D, Krämer A, Reibnegger G, Werner ER, Dierich MP, Goedert JJ, Wachter H. Neopterin and beta 2-microglobulin as prognostic indices in human immunodeficiency virus type 1 infection. *Infection* 1991;19 Suppl 2:S98-102.
70. Copeland KF, Heeney JL. T helper cell activation and human retroviral pathogenesis. *Microbiol Rev* 1996, Dec;60(4):722-42.
71. Macías J, Leal M, Delgado J, Pineda JA, Muñoz J, Relimpio F, et al. Usefulness of route of transmission, absolute CD8+ t-cell counts, and levels of serum tumor necrosis factor alpha as predictors of survival of HIV-1-infected patients with very low CD4 + T-cell counts. *Eur J Clin Microbiol Infect Dis* 2001, Apr;20(4):253-9.
72. Cozzolino M, Vidal M, Arcidiacono MV, Tebas P, Yarasheski KE, Dusso AS. HIV-Protease inhibitors impair vitamin D bioactivation to 1,25-dihydroxyvitamin D. *AIDS* 2003, Mar 7;17(4):513-20.
73. Ikezoe T, Bandobashi K, Yang Y, Takeuchi S, Sekiguchi N, Sakai S, et al. HIV-1 protease inhibitor ritonavir potentiates the effect of 1,25-dihydroxyvitamin D₃ to induce growth arrest and differentiation of human myeloid leukemia cells via down-regulation of CYP24. *Leuk Res* 2006, Aug;30(8):1005-11.

74. Gupta RP, Hollis BW, Patel SB, Patrick KS, Bell NH. CYP3A4 is a human microsomal vitamin D 25-hydroxylase. *J Bone Miner Res* 2004, Apr;19(4):680-8.
75. Gupta RP, He YA, Patrick KS, Halpert JR, Bell NH. CYP3A4 is a vitamin D-24- and 25-hydroxylase: Analysis of structure function by site-directed mutagenesis. *J Clin Endocrinol Metab* 2005, Feb;90(2):1210-9.
76. Bolland MJ, Grey A, Horne AM, Thomas MG. Osteomalacia in an HIV-infected man receiving rifabutin, a cytochrome P450 enzyme inducer: A case report. *Ann Clin Microbiol Antimicrob* 2008;7:3.
77. Brodie MJ, Boobis AR, Hillyard CJ, Abeyasekera G, Stevenson JC, MacIntyre I, Park BK. Effect of rifampicin and isoniazid on vitamin D metabolism. *Clin Pharmacol Ther* 1982, Oct;32(4):525-30.
78. Davies PD, Brown RC, Church HA, Woodhead JS. The effect of anti-tuberculosis chemotherapy on vitamin D and calcium metabolism. *Tubercle* 1987, Dec;68(4):261-6.
79. Pascussi JM, Robert A, Nguyen M, Walrant-Debray O, others. Possible involvement of pregnane X receptor# x02013; enhanced CYP24 expression in drug-induced osteomalacia. *Journal of Clinical Investigation* 2005;115(1):177.
80. Brim NM, Cu-Uvin S, Hu SL, O'Bell JW. Bone disease and pathologic fractures in a patient with tenofovir-induced fanconi syndrome. *AIDS Read* 2007, Jun;17(6):322-8, C3.
81. Gyllenstein K, Josephson F, Lidman K, Saaf M. Severe vitamin D deficiency diagnosed after introduction of antiretroviral therapy including efavirenz in a patient living at latitude 59°N. *AIDS* 2006;20:1906-7.
82. Herzmann C, Arasteh K. Efavirenz-Induced osteomalacia. *AIDS* 2009;23(2):274.
83. Melamed ML, Michos ED, Post W, Astor B. 25-Hydroxyvitamin D levels and the risk of mortality in the general population. *Arch Intern Med* 2008, Aug 11;168(15):1629-37.
84. Ginde AA, Scragg R, Schwartz RS, Camargo CA. Prospective study of serum 25-hydroxyvitamin D level, cardiovascular disease mortality, and all-cause mortality in older U.S. Adults. *J Am Geriatr Soc* 2009, Jun 22.
85. Autier P, Gandini S. Vitamin D supplementation and total mortality: A meta-analysis of randomized controlled trials. *Arch Intern Med* 2007, Sep;167(16):1730-7.

