Investigation into the mechanism and treatment of atherogenic dyslipidemia in HIV+ patients with HIV metabolic syndrome

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BSc., The University of British Columbia, 1996

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
in
THE FACULTY OF GRADUATE STUDIES
(Department of Pathology and Laboratory Medicine)

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
March, 2004

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Title of Thesis: INVESTIGATION INTO THE MECHANISM AND TREATMENT OF AHEROGENIC DYSLIPIDEMIA IN HIV+ PATIENTS WITH HIV METABOLIC SYNDROME

Degree: M. Sc. Year: 2004

Department of PATHOLOGY AND LABORATORY MEDICINE
The University of British Columbia
Vancouver, BC Canada
ABSTRACT

The use of highly active combination anti-retroviral therapy is associated with serious metabolic side effects including increased serum triglycerides, decreased HDL cholesterol as well as insulin resistance, peripheral fat loss and gain of abdominal visceral fat. There is concern that anti-retroviral therapy imposes a significant risk of heart disease in the HIV positive population.

We hypothesize that development of dyslipidemia in response to anti-retroviral therapy is associated with the presence of specific genetic polymorphisms and may relate to changes in lipoprotein lipase and hepatic lipase levels.

We have assessed a number of biochemical and genetic markers in a cohort of HIV+ subjects with dyslipidemia. In our cohort lipoprotein lipase is significantly decreased (36.03 ± 30.17 nmol/min/ml vs. 86.79 ± 63.96 nmol/min/ml, p<0.01) and hepatic lipase is significantly increased (225.15 ± 104.60 nmol/min/ml vs. 139.02 ± 40.89 nmol/min/ml, (p < 0.05) compared to healthy controls. As well, our cohort had significantly elevated serum lipids (total cholesterol 7.2 mmol/L, HDL-C 0.9 mmol/L, TG 7.8 mmol/L, TC/HDL-C 8.5). 12% of the subjects in our cohort had fasting glucose levels above 7 mmol/L, the cut-off for a diagnosis of diabetes. There was no association between the polymorphisms of LPL (Asn291Ser), ApoE (4/4, 4/3, 3/3, 3/2, 2/2), or Apo CIII (SstI G3238C) and serum lipids in our cohort.

In our cohort fibrates significantly decreased serum triglycerides (37%, p=0.028) while fibrates, in combination with statins significantly decreased serum triglycerides and total cholesterol (52%, p=0.002 and 20%, p=0.007 respectively). There were no significant changes in serum HDL, or in the ratio of total to HDL cholesterol in either of the treatment groups. Supplementation with fish oil did not improve serum lipids compared to placebo.
In conclusion, while we have not demonstrated an association between selected polymorphisms of LPL, apo E or apo C-III, we have demonstrated that LPL activity is decreased and HL activity is increased in our cohort of HIV+ patients with dyslipidemia. We believe that the combination of risk factors identified in our study, and results of previous studies, suggest that this cohort is at significantly increased risk of coronary artery disease.
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<th>Full Form</th>
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<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>Apo</td>
<td>Apolipoprotein (A, B, C,...)</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CRABP</td>
<td>Cytoplasmic retinoic-acid binding protein</td>
</tr>
<tr>
<td>DHA</td>
<td>Decosahexanoic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentanoic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fasting blood sugar</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active anti-retroviral therapy</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HDL-C</td>
<td>HDL cholesterol</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HL</td>
<td>Hepatic lipase</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate density lipoprotein</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LDL-C</td>
<td>LDL cholesterol</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>LRP</td>
<td>Low density lipoprotein receptor related binding protein</td>
</tr>
<tr>
<td>n-3 FA</td>
<td>Omega-3 fatty acid</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-nucleoside analog reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside analog reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>PI</td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptors</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element binding proteins</td>
</tr>
<tr>
<td>TC/HDL</td>
<td>Ratio of total to HDL cholesterol</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>TZD</td>
<td>Thiazolidinedione</td>
</tr>
<tr>
<td>VA-HIT</td>
<td>Veterans administration HDL-C intervention trial</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

I would like to thank Drs. Jiri Frohlich, Greg Bondy and John Hill and all the staff and students at the Healthy Heart Program at St. Paul’s Hospital. Without their support this work would never have been completed.

I would also like to thank my family, Angela and Cholmondeley for their support and encouragement throughout.

Thank you,
1 INTRODUCTION

1.1 HUMAN IMMUNODEFICENCY VIRUS

The HIV is an RNA virus of the family retroviridae which infects CD4+ T-lymphocytes, macrophages, microglial cells and dendritic cells. Infection of a cell begins with binding of viral gp-120 envelope protein with host cell CD4. Once un-coated inside the cell, viral reverse transcriptase catalyzes synthesis of a DNA copy (provirus) of the viral genome which then inserts into the host chromosomal DNA. Integrated proviral DNA can remain latent or can actively transcribe RNA leading to synthesis of new virions. Untreated infection leads to depletion of CD4+ T-lymphocytes by a variety of mechanisms including direct HIV mediated single cell killing and syncytium formation, and by HIV specific immune responses [1]. Progressive loss of CD4+ T-lymphocytes during HIV infection results in failure of innate and cell-mediated immunity rendering the patient susceptible to opportunistic infections.

The clinical course of HIV is monitored by measurement of the level of CD4+ T-lymphocytes and viral load. In Canada, once a patient is infected with HIV he is said to be HIV positive until the development of an AIDS defining illness, at which point he is classified as having AIDS. The United States include CD4 counts in their classification and an HIV+ patient is said to have AIDS if he develops an AIDS defining illness and/or CD4 count drops below 200 cells/mm$^3$. 
1.1.1 ANTI-RETROVIRAL THERAPY

HIV therapies target viral enzymes that are essential in two stages of the viral life cycle: Viral reverse transcriptase and viral protease. A typical treatment regimen utilizes the classes of drugs in combinations designed to achieve maximum viral suppression and to minimize emergence of viruses resistant to any one drug.

1.1.1.1 Reverse transcriptase inhibitors.

There are two classes of viral reverse transcriptase inhibitors. Nucleoside analog reverse transcriptase inhibitors (NRTIs) are structurally related to endogenous nucleotides and are designed to compete for reverse transcriptase. Incorporation of an NRTI into the elongating proviral DNA chain causes chain termination, thus inhibiting viral reverse transcriptase [2, 3]. Available NRTIs include Zidovudine (AZT), Lamivudine (3TC), Didanosine (ddI), Zalcitabine (ddC), Stavudine (d4T) and Abacavir. First available in the mid eighties, NRTIs provided the first options for treating HIV infection and initially resulted in substantial reductions in mortality. However, these reductions were not sustainable in the long term [4].

The second class of reverse transcriptase inhibitors is the non nucleoside analog reverse transcriptase inhibitors (NNRTIs). Drugs in this class bind specifically and non-competitively with HIV RT, or the HIV-RT-template primer-dNTP complex [5-7] resulting in the inhibition of reverse transcriptase. NNRTIs are often used in initial therapy regimens designed to avoid toxicities of protease inhibitors or to save protease inhibitors for later in infection [8]. There are three NNRTIs available: Nevirapine, Efavirenz and Delavirdine.
1.1.1.2 **Protease inhibitors:**

HIV core and structural proteins are translated as a polyprotein which must be cleaved to produce infectious virions [9]. HIV protease is a dimeric aspartyl protease [10] that targets specific amino acid sequences within this polyprotein. Inhibitors of HIV protease (PIs) are synthetic drugs, first available in 1995, that are designed to inhibit HIV protease specifically. The currently available PIs are Ritonavir, Saquinavir, Indinavir, Nelfinavir and Amprenavir.

1.1.1.3 **Combination therapy (HAART)**

Introduction of PIs allowed for the generation of highly effective combination therapy regimens commonly referred to as Highly Active Anti-Retroviral Therapy (HAART). HAART regimens, which often include two NRTIs with either a PI or an NNRTI, have resulted in dramatic and sustainable decreases in morbidity and mortality in HIV infected patients [11-17]. In one of the clearest examples, results from the Swiss HIV Cohort Study indicated that triple therapy, including a PI, reduced the likelihood of AIDS diagnosis by 42% when the CD4+ count drops below 200/uL, and reduced mortality by 65% compared to no anti-retroviral therapy [18].

The International AIDS Society – USA panel continues to advocate the use of combination anti-retroviral therapy although it recommends initiation of therapy only in symptomatic HIV+ patients (those with an HIV related opportunistic infection) and in asymptomatic patients with a CD4 count below 200 cells/uL. In asymptomatic patients with a CD4 count greater than 200 cells/uL the decision to start treatment is individual but, should be based on viral load, rate of CD4 decline, risks of drug toxicity, and on experience and wishes of the physician and the patient [8]. The desire to delay therapy stems from concerns
about the likelihood of developing drug resistance, the substantial pill and lifestyle burdens required of HAART, and from concerns about side effects of medication.

1.1.2 METABOLIC COMPLICATIONS OF HIV

1.1.2.1 Metabolic complications of HIV: the pre HAART era

Metabolic complications are common in HIV infection but their nature has changed since the introduction of HAART therapy. Prior to HAART, HIV infection was associated with lower serum total cholesterol, high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) [19-21] and with a pro-atherogenic decrease in LDL particle size [22]. Serum triglycerides (TG) increased as HIV progressed, being inversely correlated with CD4 count and positively correlated with development of opportunistic infections [19, 23] and, in patients with AIDS, positively correlated with levels of interferon α (IFNα) [20, 24]. Other studies showed increased insulin sensitivity in HIV+ patients compared with healthy controls [25, 26], a finding that is contrary to the situation in other acute infections [27].

The most significant metabolic complication of HIV, prior to HAART, was wasting syndrome. This syndrome is one of profound weight loss (greater than 10%), involving both lean body mass and adipose compartments, associated with either persistent diarrhea or chronic weakness and fever [28]. Development of wasting syndrome correlates with advancing HIV infection and progression to AIDS. While the aetiology of the syndrome itself is not completely understood, it may arise from increases in resting energy expenditure [29, 30], decreased caloric intake, gastrointestinal disturbances and increased levels of circulating cytokines related to secondary infections [31, 32].
1.1.2.2 Metabolic Complications of HIV in the HAART era: *HIV metabolic syndrome*

The metabolic picture of HIV+ patients in the HAART generation has been profoundly different. While HAART has dramatically reduced morbidity and mortality, it has also been associated with a number of metabolic side effects including mitochondrial toxicity, bone abnormalities as well as hyperlipidemia, insulin resistance and lipodystrophy collectively known as HIV metabolic syndrome.