86. Martineau AR, Wilkinson RJ, Wilkinson KA, Newton SM, Kampmann B, Hall BM, et al. A single dose of vitamin D enhances immunity to mycobacteria. *Am J Respir Crit Care Med* 2007, Jul 15;176(2):208-13.
87. Martineau AR, Griffiths CJ, Packe GE. Improved vitamin D status despite allocation to placebo in a tuberculosis treatment trial: Contamination bias? *Am J Respir Crit Care Med* 2009, Jul 15;180(2):189; author reply 189-90.
88. Scragg R, Jackson R, Holdaway IM, Lim T, Beaglehole R. Myocardial infarction is inversely associated with plasma 25-hydroxyvitamin D₃ levels: A community-based study. *Int J Epidemiol* 1990, Sep;19(3):559-63.
89. Wang TJ, Pencina MJ, Booth SL, Jacques PF, Ingelsson E, Lanier K, et al. Vitamin D deficiency and risk of cardiovascular disease. *Circulation* 2008, Jan;117(4):503-11.
90. Pilz S, März W, Wellnitz B, Seelhorst U, Fahrleitner-Pammer A, Dimai HP, et al. Association of vitamin D deficiency with heart failure and sudden cardiac death in a large cross-sectional study of patients referred for coronary angiography. *J Clin Endocrinol Metab* 2008, Oct;93(10):3927-35.
91. Kim DH, Sabour S, Sagar UN, Adams S, Whellan DJ. Prevalence of hypovitaminosis D in cardiovascular diseases (from the National Health and Nutrition Examination Survey 2001 to 2004). *Am J Cardiol* 2008, Dec 1;102(11):1540-4.
92. Pilz S, Dobnig H, Fischer JE, Wellnitz B, Seelhorst U, Boehm BO, März W. Low vitamin D levels predict stroke in patients referred to coronary angiography. *Stroke* 2008, Sep;39(9):2611-3.
93. Watson KE, Abrolat ML, Malone LL, Hoeg JM, Doherty T, Detrano R, Demer LL. Active serum vitamin D levels are inversely correlated with coronary calcification. *Circulation* 1997, Sep;96(6):1755-60.
94. Melamed ML, Muntner P, Michos ED, Uribarri J, Weber C, Sharma J, Raggi P. Serum 25-hydroxyvitamin D levels and the prevalence of peripheral arterial disease: Results from NHANES 2001 to 2004. *Arterioscler Thromb Vasc Biol* 2008, Jun;28(6):1179-85.
95. Forman JP, Bischoff-Ferrari HA, Willett WC, Stampfer MJ, Curhan GC. Vitamin D intake and risk of incident hypertension: Results from three large prospective cohort studies.

Hypertension 2005, Oct;46(4):676-82.

96. Martins D, Wolf M, Pan D, Zadshir A, Tareen N, Thadhani R, et al. Prevalence of cardiovascular risk factors and the serum levels of 25-hydroxyvitamin D in the United States: Data from The Third National Health and Nutrition Examination Survey. *Arch Intern Med* 2007;167(11):1159.
97. Reis JP, von Mühlen D, Miller ER, Michos ED, Appel LJ. Vitamin D status and cardiometabolic risk factors in the united states adolescent population. *Pediatrics* 2009, Aug 3.
98. Michos ED, Streeten EA, Ryan KA, Rampersaud E, Peyser PA, Bielak LF, et al. Serum 25-hydroxyvitamin D levels are not associated with subclinical vascular disease or C-reactive protein in the Old Order Amish. *Calcif Tissue Int* 2009, Mar;84(3):195-202.
99. Pilz S, Henry RM, Snijder MB, van Dam RM, Nijpels G, Stehouwer CD, et al. 25-Hydroxyvitamin D is not associated with carotid intima-media thickness in older men and women. *Calcif Tissue Int* 2009.
100. Reis JP, von Mühlen D, Michos ED, Miller ER, Appel LJ, Araneta MR, Barrett-Connor E. Serum vitamin D, parathyroid hormone levels, and carotid atherosclerosis. *Atherosclerosis* 2009, Jun 6.
101. Parker J, Hashmi O, Dutton D, Mavrodaris A, Stranges S, Kandala NB, et al. Levels of vitamin D and cardiometabolic disorders: Systematic review and meta-analysis. *Maturitas* 2009, Dec 21.
102. Cochrane handbook for systematic reviews of interventions version 5.0.2 [updated september 2009]. In: Higgins JPT, Green S, editors. New Reference. Available from www.cochrane-handbook.org: The Cochrane Collaboration; 2009.
103. Hsia J, Heiss G, Ren H, Allison M, Dolan NC, Greenland P, et al. Calcium/vitamin D supplementation and cardiovascular events. *Circulation* 2007, Feb 20;115(7):846-54.
104. Michos ED, Blumenthal RS. Vitamin D supplementation and cardiovascular disease risk. *Circulation* 2007, Feb 20;115(7):827-8.
105. Wallis DE, Penckofer S. Letter by Wallis and Penckofer regarding article, "calcium/vitamin D supplementation and cardiovascular events". *Circulation* 2007, Jul 17;116(3):e86; author

reply e87.

106. Zittermann A, Schleithoff SS, Koerfer R. Letter by Zitterman et al. regarding article, "calcium/vitamin D supplementation and cardiovascular events". *Circulation* 2007, Jul 17;116(3):e85; author reply e87.
107. Trivedi DP, Doll R, Khaw KT. Effect of four monthly oral vitamin D₃ (cholecalciferol) supplementation on fractures and mortality in men and women living in the community: Randomised double blind controlled trial. *BMJ* 2003, Mar 1;326(7387):469.
108. Timms PM, Mannan N, Hitman GA, Noonan K, Mills PG, Syndercombe-Court D, et al. Circulating MMP9, vitamin D and variation in the TIMP-1 response with VDR genotype: Mechanisms for inflammatory damage in chronic disorders? *QJM* 2002, Dec;95(12):787-96.
109. Gannagé-Yared MH, Azoury M, Mansour I, Baddoura R, Halaby G, Naaman R. Effects of a short-term calcium and vitamin D treatment on serum cytokines, bone markers, insulin and lipid concentrations in healthy post-menopausal women. *J Endocrinol Invest* 2003, Aug;26(8):748-53.
110. Schleithoff SS, Zittermann A, Tenderich G, Berthold HK, Stehle P, Koerfer R. Vitamin D supplementation improves cytokine profiles in patients with congestive heart failure: A double-blind, randomized, placebo-controlled trial. *Am J Clin Nutr* 2006, Apr;83(4):754-9.
111. Pilz S, Dobnig H, Nijpels G, Heine RJ, Stehouwer CD, Snijder MB, et al. Vitamin D and mortality in older men and women. *Clin Endocrinol (Oxf)* 2009, Feb.
112. Stein JH, Korcarz CE, Hurst RT, Lonn E, Kendall CB, Mohler ER, et al. Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: A consensus statement from the american society of echocardiography carotid intima-media thickness task force. Endorsed by the society for vascular medicine. *J Am Soc Echocardiogr* 2008, Feb;21(2):93-111; quiz 189-90.
113. Polak JF, Person SD, Wei GS, Godreau A, Jacobs DR, Harrington A, et al. Segment-Specific associations of carotid intima-media thickness with cardiovascular risk factors: The coronary artery risk development in young adults (CARDIA) study. *Stroke* 2010, Jan;41(1):9-15.
114. Grunfeld C, Delaney JA, Wanke C, Currier JS, Scherzer R, Biggs ML, et al. Preclinical atherosclerosis due to HIV infection: Carotid intima-medial thickness measurements from the