A review of early efficacy studies of Ritonavir (a PI) reveals the potential for lipid side effects. One study reported a 200-300% increase in serum TG and a 30 to 40% increase in total cholesterol in 32 weeks of mono-therapy [33]. Another study reported a greater than 200% increase in serum TG in approximately 63% of subjects compared to 19% of placebo treated controls [34]. Both studies demonstrated a significant benefit of ritonavir with respect to HIV infection.

As PI containing HAART use became widespread a series of anecdotal cases of hyperlipidemia, hyperglycemia and lipodystrophy were reported in HIV+ patients undergoing therapy with PI containing regimens. In each case, subjects had no prior history of dyslipidemia or diabetes [35-38]. However, given the breakthrough in HIV treatment offered by PIs, initial impressions were that “the metabolic side effects did not outweigh the improvements in survival seen with PI therapy” [39].

Carr et al. published the first study specifically reporting lipid and metabolic complications of HAART in 1998. Their study was a cross sectional comparison of 116 HIV+ subjects on PI containing regimens, 32 PI naïve HIV+ subjects and 47 healthy controls [40]. In the PI treated group, serum TG (3.3 mmol/L vs. 1.2 mmol/L p<0.01), total
cholesterol (5.9 mmol/L vs. 4.9 mmol/L p<0.0001), C-peptide (2.5 vs. 1.1 p<0.0001) and insulin (9.1 vs. 5.1 p<0.01) were significantly higher than in healthy controls while only serum TG (3.3 mmol/L vs. 1.6 mmol/L p<0.003) and total cholesterol (5.9 mmol/L vs. 4.5 mmol/L p<0.0001) were significantly higher compared to the PI naïve group. As well, peripheral lipodystrophy occurred in 64% of PI treated and 3% of PI naïve subjects. In subjects with lipodystrophy, serum TG (4.1 mmol/L vs. 1.8 mmol/L p<0.05), C-peptide (2.87 vs. 2.14 p<0.01) and insulin (10.1 vs. 7.5 p<0.05) were significantly higher and the group was more insulin resistant (2.23 vs. 1.58 p<0.05) than those without lipodystrophy.

Two retrospective studies confirmed the association of PI therapy with hyperlipidemia. Tsiodras et al. reported on a five year historical study of 221 adult subjects between 1993 and 1998 [41]. They showed an independent association between PI use and hyperlipidemia, hyperglycemia and lipodystrophy. When adjusted for multivariable models PI containing regimens were associated with a 2.8 fold higher incidence of hypercholesterolemia (p=0.001) and a 6.1 fold higher incidence of hypertriglyceridemia (p<0.001). Among the 176 patients who started PI therapy, ritonavir use was 2.6 times more likely to cause hypertriglyceridemia than other PIs (p=0.02). An important finding of this study was that development of hyperlipidemia was unrelated to improvement of immune function resulting from PI use. A second, much larger retrospective analysis of the French Aquitaine Cohort (1429 subjects) found that serum TG level could be predicted by age (p<10^{-4}), AIDS stage (p=0.001), homosexuality (p=0.003), weight of 65kg (p=0.002) and by PI use (p<10^{-4}) [42].

A prospective study followed 19 consecutive HIV+ males for an average of 22 weeks after initiating therapy with a PI containing regimen [43]. The subjects in this study had significant increases in serum TG (1.72 to 2.80 mmol/L p=0.014) and serum total cholesterol
(4.09 to 4.81 mmol/L p=0.01) and a non-significant increase in serum LDL-C. Increased very low density lipoprotein (VLDL) appeared to account for the elevation of serum TG (serum VLDL TG increased from 1.44 mmol/L to 2.42 mmol/L p=0.01). Serum HDL-C did not change significantly but there was a significant decrease in the ratio of the larger HDL₂ to smaller more atherogenic HDL₃.

Two studies in which PIs were removed from drug regimens confirmed the role of PIs in dyslipidemia associated with HIV metabolic syndrome. The first, by Carr et al., followed eighty one HIV+ subjects with lipodystrophy, randomized to continue PI therapy or to switch PIs for abacavir, nevirapine, adefovir and hydroxyurea. The data showed that switching from a PI based to an NNRTI regimen results in a significant decline in serum lipids and in intra-abdominal as well as peripheral fat with minimal effect on insulin resistance [44]. A second study interrupted HAART in 26 HIV+ men for seven weeks resulting in a significant reduction in serum TG (2.95 vs. 2.09 mmol/L, p=0.008), total cholesterol (5.02 vs. 4.11 mmol/L p=0.0001) and in LDL-C (2.95 vs. 2.48 mmol/L p=0.0013) but in no significant changes in glucose, insulin or anthropometric measurements. There was however a significant decrease in 24-hour urinary 17-hydroxycorticosteroids (15 vs. 5 mg/24 h, p<0.0001) and a significant increase in 24-hour urinary free cortisol (45 vs. 62 μg/24 h, p=0.016) [45].

1.1.2.3 Metabolic Complications of HIV in the HAART era: Lipodystrophy, mitochondrial toxicity and bone abnormalities

As well as dyslipidemia, anti-retroviral therapy has been associated with a number of other metabolic side effects including lipodystrophy, mitochondrial toxicity and osteopenia. While there is a strong correlation between PI use and dyslipidemia, NRTIs have been
implicated in the development of mitochondrial toxicity and lipodystrophy. The role of HAART in the development of bone abnormalities is less clear.

Lipodystrophy related to HAART is typically manifested by loss of peripheral fat including arm, leg, buttock and facial fat, and an increase in central adiposity. Less frequently patients develop a “buffalo hump” resulting from excessive growth of the dorso-cervical and occipital fat pads. Initially lipodystrophy was thought to be related to PI use, although several studies have reported it occurring in patients treated with PI sparing regimens [46-53]. Estimates of prevalence vary greatly between studies and depend on the site of lipodystrophy. For example, prevalence of lipodystrophy (of any type) varies from 2-84% in patients taking PIs while buffalo hump may occur in only 2-5% of subjects and increased abdominal girth in 1-56% [54].

The association between lipodystrophy and lipid complications of HAART is equally incomplete. While it seems that hyperlipidemia can occur with minimal lipodystrophy, the presence of lipodystrophy is associated with more severe hyperlipidemia and is strongly associated with the development of insulin resistance [40]. Likewise, changes in serum lipids are dependent on the specific manifestation of lipodystrophy. A study by Hadigan et al. [50] compared subjects with isolated lipoatrophy (peripheral loss), those with isolated lipohypertrophy (abdominal gain) and those with mixed lipodystrophy (peripheral loss combined with abdominal gain). Subjects with lipoatrophy had higher serum TG (6.07 vs. 3.26 mmol/L, p<0.05) and lower serum HDL-C (0.78 vs. 1.01 mmol/L, p<0.05), but had lower fasting insulin (10.8 vs. 21.4 μU/mL, p<0.05) and 2-h OGTT insulin (41.2 vs. 120.6 μU/mL, p<0.05) than subjects with mixed lipodystrophy.
It has been known for many years that use of NRTIs is associated with mitochondrial toxicity [55], the pathogenesis of which involves inhibition of mitochondrial DNA polymerase γ [56, 57]. This results in depletion of mitochondrial DNA leading to a number of distinct syndromes such as lactic acidemia, myopathy, pancreatitis, renal insufficiency and peripheral neuropathy. There is some indication that mitochondrial toxicity may also be involved in the pathogenesis of lipodystrophy [58-62] although there is evidence that PIs affect adipocyte differentiation as well, [63, 64] and thus may also cause peripheral lipodystrophy.

Bone abnormalities have long been a concern in HIV patients. Prior to the availability of HAART the major bone abnormality in HIV patients was osteonecrosis, but, as is the case with most metabolic complications, the nature of bone abnormalities changed with the advent of HAART treatment. Presently there is growing concern about osteopenia which appears to be related to the use of PI based anti-retroviral regimens. Recent studies, reviewed in [65] report osteopenia in 22% to 50% of patients on PI based regimens and osteoporosis in 3% to 22% of patients. The mechanism of bone loss is unknown but may be related to PI inhibition of vitamin D metabolism, mitochondrial toxicity, changes in cytokine levels, or may be related to HIV infection itself (reviewed in [66]).

1.1.2.4 Metabolic complications of HIV: Summary and Cardiovascular risk

In summary, HIV infection and the subsequent use of HAART is associated with a multitude of metabolic side effects including dyslipidemia, insulin resistance and lipodystrophy as well as osteopenia and mitochondrial toxicity. The term HIV metabolic syndrome collectively refers to the hypertriglyceridemia, hyperlipidemia, decreased HDL, decreased HDL and LDL particle size, insulin resistance and possibly fat distribution
changes that are associated primarily with the PI use. The phenotype of HIV metabolic
syndrome has been confirmed by several other studies \[39, 47, 67-71\]. Although the degree
to which different PI’s cause dyslipidemia differs, ritonavir has been most commonly
associated with significant hypertriglyceridemia \[37, 42, 68\].

Given the constellation of CAD risk factors common to HIV metabolic syndrome,
there is obvious concern about the long term risk of CAD in these patients. Until recently,
the bulk of evidence for increased incidence of CAD has been limited to case reports \[72-78\],
while two cohort studies have, in fact, not shown any significant increase in CAD since the
availability of HAART therapy \[79, 80\]. In the past year however, two large studies
published in the New England Journal of Medicine have shown conflicting evidence of risk.
The first retrospectively followed 36,766 HIV+ patients in the US Veterans Affairs health
network and did not show any significant increase in incidence of cardiovascular or
cerebrovascular events \[81\]. The second study prospectively followed 23,468 patients from
the DAD study group (Data Collection on Adverse Events of Anti-HIV Drugs) over 36,199
patient years \[82\]. The Authors report an adjusted relative rate of myocardial infarction (MI)
of 1.26 per year of exposure to combination anti-retroviral therapy. Despite this, the actual
rate of MI was low and the authors caution that the risk must be balanced with the benefit of
anti-retroviral treatment.