- FRAM study. *AIDS* 2009, Sep 10;23(14):1841-9.
115. Sugden JA, Davies JI, Witham MD, Morris AD, Struthers AD. Vitamin D improves endothelial function in patients with type 2 diabetes mellitus and low vitamin D levels. *Diabet Med* 2008, Mar;25(3):320-5.
116. Odueyungbo A, Smieja M, Thabane L, Smaill F, Gough K, Gill J, et al. Comparison of brachial and carotid artery ultrasound for assessing extent of subclinical atherosclerosis in HIV: A prospective cohort study. *AIDS Res Ther* 2009;6:11.
117. Forman JP, Curhan GC, Taylor EN. Plasma 25-hydroxyvitamin D levels and risk of incident hypertension among young women. *Hypertension* 2008, Nov;52(5):828-32.
118. Forouhi NG, Luan J, Cooper A, Boucher BJ, Wareham NJ. Baseline serum 25-hydroxy vitamin D is predictive of future glycemic status and insulin resistance: The medical research council Ely prospective study 1990-2000. *Diabetes* 2008, Oct;57(10):2619-25.
119. Rostand SG. Ultraviolet light may contribute to geographic and racial blood pressure differences. *Hypertension* 1997, Aug;30(2 Pt 1):150-6.
120. Krause R, Bühring M, Hopfenmüller W, Holick MF, Sharma AM. Ultraviolet B and blood pressure. *Lancet(British Edition)* 1998;352(9129):709-10.
121. Pfeifer M, Begerow B, Minne HW, Nachtigall D, Hansen C. Effects of a short-term vitamin D(3) and calcium supplementation on blood pressure and parathyroid hormone levels in elderly women. *J Clin Endocrinol Metab* 2001, Apr;86(4):1633-7.
122. Scragg R, Khaw KT, Murphy S. Effect of winter oral vitamin D₃ supplementation on cardiovascular risk factors in elderly adults. *Eur J Clin Nutr* 1995;49(9):640-6.
123. Margolis KL, Ray RM, Van Horn L, Manson JE, Allison MA, Black HR, et al. Effect of calcium and vitamin D supplementation on blood pressure: The women's health initiative randomized trial. *Hypertension* 2008, Nov;52(5):847-55.
124. Mitsuhashi T, Morris RC, Ives HE. 1,25-dihydroxyvitamin D₃ modulates growth of vascular smooth muscle cells. *J Clin Invest* 1991, Jun;87(6):1889-95.
125. Li YC. Renoprotective effects of vitamin D analogs. *Kidney Int* 2009, May 27.
126. Rashid G, Bernheim J, Green J, Benchetrit S. Parathyroid hormone stimulates the endothelial nitric oxide synthase through protein kinase A and C pathways. *Nephrol Dial Transplant*

2007, Oct;22(10):2831-7.

127. Sigmund CD. Regulation of renin expression and blood pressure by vitamin D(3). *J Clin Invest* 2002, Jul;110(2):155-6.
128. Li YC, Qiao G, Uskokovic M, Xiang W, Zheng W, Kong J. Vitamin D: A negative endocrine regulator of the renin-angiotensin system and blood pressure. *J Steroid Biochem Mol Biol* 2004, May;89-90(1-5):387-92.
129. Ullah MI, Uwaifo GI, Nicholas WC, Koch CA. Does vitamin D deficiency cause hypertension? Current evidence from clinical studies and potential mechanisms. *Int J Endocrinol* 2010;2010:579640.
130. Marchesi C, Paradis P, Schiffrin EL. Role of the renin-angiotensin system in vascular inflammation. *Trends Pharmacol Sci* 2008, Jul;29(7):367-74.
131. Greenland S. Basic problems in interaction assessment. *Environ Health Perspect* 1993, Dec;101 Suppl 4:59-66.
132. Snijder MB, Lips P, Seidell JC, Visser M, Deeg DJ, Dekker JM, van Dam RM. Vitamin D status and parathyroid hormone levels in relation to blood pressure: A population-based study in older men and women. *J Intern Med* 2007, Jun;261(6):558-65.
133. van den Bout-van den Beukel CJ, van den Bos M, Oyen WJ, Hermus AR, Sweep FC, Tack CJ, et al. The effect of cholecalciferol supplementation on vitamin D levels and insulin sensitivity is dose related in vitamin D-deficient HIV-1-infected patients. *HIV Med* 2008, Oct;9(9):771-9.
134. Lappe JM, Travers-Gustafson D, Davies KM, Recker RR, Heaney RP. Vitamin D and calcium supplementation reduces cancer risk: Results of a randomized trial. *Am J Clin Nutr* 2007, Jun;85(6):1586-91.
135. Nakamura K, Iki M. Efficacy of optimization of vitamin D in preventing osteoporosis and osteoporotic fractures: A systematic review. *Environmental Health and Preventive Medicine* 2006;11(4):155-70.
136. Cranney A, Weiler HA, O'Donnell S, Puil L. Summary of evidence-based review on vitamin D efficacy and safety in relation to bone health. *Am J Clin Nutr* 2008, Aug;88(2):513S-9S.
137. Bischoff-Ferrari HA, Dawson-Hughes B, Willett WC, Staehelin HB, Bazemore MG, Zee