Because HIV metabolic syndrome is a relatively recent development that occurs, to
varied degrees, in a subset of patients, it remains likely that we will see increased incidence
of CAD in the future. At present there is no reason to expect that the constellation of CAD
risk factors present in HIV metabolic syndrome would not lead inevitably to increased
incidence of CAD in the future. In fact, current recommendations are to monitor and treat the risk factors in HIV metabolic syndrome as you would in HIV- patients [65].

1.2 LIPID METABOLISM

1.2.1 Lipoproteins

Lipids are essential to many aspects of the body’s normal metabolism. They are the principal structural components of cell membranes, the most efficient form of energy storage and they are precursors for steroid hormones, vitamin D and bile salts. In the blood, the vast majority of lipids are transported in globular, lipid and protein rich particles called lipoproteins.

A typical lipoprotein consists of a core of non-polar lipids (triglycerides and cholesterol esters) surrounded by a monolayer of phospholipids, unesterified cholesterol and apolipoproteins (the protein component of a lipoprotein). The composition of apolipoproteins determines the metabolic fate of a lipoprotein by affecting affinity for lipid, by acting as a ligand for specific receptors, and by affecting the catalytic activity of enzymes.

There are several classes of apolipoproteins, some of which are summarized in table 1.

Table 1: Summary of selected apolipoproteins, their association with lipoproteins and their function.

<table>
<thead>
<tr>
<th>Apolipoprotein</th>
<th>Lipoprotein</th>
<th>Primary Function</th>
</tr>
</thead>
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<tr>
<td>Apo A</td>
<td>HDL</td>
<td>HDL structural protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LCAT activator.</td>
</tr>
<tr>
<td>Apo B-48</td>
<td>Chylomicrons</td>
<td>Structural in chylomicrons</td>
</tr>
<tr>
<td>Apo B-100</td>
<td>VLDL, IDL, LDL</td>
<td>Structural in VLDL, IDL and LDL. Ligand for LDL receptor.</td>
</tr>
<tr>
<td>Apo C-II</td>
<td>HDL, Chylomicrons, VLDL</td>
<td>LPL activator</td>
</tr>
<tr>
<td>Apo C-III</td>
<td>HDL, Chylomicrons, VLDL</td>
<td>LPL inhibitor</td>
</tr>
<tr>
<td>Apo E</td>
<td>Chylomicrons, VLDL, HDL</td>
<td>Ligand for B/E receptors</td>
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</tbody>
</table>
Lipoproteins can be categorized according to size, density and apolipoprotein composition. Chylomicrons (80-1200nm diameter) and VLDL (30-80nm diameter) are large, low density particles whose principle apolipoproteins are apo B-48 and apo B-100 respectively. These lipoproteins are the primary triglyceride carrying particles of the body. LDL (18-25nm) is the primary cholesterol carrying lipoprotein in humans and has a single apo B-100 molecule and predominantly carries esterified cholesterol within its core. HDL is the smallest (5-12nm) and most dense of the lipoproteins and contains apo A (A-I, A-II and A-IV) as its primary apolipoproteins. Lipoproteins within each class can be further subclassified by size and density. Sub-classification, of HDL and LDL in particular, is clinically relevant with respect to risk for developing coronary heart disease. In general small, dense HDL or LDL particles are more atherogenic than their large, less dense counterparts [83-86].

The work of Brown and Goldstein has provided an elegant model of the pathways of lipoprotein metabolism [87]. They proposed two related pathways at work: an exogenous pathway and an endogenous pathway which includes reverse cholesterol transport (Figure 1).

1.2.2 Exogenous pathway

The exogenous pathway describes the metabolism of dietary fat beginning with the uptake of fatty acids and cholesterol in the gut. In the case of cholesterol, uptake occurs via a recently discovered specific transporter in intestinal enterocytes [88]. Once absorbed, TG and free cholesterol are then packaged into chylomicrons which are released into circulation via the thoracic duct. Initially chylomicrons contain apolipoproteins B-48 and A-I, A-II and A-IV [89, 90] but, once in the plasma, they can acquire apo C’s (C-I, C-II and C-III) and apo E by exchange with HDL [91, 92]. In the circulation chylomicrons encounter lipoprotein
lipase (LPL), an enzyme that hydrolyzes much of the core TGs within chylomicrons. The particle, now known as a chylomicron remnant, loses phospholipids, apo A (I and IV) and apo C (I, II and III) to HDL in exchange for apo E and cholesterol esters [93], and is rapidly cleared from circulation by binding to either the apo E receptor or to the LDL receptor related protein in the liver [94].

1.2.3 Endogenous pathway

The endogenous pathway begins with the synthesis and secretion of VLDL from the liver. Initially, the primary apolipoprotein associated with VLDL is apo B-100 however, like chylomicrons, VLDL acquires apo C (I, II and III) and apo E from HDL in exchange for core TG [92]. The fate of VLDL closely resembles that of chylomicrons in that it is delipidated by LPL and converted to VLDL remnants which can be taken up by the hepatic apo B/E receptor. A point of departure lies in the potential for further delipidation by hepatic lipase (HL) resulting in intermediate density lipoprotein (IDL). These particles are either rapidly removed from circulation by the LDL receptor or, through loss of triglycerides, apo C and apo E, eventually form LDL particles [94].

LDL particles are responsible for trafficking cholesterol to the liver or to peripheral tissues where it is removed from circulation by the binding to the LDL receptor. This receptor is found on every cell type, but at the highest levels in steroidogenic and hepatic tissues.

1.2.4 Reverse Cholesterol Transport

Removal and recycling of cholesterol from peripheral tissues involves the reverse cholesterol transport process, in which HDL is the major player. HDL particles arise either
as nascent particles or apolipoproteins from hepatocytes or the intestine, or they can arise as byproducts of delipidation by LPL and HL, which remove core triglyceride from chylomicrons and VLDL leaving excess surface area rich in apo A-I and apo C (C-I, C-II and C-III). The primary apolipoprotein of HDL is apo A-I.

Reverse cholesterol transport begins when cholesterol, transported out of cells by ABC A-I, is taken up by small immature preβ-HDL particles. The cholesterol is esterified by lecithin cholesterol-acyl transferase (reviewed in [95]) and moved to the core of what, by now, is a mature HDL particle. The lipid within HDL is removed from circulation either by direct uptake of HDL particles or via cholesterol ester transfer protein, which exchanges HDL-C for TGs in LDL, VLDL and chylomicron remnants. These particles are taken up by the liver as described above. The TGs within the remaining HDL particles are hydrolyzed by HL, thus recycling the HDL particles by creating small apo A-I containing particles. This process has recently been reviewed by Sviridov and Nestel [96].

1.3 ROLE OF DYSLIPIDEMIA IN DISEASE

1.3.1 Atherosclerosis.

Atherosclerosis is the pathological basis of coronary artery disease (CAD) and is multifactorial, having substantial contribution by both genetic and environmental factors. In his “response to injury hypothesis” Russell Ross [97] proposes that an atherosclerotic plaque progresses from initial injury to the vascular endothelium which leads to endothelial dysfunction which increases endothelial permeability to lipids and leukocytes. A “vicious cycle” of lipid accumulation, inflammation and fibromuscular hyperplasia follows, leading eventually to a mature, lipid rich plaque. The core of the plaque contains foam cells, which
are macrophages rich in oxidised LDL, and is covered by a thickened fibrous cap of smooth muscle cells. Rupture of the fibrous cap results in thrombosis which may considerably occlude the arterial lumen resulting in angina or a myocardial infarction (MI).

There are several modifiable and non-modifiable risk factors for CAD which can be sub-divided into three broad categories [98]: Major or causal risk factors including elevated serum total and LDL-C, low serum HDL-C, smoking, hypertension, diabetes, physical inactivity, obesity and age, conditional risk factors such as elevated serum TG, decreased LDL particle size, elevated serum homocysteine and lipoprotein (a) and inflammatory markers such as C-reactive protein, and predisposing risk factors which include abdominal obesity and family history for CAD.

Hyperlipidemia, particularly elevated LDL-C and decreased HDL-C, is a well established risk factor for CAD. This has been demonstrated by epidemiological, pathological, genetic studies and in clinical trials [99-101]. The Framingham study shows that decreased serum HDL-C is associated with increased risk of CAD at all levels of LDL-C [99] while the Helsinki Heart study and the VA-HIT study show that a 1% increase in serum HDL-C is associated with a 3% decrease in the risk of CAD [102]]. Until recently, the causative role of elevated serum TG has been less clear.

In a meta-analysis of seventeen population based studies of over 46,000 men and 10,800 women the relative risk of CAD caused by a 1 mmol/L increase in serum TG was 1.32 in men and 1.76 in women. Adjusting for other risk factors including serum HDL, which inversely correlates with serum TG, the relative risk was 1.14 in men and 1.37 in women [103, 104]. The mechanism involved in increased risk relates to the association
between TG levels and LDL particle size. Increased serum TG results in an increase in TG rich remnants (VLDL remnants) which cannot be efficiently cleared from circulation. This leads to production of TG rich, cholesterol depleted LDL particles which undergo hydrolysis by HL resulting in atherogenic small dense LDL particles (reviewed by [105]).

The Canadian guidelines for the management and treatment of dyslipidemia are a consensus approach to management of lipid risk factors for CAD in Canada [106]. The guidelines estimate percentage risk based on age, total cholesterol, HDL-C, systolic blood pressure and smoking status using the Framingham risk tables [98, 107]. Treatment target levels are based on ten year risk of CAD (table 2). For patients in low and moderate risk groups the recommendations are for an initial trial of lifestyle and diet modification followed by appropriate lipid lowering medication if necessary. Those at high and very high risk who fail to meet targets are recommended to start lipid lowering medication along with lifestyle modifications immediately.