- RY, Wong JB. Effect of vitamin D on falls: A meta-analysis. *JAMA* 2004, Apr 28;291(16):1999-2006.
138. Jackson C, Gaugris S, Sen SS, Hosking D. The effect of cholecalciferol (vitamin D₃) on the risk of fall and fracture: A meta-analysis. *QJM* 2007, Apr;100(4):185-92.
139. Papadimitropoulos E, Wells G, Shea B, Gillespie W, Weaver B, Zytaruk N, et al. Meta-analyses of therapies for postmenopausal osteoporosis. VIII: Meta-Analysis of the efficacy of vitamin D treatment in preventing osteoporosis in postmenopausal women. *Endocr Rev* 2002, Aug;23(4):560-9.
140. Bischoff-Ferrari HA, Willett WC, Wong JB, Giovannucci E, Dietrich T, Dawson-Hughes B. Fracture prevention with vitamin D supplementation: A meta-analysis of randomized controlled trials. *JAMA* 2005, May 11;293(18):2257-64.
141. Bischoff-Ferrari HA, Willett WC, Wong JB, Stuck AE, Staehelin HB, Orav EJ, et al. Prevention of nonvertebral fractures with oral vitamin D and dose dependency: A meta-analysis of randomized controlled trials. *Arch Intern Med* 2009;169(6):551.
142. DIPART (Vitamin D Individual Patient Analysis of Randomized Trials) Group. Patient level pooled analysis of 68 500 patients from seven major vitamin D fracture trials in US and Europe. *BMJ* 2010;340:b5463.
143. Brown TT, Qaqish RB. Antiretroviral therapy and the prevalence of osteopenia and osteoporosis: A meta-analytic review. *AIDS* 2006, Nov 14;20(17):2165-74.
144. Triant VA, Brown TT, Lee H, Grinspoon SK. Fracture prevalence among human immunodeficiency virus (HIV)-infected versus non-HIV-infected patients in a large U.S. Healthcare system. *J Clin Endocrinol Metab* 2008, Sep;93(9):3499-504.
145. Rothman KJ. *Modern epidemiology*. Boston: Little Brown; 1986.
146. Hernán MA, Hernandez-Diaz S, Robins JM. A structural approach to selection bias. *Epidemiology* 2004;15(5):615.
147. Hulley SB, Cummings SR, Browner WS, Grady DG, Newman TB. *Designing clinical research*. Third ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2007.
148. Rothman KJ, Greenland S. Causation and causal inference in epidemiology. *Am J Public Health* 2005;95 Suppl 1:S144-50.

149. Vieth R. How to optimize vitamin D supplementation to prevent cancer, based on cellular adaptation and hydroxylase enzymology. *Anticancer Res* 2009, Sep;29(9):3675-84.
150. Delgado-Rodríguez M, Llorca J. Bias. *J Epidemiol Community Health* 2004, Aug;58(8):635-41.
151. Greenland S, Pearl J, Robins JM. Causal diagrams for epidemiologic research. *Epidemiology* 1999;37-48.
152. Lonn EM, Yusuf S, Doris CI, Sabine MJ, Dzavik V, Hutchison K, et al. Study design and baseline characteristics of the study to evaluate carotid ultrasound changes in patients treated with ramipril and vitamin E: SECURE. *Am J Cardiol* 1996, Oct 15;78(8):914-9.
153. Anand SS, Yusuf S, Vuksan V, Devanesen S, Teo KK, Montague PA, et al. Differences in risk factors, atherosclerosis, and cardiovascular disease between ethnic groups in Canada: The study of health assessment and risk in ethnic groups (SHARE). *Lancet* 2000, Jul 22;356(9226):279-84.
154. Held C, Sumner G, Sheridan P, McQueen M, Smith S, Dagenais G, et al. Correlations between plasma homocysteine and folate concentrations and carotid atherosclerosis in high-risk individuals: Baseline data from the homocysteine and atherosclerosis reduction trial (HART). *Vasc Med* 2008, Nov;13(4):245-53.
155. Lonn EM, Gerstein HC, Sheridan P, Smith S, Diaz R, Mohan V, et al. Effect of ramipril and of rosiglitazone on carotid intima-media thickness in people with impaired glucose tolerance or impaired fasting glucose: STARR (study of atherosclerosis with ramipril and rosiglitazone). *J Am Coll Cardiol* 2009, Jun 2;53(22):2028-35.
156. Carter GD. 25-Hydroxyvitamin D assays: The quest for accuracy. *Clin Chem* 2009, Jul;55(7):1300-2.
157. Evaluation of the elecsys vitamin D₃ 25-OH assay on the roche modular analytics e170 immunoassay analyser; Australasian Association of Clinical Biochemists Annual Scientific Conference 2007. Melbourne, Australia: 2007.
158. Hernán MA, Hernández-Díaz S, Werler MM, Mitchell AA. Causal knowledge as a prerequisite for confounding evaluation: An application to birth defects epidemiology. *Am J Epidemiol* 2002, Jan 15;155(2):176-84.