Table 2: Target values for treatment of lipid disorders based on the Canadian guidelines.[106]

<table>
<thead>
<tr>
<th>Risk Level</th>
<th>LDL-C mmol/L</th>
<th>Target Values</th>
<th>TC : HDL ratio</th>
<th>TG mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Very high risk</strong></td>
<td>&lt; 2.5</td>
<td>&lt; 4</td>
<td>&lt; 2</td>
<td></td>
</tr>
<tr>
<td>10 yr risk &gt; 30%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>or history of CAD or diabetes.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>High risk</strong></td>
<td>&lt; 3</td>
<td>&lt; 5</td>
<td>&lt; 2</td>
<td></td>
</tr>
<tr>
<td>10 yr risk 20%-30%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Moderate risk</strong></td>
<td>&lt; 4</td>
<td>&lt; 6</td>
<td>&lt; 2</td>
<td></td>
</tr>
<tr>
<td>10 yr risk 10%-20%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Low risk</strong></td>
<td>&lt; 5</td>
<td>&lt; 7</td>
<td>&lt; 3</td>
<td></td>
</tr>
<tr>
<td>10 yr risk &lt; 10%</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
1.3.2 Syndrome X

Syndrome X, otherwise known as metabolic syndrome, describes a cluster of CAD risk factors including, hypertriglyceridemia, low serum HDL-C, insulin resistance, abdominal obesity, hypertension, and a preponderance of small dense LDL particles [106, 108, 109]. There are two commonly used classification systems for syndrome X (table 3). One devised by the World Health Organization (WHO) utilizes a number of criteria including insulin resistance, waist to hip ratio etc. A second definition, devised by the American National Cholesterol Education Program’s expert panel on detection, evaluation and treatment of high blood cholesterol in adults (ATP III)[110] utilizes widely available, commonly used, and well understood tests including waist circumference, blood pressure and serum levels of triglycerides, HDL and glucose.

Table 3: Summary of WHO and ATPIII case definition for metabolic syndrome in men.

<table>
<thead>
<tr>
<th>World Health Organization</th>
<th>NCEP ATP III</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Either:</strong></td>
<td></td>
</tr>
<tr>
<td>• Insulin resistance</td>
<td>• abdominal obesity (&gt;102 cm)</td>
</tr>
<tr>
<td>• FBS ≥ 6.1 mmol/L</td>
<td>• serum TG ≥1.7mmol/L</td>
</tr>
<tr>
<td>• 2hr OGGT 7.8-11 mmol/L</td>
<td>• serum HDL &lt;1mmol/L</td>
</tr>
<tr>
<td>• Type 2 diabetes</td>
<td>• Blood pressure ≥130 systolic or ≥85 diastolic</td>
</tr>
<tr>
<td>• 2hr OGGT ≥ 11.1 mmol/L</td>
<td>• fasting blood sugar ≥6.1mmol/L</td>
</tr>
</tbody>
</table>

| And two of:               |             |
| • Blood pressure ≥140 systolic and/or ≥90 diastolic and/or taking anti-hypertensives | |
| • Serum TG ≥1.7mmol/L and/or serum HDL <0.9mmol/L | |
| • WHR >0.9 and/or BMI >30 | |
| • Urine albumin/creatinine ratio ≥30mg/g | |

Syndrome X has been shown to confer significant risk for CAD and diabetes [111-113]. In fact, an analysis of the Quebec Cardiovascular Study showed that, when considered
simultaneously, the triad of hyperinsulinemia, hyperapolipoprotein B, and small dense LDL was more predictive of CAD in men than the lipid triad of elevated plasma LDL-C, TG and decreased HDL-C [114].

The value of measuring waist circumference has been born out by of evidence that abdominal obesity, particularly elevated visceral adipose tissue, commonly associates with metabolic syndrome and is associated with elevated risk for CAD. One study reported that greater than 80% of men with waist circumference ≥ 90 cm and with TG ≥ 2.0 mmol/L also had the metabolic triad of hyperinsulinemia, hyperapolipoprotein B and small dense LDL. In that study, men with elevated waist circumference and serum TG were at significantly higher risk of CAD than men with waist circumference ≤ 90 cm and low serum TG (odds ratio 3.6, p<0.03) [115].

HAART metabolic syndrome often includes elevated serum TG and total cholesterol, decreased serum HDL, insulin resistance and a tendency towards central adiposity. Knowing this, the comparison with syndrome X is self evident, again raising the concern of the potential for increased risk of CAD in HAART metabolic syndrome.

1.4 BIOCHEMICAL AND GENETIC FACTORS IN TG METABOLISM

The most significant metabolic side effect of antiretroviral therapy is increased serum TG. A number of biochemical, genetic, and environmental factors play a role in regulating the level of serum TG in the blood. Among the genetic causes of elevated TG are mutations resulting in deficiency of apo C-II, polymorphisms of apo E, apo C-III, apo A-V genes and
polymorphisms or mutations within the LPL or HL genes. Environmental factors include alcohol, diet, drugs and obesity, which is often associated with diabetes.

LPL and HL are two key enzymes in TG metabolism. Both are serine esterases belonging to the lipase family, which also includes pancreatic (PL) and endothelial lipase. LPL and HL are bound to membrane proteoglycans on the endothelial surface of the circulatory system [116] and can be released into the plasma by injection of a bolus of heparin. While the two enzymes are structurally similar, key differences exist with respect to their catalytic activity and roles in lipoprotein metabolism [117].

1.4.1 Lipoprotein lipase

LPL is primarily synthesized in muscle and adipose tissue although its mRNA has been found in a variety of other tissues (reviewed in [118]). LPL catalyzes the initial steps in the exogenous and the endogenous lipoprotein pathways by hydrolyzing TG from the core of chylomicrons and VLDL to produce remnant (IDL) particles that can be cleared from circulation or, in the case of VLDL, can be further catabolized. The excess VLDL and chylomicron surface area produced by hydrolysis contributes to the HDL pool. Free fatty acids formed by hydrolysis are released into circulation to be taken up by muscles, including the myocardium, and used for energy, for storage, or in a structural capacity. Apo C-II is required for activation of LPL [119]. In fact, this is an important difference between LPL and HL, which does not require a co-factor. Apo C-II binds to the surface of HDL particles from which it is transferred to VLDL particles and chylomicrons [120].

LPL activity is regulated by a number of factors. Elevated glucose, which stimulates insulin secretion, increases LPL activity, while prolonged fasting results in decreased LPL
activity in adipocytes. LPL is inhibited by apo C-III [121, 122]. In fact plasma and VLDL concentration of apoC-III is increased in hypertriglyceridemic subjects with reduced VLDL catabolism [123].

Deficiency of LPL, resulting either from genetic defect of LPL or deficiency of apo C-II, has profound clinical consequences. Individuals with homozygous LPL deficiency develop familial chylomicronemia. The result is an accumulation of chylomicrons and VLDL causing serum TG elevations up to the 100 mmol/L range. Serum LDL-C is very low in LPL deficiency due to ineffective processing of VLDL particles. Affected individuals present with eruptive xanthomas and lipaemia retinalis, abdominal pain due to pancreatitis, failure to thrive and hepatosplenomegaly. While genetic disorders resulting in complete deficiency of LPL activity, either from LPL or apo C-II deficiency are extremely rare in most populations there are a number of more common polymorphisms which affect LPL activity.

Three common polymorphisms within LPL, that affect the level of serum triglycerides are known: Asp9Asn (D9N) and Asn291Ser (N291S) which occur at roughly 1-6% in healthy white subjects [124] have been associated with moderately increased serum TG (roughly 20% and 31%) while Ser447stop (S447X) whose frequency ranges from 11-23% [125] decreases serum TG by approximately 8%. Biochemical analysis of each polymorphism indicates that D9N results in a decrease in LPL secretion [126] while the N291 polymorphism affects the stability of LPL dimers, thus reducing LPL activity [127].
1.4.2 Hepatic lipase

HL is synthesized in the liver and binds to the surface of hepatocytes and hepatic endothelial cells [128, 129], although it is also found in the adrenal gland and ovary. Like LPL, HL is thought to be catalytically active as a head to tail dimer [130].

HL hydrolyzes TGs and phospholipids in VLDL remnants and in HDL particles. By its action on VLDL, HL contributes to the formation of IDL and potentially LDL particles. HL also plays a central role in HDL metabolism both by its hydrolytic activity and by binding to and facilitating HDL uptake by the liver. Hydrolysis of phospholipid and TG within HDL particles contributes to reduction of HDL particle size forming preβ-HDL, a cholesterol poor, phospholipid-apo A-I complex that is the starting point for reverse cholesterol transport. Therefore, by its action on HDL, HL catalyses a necessary step in reverse cholesterol transport.

HL activity is associated with lipoprotein changes that are typically considered to be atherogenic. Transgenic models (reviewed in [131]) show that increased HL activity decreases HDL-C and HDL particle size [132-134], while HL deficiency results in increased levels of phospholipid and apo E rich HDL particles [135]. In clinical studies HL activity inversely correlates with serum HDL-C levels (large buoyant HDL-2 in particular) [136-138] and Zambon et al. [139] showed a positive correlation between increased hepatic lipase activity and coronary stenosis related to increased LDL density. These changes in lipoprotein quality and quantity are all known to occur in HAART metabolic syndrome.
1.4.3 HL and LPL in HAART metabolic syndrome.

Given the nature of the dyslipidemia in HAART treated patients, both LPL and HL are appealing candidate enzymes. In fact, in PI treated cultured adipocytes LPL activity has been shown to decrease [140]. Two human studies of HIV+ patients have shown decreased LPL activity with treatment with PI containing regimens [141, 142]. Another study of healthy HIV negative subjects treated with Ritonavir [143] reported no change in LPL activity. Each study reported a decrease in HL activity. The conclusions drawn from each study are that it is unlikely that changes in LPL or HL activity can account for the full extent of the lipid disorders, although it is clear that LPL and HL can play a contributory role.

1.4.4 Apolipoprotein E

Apolipoprotein E (apo E) is a 34 kD glycoprotein associated with chylomicrons, VLDL and HDL. It is synthesized in the liver and to a lesser extent in a number of peripheral tissues including the kidney and adrenal gland [144]. By binding the apo E and LDL receptors (apo B, E receptors) in the liver and peripheral tissues apo E mediates clearance of chylomicron and VLDL remnants as well as apo E containing HDL from circulation. In this capacity apo E plays a major role in regulating the metabolism of these lipoproteins.