159. Robins JM, Greenland S. The role of model selection in causal inference from nonexperimental data. *Am J Epidemiol* 1986, Mar;123(3):392-402.
160. Merchant AT, Pitiphat W. Directed acyclic graphs (DAGS): An aid to assess confounding in dental research. *Community Dent Oral Epidemiol* 2002, Dec;30(6):399-404.
161. Shrier I, Platt RW. Reducing bias through directed acyclic graphs. *BMC Med Res Methodol* 2008;8:70.
162. Pearl J. *Causality: Models, reasoning, and inference*. First ed. Cambridge, UK: Cambridge University Press; 2000.
163. Pearl J. More on Pearl's and Rubin's frameworks for causal inference. *Statistical Modeling, Causal Inference, and Social Science Blog* 7 July 2009: Available from: http://www.stat.columbia.edu/~cook/movabletype/archives/2009/07/more_on_pearls.html. Accessed 5 August 2009.
164. Pearl J. "The art and science of cause and effect." Lecture given as part of UCLA 81st faculty lecture series, 29 oct. 1996. UCLA.
165. Christenfeld NJ, Sloan RP, Carroll D, Greenland S. Risk factors, confounding, and the illusion of statistical control. *Psychosom Med* 2004;66(6):868-75.
166. Greenland S, Robins JM. Confounding and misclassification. *Am J Epidemiol* 1985;122(3):495-506.
167. Marshall JR, Hastrup JL. Mismeasurement and the resonance of strong confounders: Uncorrelated errors. *Am J Epidemiol* 1996, May 15;143(10):1069-78.
168. Marshall JR, Hastrup JL, Ross JS. Mismeasurement and the resonance of strong confounders: Correlated errors. *Am J Epidemiol* 1999, Jul 1;150(1):88-96.
169. Fewell Z, Davey Smith G, Sterne JA. The impact of residual and unmeasured confounding in epidemiologic studies: A simulation study. *Am J Epidemiol* 2007, Sep 15;166(6):646-55.
170. Marshall J. Invited commentary: Fewell and colleagues--fuel for debate. *Am J Epidemiol* 2007, Sep 15;166(6):656-8; discussion 659-61.
171. McCarty CA. Sunlight exposure assessment: Can we accurately assess vitamin D exposure from sunlight questionnaires? *Am J Clin Nutr* 2008, Apr;87(4):1097S-101S.
172. Lawlor DA, Davey Smith G, Kundu D, Bruckdorfer KR, Ebrahim S. Those confounded

- vitamins: What can we learn from the differences between observational versus randomised trial evidence? *Lancet* 2004, May 22;363(9422):1724-7.
173. Gorber SC, Schofield-Hurwitz S, Hardt J, Levasseur G, Tremblay M. The accuracy of self-reported smoking: A systematic review of the relationship between self-reported and cotinine-assessed smoking status. *Nicotine Tob Res* 2009, Jan;11(1):12-24.
174. Najjar SS. IMT as a risk marker: The plot thickens. *J Am Soc Echocardiogr* 2009, May;22(5):505-7.
175. Lorenz MW, Markus HS, Bots ML, Rosvall M, Sitzer M. Prediction of clinical cardiovascular events with carotid intima-media thickness: A systematic review and meta-analysis. *Circulation* 2007, Jan 30;115(4):459-67.
176. Wang Y, Jacobs EJ, McCullough ML, Rodriguez C, Thun MJ, Calle EE, Flanders WD. Comparing methods for accounting for seasonal variability in a biomarker when only a single sample is available: Insights from simulations based on serum 25-hydroxyvitamin d. *Am J Epidemiol* 2009, Jul 1;170(1):88-94.
177. Shrout PE, Fleiss JL. Intraclass correlations: Uses in assessing rater reliability. *Psychol Bull* 1979, Mar;86(2):420-8.
178. POSTRCSPLINE: Stata module containing post-estimation commands for models using a restricted cubic spline [computer program]. <http://ideas.repec.org/c/boc/bocode/s456928.html>: Buis, Maarten L; 2009.
179. Freedman BI, Wagenknecht LE, Hairston KG, Bowden DW, Carr JJ, Hightower RC, et al. Vitamin D, adiposity, and calcified atherosclerotic plaque in African-Americans. *J Clin Endocrinol Metab* 2010, Mar;95(3):1076-83.
180. Pérez-Castrillón JL, Abad Manteca L, Vega G, Del Pino Montes J, de Luis D, Duenas Laita A. Vitamin D levels and lipid response to atorvastatin. *Int J Endocrinol* 2010;2010:320721.
181. Pérez-Castrillón JL, Vega G, Abad L, Sanz A, Chaves J, Hernandez G, Dueñas A. Effects of atorvastatin on vitamin D levels in patients with acute ischemic heart disease. *Am J Cardiol* 2007, Apr 1;99(7):903-5.