In humans the apo E gene is polymorphic, having three isoforms (ε2, ε3 and ε4) resulting from single amino acid substitutions at two sites (aa112 and aa158) [145]. Carriers of the apo E2 allele have lower serum total and LDL cholesterol and increased serum TG while carriers of apo E4 have increased serum total cholesterol, LDL cholesterol and TG compared apo E3 carriers [146, 147]. As well as the effect on serum lipids the presence of either of the two minor alleles may confer increased risk for CAD [148, 149] and other diseases including Alzheimers [150].
The apo E gene is a locus with a substantial environmental interaction. For example, the effects of apo E on serum lipids are strongest in populations with a “westernized” diet and lifestyle. As well, certain alleles has been shown to affect response of serum lipids to interventions such as diet management (E4 most responsive) (reviewed in [151]) and statin treatment (E2 most responsive, E4 least responsive), although interestingly, this association has only been noted in males (reviewed in [152]). There is evidence of an association between Apo E2 and E4 and the development of hypertriglyceridemia secondary to treatment with isotretinoin [153]. The effect of salmon oil supplementation on serum TG is also influenced by apo E genotype, with carriers of E4 showing the best response in one randomized controlled trial [154]. In one study of the effect of statin on LDL-C in 328 volunteers, male carriers of E2 averaged a 44% decrease in LDL-C compared to 37% in E3 and 34% in E4 carriers. No such interaction was seen in females [155].

Given the impact of apo E genotype on lipid responses to drugs and environmental influences, Apo E is an appealing candidate for analysis in relation to HAART. Thus far the association has not been entirely clear. A few reports suggest an association between apo E2 and apo E4 with hypertriglyceridemia in PI treated individuals [156-158] while some cohort studies have found no such association [159] [43].

1.4.5 Apolipoprotein C-III.

The gene for Apo C-III resides in 11q23 in the apolipoprotein cluster which also includes apo A-I and A-IV. The apo C “family”, which includes apo C-III, C-I and C-II, associates with chylomicrons, VLDL, and HDL. Apo C-III reduces that capacity to clear TG rich lipoproteins by decreasing apo E and abolishing apo B mediated binding to the LDL
receptor, inhibiting binding of TG rich particles to the endothelial surface, and specifically inhibiting catabolism by LPL [119, 121, 160, 161].

A close correlation exists between the level of apo C-III containing lipoproteins and serum TG levels [162-164]. As well, apo C-III level is associated with syndrome X [165]. In HIV, Bonnet et al. reported a 2-3 fold increase in apo C-III and apo E associated in PI treated patients[166]. The increase was strongly correlated with development of lipodystrophy and, following treatment with fibrates, TG, apo C-III and apo E all returned to a normal level within two months[166].

A number of polymorphisms have been described in the apo C-III gene but there are three relatively common polymorphisms known to affect serum triglyceride levels. Two polymorphisms within an insulin response element abolish response of the CIII promoter to insulin thus significantly affecting postprandial lipid metabolism. A third polymorphism, the SstI (G3238C) in the 3′ untranslated region of exon four, has been associated with hypertriglyceridemia and increased incidence risk of CAD (reviewed in [167]. In HIV, one study suggested that carriers of certain haplotypes of apo CIII and apo E polymorphisms may identify a predisposition to developing dyslipidemia with HAART treatment [168].

1.5 TREATMENT OF HYPERTRIGLYCERIDEMIA

1.5.1 Pharmacological interventions

After excluding secondary causes and when lifestyle and diet modification fail, the Canadian guidelines recommend the use of fibrates and/or niacin for treatment of elevated serum TG [106]. Niacin is a B complex vitamin which has been associated with a reduction
in serum TG of 20% to 50%, in LDL-C of 5%-25% and an increase in HDL-C of 15%-35%
[169]. Its mechanism is unclear but is possibly related to decreased lipolysis in adipose
tissue or to decreased hepatic synthesis of apo-B containing particles. In therapeutic doses
niacin is often poorly tolerated and has been shown to worsen glycemic control in patients
with insulin resistance, which is common in HIV metabolic syndrome. For these reasons
niacin is generally not the drug of choice in HIV metabolic syndrome [169].

The “front line” medications for hypertriglyceridemia are fibrates which have been
shown to decrease serum TG by 20% to 50% and increase serum HDL-C by 10% to 20%
[169]. Clinical trials have shown fibrates to be beneficial not only by reduction in serum TG
but also in the reduction of CAD incidence and atherosclerotic lesion development. The VA-
HIT trial randomized patients with CAD to receive either gemfibrozil or placebo. Over five
years HDL-C increased 6% while serum TG decreased by 31% and total cholesterol by 4%
while the rates of MI decreased by 22% in the treatment group compared to control [101].
The five year Helsinki Heart Study of 4081 males showed a 35% reduction in serum TG and
a 34% reduction in the incidence of CAD. The effects were most dramatic in overweight
patients with significantly increased serum TG and low HDL-C in whom a 78% reduction in
the incidence of CAD was noted in the fibrate treated group compared to placebo [102]. In
the BECAIT (Bezafibrate Coronary Atherosclerosis Intervention Trial) study, among 92
young male MI survivors, fibrate use was associated with a 31% reduction in serum TG and
a significant reduction in the progression of coronary atherosclerosis compared with placebo
[170]. Finally, results of the DAIS (Diabetes Atherosclerosis Intervention Study) study
indicate that fenofibrate significantly improved the lipid profile and effectively decreased
angiographic progression of CAD [171].
Fibrates lower serum TG and increase serum HDL-C, at least in part through a mechanism involving a group of nuclear receptors known as peroxisome proliferator-activated receptors (PPARs). Fibrates are known to activate PPARs which can affect the expression of genes involved in lipoprotein metabolism. In particular, activation of PPARα by fibrates decreases VLDL production by stimulating oxidation of free fatty acids in the liver and skeletal muscle [172, 173], and decreases synthesis of apoC-III [174, 175], a specific inhibitor of LPL. PPARα has also been shown to directly increase synthesis of LPL in the liver in response to fibrate treatment [176]. As well, serum HDL-C increases in response to fibrate treatment by a PPAR mediated increase in synthesis apoA-I [177] and apoA-II [178], two apoproteins important to HDL production.

1.5.2 Omega-3 fatty acids

Fish oil rich in the omega-3 fatty acids (n-3 FAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) has a well established serum TG lowering effect. Interestingly, the initial interest in the benefits of n-3 FAs was based on studies finding a low incidence of CAD in Greenland Eskimos, despite their diet which is very rich in fat. A meta-analysis of 36 placebo-controlled crossover and 29 placebo controlled parallel studies showed that serum TG decreased by 25-30%, HDL-C rose by 1-3%, LDL-C rose by 5-10% while serum total cholesterol was unaffected [179]. The TG lowering effect was more significant in hypertriglyceridemic subjects and was sustained as long as the subject continues to take a therapeutic dose, [180] recommended to be 3-4 grams of DHA and EPA per day.

The mechanism behind the hypotriglyceridemic effect of polyunsaturated n-3 FAs relates to the effect on expression of regulatory genes, PPARα in particular. In this way n-3 fatty acids cause decreases in serum TG by a similar mechanism as fibrates (reviewed in
The beneficial effects of n-3 FA consumption extend beyond the effect of lowering TG. In fact n-3 FAs are also known to have anti-hypertensive, anti-inflammatory, anti-arrhythmic, and anti-coagulative properties (reviewed briefly in [182]). As well, n-3 FA consumption has been shown to shift the distribution of LDL toward larger, less atherogenic particle sizes [183] and has been shown to decrease overall mortality and sudden death in patients with CAD [184].

n-3 FAs have been used in HIV+ patients in the past, although only with the aim of improving immune parameters, which was not successful [185]. Treatment of HAART associated dyslipidemia with n-3 FAs is an appealing alternative for a number of reasons. N-3 FAs are primarily used in hypertriglyceridemia, which is the major lipid side effect of HAART. As well, the perception that they are more a dietary supplement than a drug is appealing to the HIV+ population that typically has a large pill burden.
1.6 HYPOTHESES

The primary hypothesis of this thesis was as follows: **The underlying causes for the development of dyslipidemia in HIV metabolic syndrome relate to, among others, polymorphisms of apo E, apo C-III, LPL and to decreased lipoprotein catabolism due to decreased LPL activity.** Related to this hypothesis, we tested the lipid lowering effect of a combination of dietary counselling and n-3 FA supplementation, and the effect of treatment with statins and fibrates in patients with HIV metabolic syndrome.

1.7 SPECIFIC AIMS

- Recruit approximately 100 consecutive dyslipidemic HIV+ subjects from the lipid clinic in the infectious disease center at St. Paul’s Hospital.

- To characterize this cohort with respect to waist circumference, self report of lipodystrophy, serum lipid levels, medication use and HIV status.

- To determine the association effect of use of lipid lowering medication and HAART regimens including nevirapine on serum lipid levels.

- To measure LPL and HL activity in post heparin plasma from a subset of patients.

- To collect and isolate DNA from these subjects for genetic analysis of polymorphisms of apo E, LPL and apo C-III.

- To characterize lipid levels in HIV positive patients treated with and without n-3 FA supplementation in the context of a randomized controlled clinical trial.
2 METHODS

2.1 RECRUITMENT

All subjects were recruited from the St. Paul's Hospital Infectious Disease Center HIV metabolic clinic to which they had been referred for treatment of dyslipidemia or lipodystrophy. All participants signed an informed consent that was approved by the St. Paul's Hospital research ethics boards. Height, weight and waist circumference was measured and the subjects were asked to complete a questionnaire regarding presence and severity of lipodystrophy and to provide a blood sample. Blood was collected in the St. Paul’s lab into a tube containing EDTA following a 12 hour overnight fast. Samples were immediately placed on ice and centrifuged at 3000rpm for 15 minutes at 4° C to separate the plasma from the cells. Plasma and cells were stored at −70° C.

Waist circumference was measured as previously described [186]. The average of two successive measurements was recorded. Briefly circumference was measured at the point of maximal narrowing of the trunk as view from the anterior with the participant standing upright following normal expiration. When this point was not apparent, measurement was done at the level of the 12th rib.

2.2 LIPASE ANALYSIS

A number of patients were selected by their physician for assessment of lipase levels. These patients had severe (serum TG>4.5) or persistent (elevated TG despite lipid lowering therapy) hypertriglyceridemia. Ten minutes after subjects received a bolus of heparin (60 U/Kg) a sample of blood was taken into a 7ml tube containing EDTA. Blood was placed on
ice and centrifuged immediately at 3000 rpm for 15 minutes at 4 degrees to separate plasma from cells. Plasma was stored in aliquots at −70° C until used for analysis.

Lipase analysis was done according to a method modified from that of Nilsson-Ehle and Ekman [187]. Briefly, substrate was produced by drying under nitrogen, a mixture of triolein (7.5 mg in chloroform), phosphatidylcholine (1.0 mg in ethanol) and [3H]triolein (50 μCi) (Amersham pharmacia). 2.1 ml of 0.2 M Tris-HCl, pH 8.8 and 0.4 ml of 1% BSA in 0.2 M Tris-HCl, pH 8.8 was added and an emulsion was produced by sonication on ice for 8 min at 50% pulse. 0.5 ml of 4% BSA in 0.2 M Tris-HCl was added to the mix for a final volume of 5 ml.

10 μL of sample of plasma was added to 90 μL of the appropriate buffer (0.05 M Tris-HCl, 0.02 M NaCl, pH 8.8 for LPL and 0.05 M Tris-HCl, 2 M NaCl, pH 8.8 for HL) and 100 μL of substrate was added. The mixture was incubated at 37°C for 30 minutes after which the reaction was stopped by adding chloroform:methanol:heptane (1.25:1.41:1) and the fatty acids were extracted by adding 0.1 M H3BO3, 0.1 M K2CO3, pH 10.5, vortexing and centrifuging at 3000 rpm for 10 min. The radioactivity in a 1.0 ml aliquot from the upper (methanol:water) phase was determined with a liquid scintillation counter using ACS (Amersham, Oakville Ontario) as scintillant.

Each sample was assayed in triplicate using a pooled batch of substrate. Precision was monitored using pooled serum controls run in triplicate in each assay. The intra-assay C.V. was 9% for total lipase and 16% for HL.
2.3 GENETIC ANALYSES

DNA was extracted from whole blood leukocytes or whole blood using the DNA Isolation Kit for Blood / Bone Marrow / Tissue (Roche Molecular Biochemicals). Samples were then genotyped for Apo CIII Sst I and LPL N291S by PCR followed by restriction endonuclease digestion. Polymorphisms of apo E were detected using the LightCycler (Roche Molecular Biochemicals). The individual PCR reactions are described more completely below.

2.3.1 Apo C-III SStI polymorphism

A 572 nucleotide target sequence in the 3’ untranslated region of exon 4 of the apo C-III gene was amplified using 5’-CCT GGA GTC TGT CCA GTG CCC ACC CAC A-3’ as the upstream primer and 5’-GAT TCC TGC CTG AGG TCT CAG GGC TGT CGT-3’ as the downstream primer. Amplifications were carried out in 0.2 mmol/L dNTPs, 5 µg BSA, 1 µmol/L of each primer, 1.5 U of HotStarTaq DNA polymerase (Qiagen) and 5 µL of 10x PCR buffer (Qiagen) in a total volume of 50 µL. 10x concentrated PCR buffer contains Tris-Cl, KCl, (NH₄)₂SO₄ and 15 mmol/L MgCl₂, pH 8.7. HotStarTaq DNA Polymerase is stored in 20 mM Tris-Cl, 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.5% (v/v) Nonidet P-40, 0.5% (v/v) Tween 20, 50% glycerol (v/v), stabilizer, pH 9.0. Following initial denaturation (15 minutes at 95°C), a touchdown protocol was used which included 20 cycles of touchdown PCR, 95°C (45 sec), 70°C-60°C (1 minute 30 sec), 72°C (1 minute 30 sec), followed by an additional 10 cycles of 95°C (45 sec), 60°C (1 minute 30 sec), 72°C (1 minute 30 sec) and a final extension step of 1 minute at 72°C. 15 µL of the PCR product was digested with 10 U Sac-I (New England Biolabs equivalent of Sst I) according to manufacturers instructions in a total volume of 25 µL for 2 hours at 37°C. Fragments were
separated by electrophoresis in a 2% agarose gel containing ethidium bromide, and were visualized on a transiluminiator (Eagle Eye).

### 2.3.2 LPL 291 polymorphism

A 280 nucleotide target sequence of the LPL gene was amplified using 5'-GCC GAG ATA CAA TCT TGG TA-3' as the upstream primer and 5'-ATA ATA TAA AAT AAT AAT ACT GCT TCT TTT GGC TCT GAC TGT A-3' as the downstream primer [Hoffer, 1996 #62]. Amplifications were carried out in 50 nmol/L dNTPs, 0.25 mmol/L MgCl₂, 1 μmol/L of each primer, 1.5 U of HotStarTaq DNA polymerase (Qiagen) and 5 μL of 10x PCR buffer (Qiagen) in a total volume of 50 μL. 10x concentrated PCR buffer contains Tris-Cl, KCl, (NH₄)₂SO₄ and 15 mmol/L MgCl₂, pH 8.7. HotStarTaq DNA Polymerase is stored in 20 mM Tris-Cl, 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.5% (v/v) Nonidet P-40, 0.5% (v/v) Tween 20, 50% glycerol (v/v), stabilizer, pH 9. Following initial denaturation (15 minutes at 95°C), a touchdown protocol was used which included 20 cycles of 95°C (1 minute), 60°C to 50°C (45 sec), 72°C (30 sec) followed by an additional 20 cycles of 95°C (1 min), 50°C (45 sec), 72°C (30 sec) and a final extension of 72°C for 10 minutes. 15μL of the PCR reaction was digested with (#units) RSA I according to the manufacturers protocol (New England Biolabs), in a total volume of 25μL for 2 hours at 37°C. Fragments were separated by electrophoresis in a 3% agarose gel containing ethidium bromide, and were visualized on a transiluminiator (Eagle Eye).

### 2.3.3 Apo E polymorphism

Genotyping for polymorphisms at nucleotides 112 and 158 of the apo E gene was performed using the LightCycler Apo E Mutation Detection Kit (Roche Molecular
Biochemicals). The genotyping was done according to the manufacturers recommended procedure. This procedure was done by the clinical lab at St. Paul's Hospital.

2.4 STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS version 8 statistical software package (SPSS, Inc.). Both parametric (t-test) and non-parametric (Kruskall-Walis) analyses were done depending on the normality of the data distribution. In the case of comparisons involving serum triglyceride and total cholesterol, both of which had significantly skewed distributions, analyses were done following a logarithmic transformation.
3 RESULTS

3.1 DESCRIPTIVES

One hundred and seven subjects were recruited between December 2000 and May 2001. Table 4 summarizes descriptive statistics including age, height, weight, BMI and waist circumference in the study population.

Table 4: Descriptive statistics of cohort.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Range</th>
<th>Std. Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>49</td>
<td>30 - 78</td>
<td>9.0</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>92.6</td>
<td>72.8 - 115.7</td>
<td>8.7</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.7</td>
<td>1.5 - 1.9</td>
<td>0.1</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>75.5</td>
<td>42.4 - 111.1</td>
<td>11.7</td>
</tr>
<tr>
<td>BMI (Kg/m$^2$)</td>
<td>25.0</td>
<td>19.3 - 34.6</td>
<td>3.2</td>
</tr>
</tbody>
</table>

Table 5 summarizes the levels of serum lipids and fasting glucose at the time of recruitment. At that time a number of the subjects were already taking medication to lower their serum lipids. In order to get a more complete picture of the dyslipidemia resulting from antiretroviral therapy, lipid levels prior to the introduction of lipid medication use were recorded (see table 6).

Table 5: Serum lipid and glucose levels at time of recruitment.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Min</th>
<th>Max</th>
<th>Mean</th>
<th>Std. Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting Blood Sugar</td>
<td>86</td>
<td>4.2</td>
<td>15.8</td>
<td>6.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Total Cholesterol</td>
<td>105</td>
<td>3.4</td>
<td>13.8</td>
<td>6.9</td>
<td>1.9</td>
</tr>
<tr>
<td>HDL Cholesterol</td>
<td>103</td>
<td>0.3</td>
<td>1.9</td>
<td>0.95</td>
<td>0.3</td>
</tr>
<tr>
<td>Serum Triglycerides</td>
<td>105</td>
<td>0.9</td>
<td>38.5</td>
<td>7.00</td>
<td>6.2</td>
</tr>
<tr>
<td>TC/HDL</td>
<td>103</td>
<td>2.8</td>
<td>26.7</td>
<td>8.2</td>
<td>4.4</td>
</tr>
</tbody>
</table>
Of the eighty subjects with recorded fasting blood sugars prior to the use of lipid lowering medication, thirteen had a FBS higher than 7 mmol/L. In that subset, serum triglycerides were significantly higher (11.3 mmol/L vs. 7.0 mmol/L) but there was no statistically significant difference in any of other lipid parameter (see table 7).

**Table 6: Serum lipids and glucose prior to initiation of lipid lowering medication.**

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Min</th>
<th>Max</th>
<th>Mean</th>
<th>Std. Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting Blood Sugar</td>
<td>80</td>
<td>4.1</td>
<td>15.8</td>
<td>6.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Total Cholesterol</td>
<td>95</td>
<td>3.5</td>
<td>13.1</td>
<td>7.2</td>
<td>2.0</td>
</tr>
<tr>
<td>HDL Cholesterol</td>
<td>89</td>
<td>0.2</td>
<td>1.7</td>
<td>0.9</td>
<td>0.3</td>
</tr>
<tr>
<td>Serum Triglycerides</td>
<td>95</td>
<td>0.9</td>
<td>31.4</td>
<td>7.8</td>
<td>5.8</td>
</tr>
<tr>
<td>TC/HDL</td>
<td>88</td>
<td>3.5</td>
<td>26.5</td>
<td>8.5</td>
<td>3.7</td>
</tr>
</tbody>
</table>

**Table 7: Serum lipids prior to use of lipid lowering medication stratified by fasting blood sugar above and below 7 mmol/L.**

<table>
<thead>
<tr>
<th>FBS (mmol/L)</th>
<th>N</th>
<th>Mean (mmol/L)</th>
<th>Std. Dev</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥7</td>
<td>13</td>
<td>7.8</td>
<td>2.4</td>
<td>0.37*</td>
</tr>
<tr>
<td>&lt;7</td>
<td>67</td>
<td>7.8</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥7</td>
<td>13</td>
<td>0.88</td>
<td>0.23</td>
<td>0.54</td>
</tr>
<tr>
<td>&lt;7</td>
<td>61</td>
<td>0.93</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥7</td>
<td>13</td>
<td>11.3</td>
<td>7.1</td>
<td>0.007*</td>
</tr>
<tr>
<td>&lt;7</td>
<td>66</td>
<td>7.0</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>TC/HDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥7</td>
<td>13</td>
<td>9.6</td>
<td>5.5</td>
<td>0.228</td>
</tr>
<tr>
<td>&lt;7</td>
<td>61</td>
<td>8.2</td>
<td>3.2</td>
<td></td>
</tr>
</tbody>
</table>

*p-value calculated on log transformed data.