Appendix A

Medline search strategy for studies relevant to CVD, vitamin D, and HIV-in-

fection. This was initially applied to OVID Medline (R), 1956 to April 2009. The strategy was later adapted to EMBASE 1988 to 2009 Week 17 (April).

1. exp Vitamin D Deficiency/
2. exp Vitamin D/
3. vitamin d.mp.
4. 7-dehydrocholesterol.mp.
5. exp Receptors, Calcitriol/
6. calcitriol.mp.
7. exp Cholecalciferol/
8. 25-Hydroxyvitamin D3 1-alpha-Hydroxylase/
9. 25-hydroxycholecalciferol.mp.
10. 6 or 3 or 7 or 9 or 2 or 8 or 1 or 4 or 5
11. exp HIV/
12. HIV Infections/
13. exp Anti-HIV Agents/
14. 11 or 13 or 12
15. 10 and 14
16. exp Cardiovascular Diseases/
17. carotid IMT.mp.
18. carotid intima medial thickness.mp.
19. intima-media thickness.mp.
20. exp Arteriosclerosis/
21. 18 or 19 or 16 or 17
22. 18 or 19 or 17 or 20
23. 21 and 10 and 14
24. hiv.mp. [mp=title, original title, abstract, name of substance word, subject heading word]
25. 11 or 24 or 13 or 12
26. 25 and 10
27. 21 and 10
28. 22 and 10
29. from 28 keep 1-10

- 30. from 29 keep 1-10
- 31. 6 or 1 or 3 or 7 or 9 or 2
- 32. 16 and 31
- 33. limit 32 to (humans and english)
- 34. from 33 keep 2-3,5,9-10,15,25,31,35,40,56,59-61,70-72,83,87,95,97
- 35. from 33 keep 102,111,115,117,122,126-128,131,138,146,150,185,194,196-197
- 36. from 33 keep 317,321,323,328,358,376-378,384-386,393,397
- 37. from 33 keep 406,412,415,417-418,444,446,461,463,471,474,479,481,486-488,495
- 38. from 33 keep 506,509,515,527,535,548-549,584,588
- 39. from 33 keep 604,622,624,630,634,648,654-656,673,676,691-692,696,698
- 40. from 33 keep 734,739,760,771,780,796
- 41. from 33 keep 809,811,813,898
- 42. from 33 keep 941,977,981,989
- 43. from 33 keep 1063

Appendix B

Medline search strategy for studies relevant to CVD and vitamin D without specification of HIV-infection. The initial search utilized OVID Medline (R), 1956 to March 2009.

1. exp Vitamin D Deficiency/
2. exp Vitamin D/
3. vitamin d.mp.
4. 7-dehydrocholesterol.mp.
5. exp Receptors, Calcitriol/
6. calcitriol.mp.
7. exp Cholecalciferol/
8. 25-Hydroxyvitamin D3 1-alpha-Hydroxylase/
9. 25-hydroxycholecalciferol.mp.
10. 6 or 3 or 7 or 9 or 2 or 8 or 1 or 4 or 5
11. exp HIV/
12. HIV Infections/
13. exp Anti-HIV Agents/
14. 11 or 13 or 12
15. 10 and 14
16. exp Cardiovascular Diseases/
17. carotid IMT.mp.
18. carotid intima medial thickness.mp.
19. intima-media thickness.mp.
20. exp Arteriosclerosis/
21. 18 or 19 or 16 or 17
22. 18 or 19 or 17 or 20
23. 21 and 10 and 14
24. hiv.mp. [mp=title, original title, abstract, name of substance word, subject heading word]
25. 11 or 24 or 13 or 12
26. 25 and 10
27. 21 and 10
28. 22 and 10