Waist circumference, weight, BMI and HDL-cholesterol were all normally distributed.

Serum triglycerides, total cholesterol, total cholesterol to HDL-C ratio and fasting blood
sugar were all positively skewed necessitating log transformation prior to statistical analysis (Figures 2-9).

The anti-retroviral regimens of ninety seven subjects were recorded. At the time of recruitment 91% of subjects were on regimens containing NRTIs, 68% were on NNRTIs and 89% were on PI containing regimens. 84% of subjects were taking a regimen containing PIs and NRTIs, 63% were taking NRTI / NNRTI combinations, 62% were taking NRTI / PI combinations and 57% were on regimens containing all three drug classes.

### 3.2 LIPASE ACTIVITIES

In the subset of subjects (n=29) in whom lipase activity was measured, serum triglycerides were 9.4 ± 6.2 mmol/L, total cholesterol was 7.1 ± 1.9 mmol/L and HDL cholesterol was 0.8 ± 0.2 mmol/L. In the control population (n=11) serum triglycerides were 1.3 ± 0.7 mmol/L, total cholesterol was 4.6 ± 0.5 mmol/L and HDL cholesterol was 1.4 ± 0.3 mmol/L.

<table>
<thead>
<tr>
<th></th>
<th>Healthy Controls n=11</th>
<th>HIV+ subjects n=29</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>Min</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>Max</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>St. Dev</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>HDL Cholesterol</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Serum Triglycerides</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>Min</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>Max</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>St. Dev</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Min</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>Max</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>St. Dev</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Min</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Max</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>St. Dev</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Lipoprotein lipase activity in the HIV+ subset was 36.03 ± 30.17 nmol/min/ml (0 to 116.57 nmol/min/ml) compared to 86.79 ± 63.96 nmol/min/ml (23.06 to 236.16 nmol/min/ml) in the healthy control group. Using the non-parametric Kruskal-Wallis test
this difference was statistically significant ($p < 0.01$) (Figure 10). Lipoprotein lipase activity was inversely correlated with serum triglycerides ($R^2 = 0.34$) and was positively correlated with serum HDL-cholesterol ($R^2 = 0.26$) (Figure 11-12).

Hepatic lipase activity in the subset was $225.15 \pm 104.60$ nmol/min/ml (78.39 to 501.05 nmol/min/ml) compared to $139.02 \pm 40.89$ nmol/min/ml (72.08 to 204.84 nmol/min/ml) in the healthy control group. Again, this difference was found to be statistically significant by the Kruskal-Wallis test ($p < 0.05$) (Figure 13). Hepatic lipase activity was inversely correlated with level of serum HDL-cholesterol ($R^2 = 0.129$) (Figure 14).

### 3.3 Frequency of LPL N291S Polymorphism

Seventy seven individuals were screened for the N291S polymorphism in the LPL gene. Three were carriers of the rare allele and all were heterozygous. The frequency of this polymorphism (1.9%) falls within the range of previously published findings which reported frequencies ranging from 1% to 7% (Wittrup et al, Circulation 1999, 99; 2901-2907). There was no change in any serum lipid marker in carriers of this polymorphism.

### 3.4 Frequency of Apo E Polymorphisms

Seventy eight subjects were screened for polymorphisms of the apo E gene using the LightCycler Apo E gene kit (Roche). The apo E3 allele was the most common with a frequency of 76.0% compared with the two rarer apo E2 and E4 alleles at 12.7% and 11.4% respectively. Of the seventy eight subjects screened, sixteen were E2/E3, forty five were E3/E3, fourteen were E3/E4, four were E2/E4 and there were no E2/E2 or E4/E4 subjects. The polymorphisms were in Hardy-Weinberg equilibrium and the allele frequencies were
similar to those in previous reports. There was no significant effect of genotype on serum lipid markers.

3.5 **FREQUENCY OF APO CIII SST-I POLYMORPHISM**

The SST-I polymorphism in the apo CIII gene were screened for in seventy seven individuals. The rare allele was found to have a frequency of 7.7% and was found to be in Hardy-Weinberg equilibrium. This frequency is consistent with previous reports [188, 189]. Although not significant, there was a trend for carriers of the rare allele to have elevated serum triglycerides ($p = 0.069$) and decreased serum HDL-C. There was no difference in allele frequency in subjects with serum triglycerides above the group mean (7.83 mmol/L) compared with subjects with serum triglycerides below the group mean.

3.6 **FREQUENCY OF LIPODYSTROPHY AND ITS EFFECT ON LIPIDS**

The presence of lipodystrophy was assessed by self report questionnaire. In summary, of the one hundred and seven subjects who completed the questionnaire 82% reported a loss of peripheral fat, 5% reported gain of fat in at least one body area, excluding the abdomen. 50% of subjects reported gain of abdominal fat while 25% reported no change and 25% reported a net decrease in abdominal fat. 25% of the subjects reported gain of fat in the neck.

Of the subjects that reported lipodystrophy, 45% of subjects reported to only suffer from lipoatrophy, 10% of subjects suffer only from lipohypertrophy and 46% of subjects suffer from mixed lipodystrophy. There was no significant difference in serum lipid markers between groups any of these sub-groups.
3.7 EFFECT OF NEVIRAPINE ON SERUM HDL-C

Nevirapine is an NNRTI that does not appear to cause dyslipidemia and may, in fact, raise HDL-C. We studied the effects of nevirapine in our cohort. Seventy one subjects who were taking HAART including a PI and were not taking any lipid lowering medication were selected. The regimens of thirty five of these patients also included nevirapine. All subjects had been taking their respective medications for at least three months.

HDL-cholesterol was 0.98 ± 0.28 mmol/L (0.62 to 1.71 mmol/L) in the nevirapine treated group compared with 0.84 ± 0.24 mmol/L (0.20 to 1.32 mmol/L) in the group whose regimens did not include nevirapine. This difference was statistically significant (p<0.05 students t-test) (Figure 15). There was no significant difference in any of serum triglycerides, total cholesterol or serum total to HDL-C ratio.

3.8 EFFECT OF LIPID LOWERING MEDICATION

Fibrates are known to lower serum triglyceride. We carried out a sub analysis of sixteen subjects who received only fibrates as a treatment for their dyslipidemia. Paired data from before and after fibrate therapy were analyzed and the results indicate that treatment with fibrates significantly reduces serum triglyceride levels (p = 0.028). Serum triglycerides decreased by 37% from 6.89 mmol/L to 4.81 mmol/L. While serum total cholesterol decreased (5%) and serum HDL-C increased (8%), neither change reached the level of statistical significance.
Table 9: Serum lipids before and after therapy with fibrates alone.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>N</th>
<th>Std. Deviation</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log Serum Triglycerides</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before</td>
<td>1.93</td>
<td>16</td>
<td>0.60</td>
<td>0.028</td>
</tr>
<tr>
<td>After</td>
<td>1.57</td>
<td>16</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>Log Total Cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before</td>
<td>2.01</td>
<td>16</td>
<td>0.25</td>
<td>0.429</td>
</tr>
<tr>
<td>After</td>
<td>1.95</td>
<td>16</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before</td>
<td>0.96</td>
<td>16</td>
<td>0.35</td>
<td>0.289</td>
</tr>
<tr>
<td>After</td>
<td>1.04</td>
<td>16</td>
<td>0.42</td>
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</tr>
<tr>
<td>Total / HDL ratio</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>before</td>
<td>8.27</td>
<td>15</td>
<td>3.29</td>
<td>0.233</td>
</tr>
<tr>
<td>After</td>
<td>7.45</td>
<td>15</td>
<td>3.07</td>
<td></td>
</tr>
</tbody>
</table>

Seven individuals were treated with statins alone and there was no statistically significant difference in any lipid marker. Mean serum total cholesterol was decreased and serum HDL cholesterol was increased but neither reached statistical significance.

Table 10: Serum lipids before, and after therapy with Statins alone

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>N</th>
<th>Std. Deviation</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log Serum Triglycerides</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before</td>
<td>1.96</td>
<td>6</td>
<td>.50</td>
<td>0.980</td>
</tr>
<tr>
<td>after</td>
<td>1.95</td>
<td>6</td>
<td>.51</td>
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</tr>
<tr>
<td>Log Total Cholesterol</td>
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<td></td>
</tr>
<tr>
<td>before</td>
<td>2.06</td>
<td>7</td>
<td>.18</td>
<td>0.155</td>
</tr>
<tr>
<td>after</td>
<td>1.93</td>
<td>7</td>
<td>.20</td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>before</td>
<td>1.05</td>
<td>6</td>
<td>.28</td>
<td>0.699</td>
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<tr>
<td>after</td>
<td>1.10</td>
<td>6</td>
<td>.26</td>
<td></td>
</tr>
<tr>
<td>Total / HDL ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before</td>
<td>8.10</td>
<td>6</td>
<td>1.75</td>
<td>0.367</td>
</tr>
<tr>
<td>after</td>
<td>7.00</td>
<td>6</td>
<td>1.67</td>
<td></td>
</tr>
</tbody>
</table>

Fourteen subjects were treated with a combination of fibrate and statin. In these subjects combination therapy resulted in a highly significant decrease in serum triglycerides (52%, $p = 0.002$) and in serum total cholesterol (20%, $p = 0.007$). The 9% increase in serum HDL-C did not reach statistical significance.
Table 11: Serum lipids before, and after therapy with Statin and Fibrate.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>N</th>
<th>Std. Deviation</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Log Serum Triglycerides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before</td>
<td>1.87</td>
<td>14</td>
<td>.68</td>
<td>0.002</td>
</tr>
<tr>
<td>After</td>
<td>1.14</td>
<td>14</td>
<td>.50</td>
<td></td>
</tr>
<tr>
<td><strong>Log Total Cholesterol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before</td>
<td>1.94</td>
<td>14</td>
<td>.27</td>
<td>0.007</td>
</tr>
<tr>
<td>After</td>
<td>1.72</td>
<td>14</td>
<td>.23</td>
<td></td>
</tr>
<tr>
<td><strong>HDL cholesterol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before</td>
<td>.84</td>
<td>11</td>
<td>.24</td>
<td>0.267</td>
</tr>
<tr>
<td>After</td>
<td>.92</td>
<td>11</td>
<td>.41</td>
<td></td>
</tr>
<tr>
<td><strong>Total / HDL ratio</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before</td>
<td>9.36</td>
<td>11</td>
<td>3.09</td>
<td>0.115</td>
</tr>
<tr>
<td>After</td>
<td>7.37</td>
<td>11</td>
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THE TRIGLYCERIDE LOWERING EFFECTS OF OMEGA-3 FATTY ACIDS IN HIV INFECTED ADULTS ON HAART

This study was a double blind, placebo controlled trial of the effect of supplementation with salmon oil capsules rich in n-3 FAs. It was designed and managed by the Oak Tree Clinic at BC Women’s and Children’s Hospital in collaboration with the Atherosclerosis specialty lab at St. Paul’s Hospital. Analysis of serum lipids was done at the Atherosclerosis Specialty Lab.