- 29. from 28 keep 1-10
- 30. from 29 keep 1-10
- 31. 6 or 1 or 3 or 7 or 9 or 2
- 32. 16 and 31
- 33. limit 32 to (humans and english)
- 34. from 33 keep 2-3,5,9-10,15,25,31,35,40,56,59-61,70-72,83,87,95,97
- 35. from 33 keep 102,111,115,117,122,126-128,131,138,146,150,185,194,196-197
- 36. from 33 keep 317,321,323,328,358,376-378,384-386,393,397
- 37. from 33 keep 406,412,415,417-418,444,446,461,463,471,474,479,481,486-488,495
- 38. from 33 keep 506,509,515,527,535,548-549,584,588
- 39. from 33 keep 604,622,624,630,634,648,654-656,673,676,691-692,696,698
- 40. from 33 keep 734,739,760,771,780,796
- 41. from 33 keep 809,811,813,898
- 42. from 33 keep 941,977,981,989
- 43. from 33 keep 1063
- 44. from 33 keep
201-203,210,214,219,225-226,231,233-234,236,242-243,245,247,254,258-259,263,271-272,286-287,290,296,299-300
- 45. 35 or 39 or 40 or 36 or 41 or 38 or 42 or 34 or 37 or 43 or 44

Appendix C

The Canadian HIV Vascular Study: Baseline Nursing Questionnaire

Site #	Patient ID	Initials	Year/Month/Day

The following questions collect information related to your vitamin D status.

1. Which ONE of the following best describes your SKIN COLOUR?

- ☐ **Very fair** (my skin burns easily, does not tan)
☐ **Fair** (my skin burns easily but I can develop a slight tan)
☐ **Light** (my skin is light during the winter but I can tan easily in the summer)
☐ **Medium** (e.g. always at least somewhat brown or olive, rarely burns)
☐ **Dark to black**

2. What types of SUPPLEMENTS do you take each day? (circle all that apply)

Multivitamin Calcium WITH vitamin D Cod LIVER oil Vitamin D alone None
 Calcium Without vitamin D

3. On average, how many glasses of MILK do you drink each DAY? Cow's ____ /day
 Rice/Soy ____/day
4. On average, how many servings of SALMON or TUNA do you eat each WEEK? ____/week

The following questions relate to seasonal changes in vitamin D status

5. During the COLDER MONTHS of the year (NOVEMBER through APRIL) do you:

- a) Supplement with extra vitamin D? ☐ Yes ☐ No
 b) Use a TANNING BED or SUNLAMP? ☐ Yes ☐ No
 c) TRAVEL to a WARM SOUTHERN CLIMATE most years? ☐ Yes ☐ No
 d) If yes to (c), do you develop a SUNTAN on your arms or legs? ☐ Yes ☐ No

6. During the WARMER MONTHS of the year (MAY through OCTOBER):

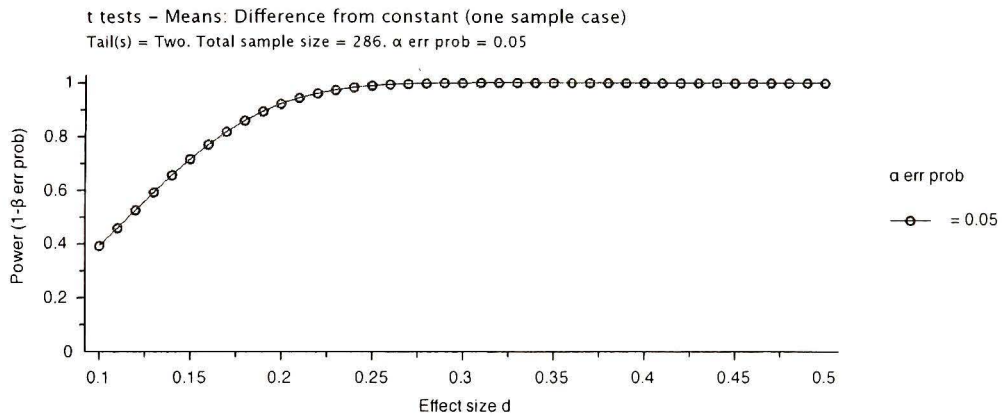
- a) How many days per week are you outside for at least 30 minutes (BETWEEN 9 AM TO 5PM)? ____ days/week
 b) Do you regularly use sunscreen ☐ Yes ☐ No
 c) When outside are your limbs (arms and legs) usually covered? ☐ Yes ☐ Partial ☐ No

The following questions relate to your ability to absorb dietary vitamin D

7. During a TYPICAL week, do you experience diarrhea or very loose stools? And if yes, how many days does this typically occur? ☐ Yes ☐ No
 ____ days/week

Appendix D

Figure A. Power analysis



The power analysis graph was created using G*Power 3.0.10