3.9.1 OMEGA-3 STUDY METHODS

3.9.1.1 Recruitment

Subjects were recruited from HIV clinics at the Oak Tree Clinic, the Spectrum clinic and the metabolic clinic at the Infectious Disease Center at St. Paul’s Hospital. All subjects provided informed consent, approved by the University of British Columbia, St. Paul’s Hospital and the BC Children’s and Women’s Hospital research ethics boards. Inclusion / Exclusion criteria were as follows:

Inclusion Criteria:

- 18 years of age or older
- HIV+ positive
- Subjects on PI containing HAART must have been on regimen for at least 8 weeks.
- Ability to swallow nine capsules per day for 12 weeks.
- Serum triglycerides ≥ 3 mmol/L (12 hour fasting and no ETOH for 72 hours).
- Patients with triglycerides ≥ 3 mmol/L who were on stable lipid-lowering medications > 2 months were also considered eligible.
Exclusion Criteria:

- Had an acute opportunistic infection
- A history of fat malabsorption (must be ruled out in those with diarrhea)
- Hypothyroid or poorly controlled diabetes.
- Are pregnant or lactating.
- Initiated lipid lowering medication or, in the case of subjects on lipid lowering medication, change lipid lowering medication during the course of the study.
- Sensitivity or intolerance to fish oils.

3.9.1.2 Study protocol

The study lasted 16 weeks. Dietary counselling appropriate to the clinical condition was given at baseline in order to stabilize the subject’s diet. At week 4 participants that still met entry criteria were randomized to receive either 300 mg (t.i.d) n3/day or placebo (olive oil) for 12 weeks. The capsules used were North Sea Salmon Oil 1000 mg HFD 41 (Enerex Botanicals Ltd, Burnaby, BC) which contain 30% EPA / DHA (180 mg EPA, 120 mg DHA). Participants were asked to take nine capsules daily (three per meal) for the 12 week period.

At enrolment, anthropometric measures including height, weight, waist circumference, caliper skin folds and a bioimpedance assay (BIA) for body fat percentage were done. Participants were also asked to complete a 3 day food record and provided a sample of fasting blood following enrolment. At week 4, participants provided a sample of fasting blood and a 3 day food record and had weight measured. At week 10 participants were contacted and asked to provide another sample of fasting blood and to report any adverse events from the capsules. At week 16, at the close of the trial, participants were again assessed for the same anthropometric measures as at baseline and were asked to provide a 3 day food record and a sample of fasting blood. Routine testing of markers of HIV infection
(CD4 count and viral load) as well as coagulation testing by INR were performed by the hospital lab on each blood sample collected throughout the study.

3.9.1.3 Blood collection and storage

All blood samples were collected either at the St. Paul’s Hospital or at the BC Children’s and Women’s Hospital laboratory. Fasting blood samples were collected in a 10 ml tube containing EDTA. Blood was kept on ice and centrifuged at 3000 rpm for 15 minutes at 4°C. Aliquots of plasma were stored at −70°C until the conclusion of the study for analysis in batches.

3.9.1.4 Lipid analyses

Plasma total cholesterol, HDL cholesterol and triglycerides were measured in our lab. Samples were analyzed in duplicate by enzyme colourimetric method (Bayer Corp, Tarrytown NY) using a Technicon RA-1000 (Bayer Corp, Tarrytown NY) automated analyzer. HDL cholesterol was measured after precipitation of apolipoprotein B containing lipoproteins with phosphotungstic acid and MgCl₂. Average inter-assay C.V. for the cholesterol and triglyceride was below 3%. For HDL the average inter-assay C. V. was 3.3%.

3.9.2 OMEGA-3 STUDY RESULTS

Of the 34 subjects initially enrolled in the study twenty were included in the final analysis, ten were excluded due to baseline serum TG levels less than 3 mmol/L, three did not complete the protocol and one was excluded due to a lab error. There were no differences between the n-3 and placebo groups at baseline in age, BMI, serum lipids, dietary intake, duration on HAART, time since HIV diagnosis or CD4 count. Dietary counselling
received at enrolment had a non-significant but favourable effect on serum TG, total cholesterol, LDL-C and HDL-C. Serum triglycerides decreased from 5.3 mmol/L to 4.8 mmol/L, total cholesterol decreased from 6.0 mmol/L to 5.7 mmol/L and serum HDL-C increased from 0.88 mmol/L to 0.93 mmol/L (Figures 16-18). During the treatment phase there was no statistically significant change in any of the study parameters. Over the length of the study, including both the diet stabilization phase and the treatment phase, serum TG decreased by 25% in the n-3 group compared with 16% for the placebo group (Figures 20-23). The difference between the groups did not reach statistical significance but does demonstrate a potential benefit of both fish oil supplementation and dietary counselling in HIV+ patients suffering from treatment associated dyslipidemia.

3.9.3 OMEGA-3 STUDY DISCUSSION

Salmon oil is an intriguing treatment alternative for HAART dyslipidemia. In particular because of its perception as a natural health product rather than a drug it may prove a popular choice in a population that typically has a very high pill burden. As well, salmon oil has been shown to have a wealth of beneficial effects beyond its lipid lowering capabilities (reviewed briefly in [182]). The mechanism of action of salmon oil is similar to that of fibrates in that both are PPARα agonists (reviewed in [181]). Fibrates are the front line of therapy for hypertriglyceridemia and have been shown to improve serum lipids as well as decrease the incidence of CAD in HIV- populations, likewise for salmon oil. As far as we understand this trial was the first placebo controlled trial of salmon oil supplementation in the HIV+ population and it was hoped that the study would conclusively support the use of salmon oil as a treatment alternative in this population.
The trial was conducted in collaboration with the Oak Tree clinic at BC Women’s and Children’s hospital. The bulk of patient recruitment and study management was at Oak Tree while lipid analysis and lab procedures were managed through St. Paul’s Hospital. Based on initial power calculations the goal of the study was to recruit fifty subjects into a double blinded placebo controlled trial. Unfortunately, for a number of reasons, recruitment for the study was difficult and was cut short, leaving a total of twenty subjects for final analysis. This limited the statistical power of the study but did allow a demonstration of trends in lipid profiles following diet counselling and treatment with salmon oil.

While a favourable trend to improved serum lipids in response to counselling by a dietician was noted, interpretation of the effect of treatment was more complicated. Results at week ten (six weeks into treatment) indicated a decrease in total cholesterol, serum triglycerides and HDL-cholesterol, and an increase in LDL-cholesterol in the salmon oil treated group compared to control. By week sixteen there was no difference between the groups in any lipid parameter. Nonetheless, treatment with salmon oil did result in a favourable improvement in serum triglycerides compared to placebo (12% vs. 9% decrease). LDL cholesterol increased slightly over the course of the treatment; however, as has been shown in previous studies the increase may be related to increased LDL particle size and thus may not represent a significant increase in risk of atherosclerosis [183].

There are a number of explanations for the lack of significance. However, this study does suggest value of diet counselling and does provide some evidence that salmon oil, in combination with diet counselling can be used in HIV+ population. The difficulties encountered in this study highlight the difficulties typical to diet studies, and studies in the HIV+ population in particular. Both the pill burden and difficulty recruiting subjects fitting
strict eligibility criteria made recruitment difficult. As well, comparison with the placebo
control was complicated by a number of patient factors. If one were to improve the study it
would be necessary to improve tolerability of the salmon oil and to include a cross-over
design to remove confounding factors in analysis.
4 DISCUSSION

4.1 Cohort Characteristics

One hundred and eight participants were recruited into this study. One participant was excluded from analysis because of a diagnosis of familial hypercholesterolemia, a lipid disorder unrelated to HIV or therapy with anti-retroviral drugs. The cohort was composed predominantly of Caucasian males and none of the subjects had been diagnosed with any lipid disorders prior to the diagnosis of HIV or the initiation or anti-retroviral therapy.

Waist circumference in this cohort was comparable to the average waist circumference in a cohort of cardiac rehab patients in our center (92.6±8.7cm in HIV+ vs. 92.7±11cm in cardiac rehab), however, average BMI in the HIV+ cohort was lower than in the cardiac rehab group (25.0±3.2Kg/m$^2$ in HIV+ vs. 27.0±3.7Kg/m$^2$ in cardiac rehab) [190]. When compared to a cohort of apparently healthy Caucasian males from a second study, the BMI of the HIV+ group was comparable (25.0±3.2Kg/m$^2$ in HIV+ vs. 25.5±3.6Kg/m$^2$ in healthy males) at a slightly higher average waist circumference (92.6±8.7cm in HIV+ vs. 88.7±9.7cm in healthy males) [191]. Taken together, these observations suggest an increase in central adiposity in the HIV+ group as compared with both cardiac rehab patients and healthy male volunteers. A fat distribution of this nature is commonly seen in metabolic syndrome and is known to confer greater risk of CAD in the HIV- population [86, 106, 192].

Lipodystrophy was assessed by a self report questionnaire adapted from Carr et al. [193]. The questionnaire breaks questions about the body up into sections including face, front and side of neck, back or base of neck, arms, breasts, waist, buttocks and legs. The subject is asked to rate the extent of fat loss or gain in each of these sections as none, mild,
moderate or severe. The accuracy of the questionnaire has subsequently been validated by comparing self report with physician reported lipodystrophy [193]. The typically observed phenotype of lipodystrophy in HAART treated patients is one of combined peripheral fat loss with gain of central adiposity and, less frequently, gain of posterior neck fat, the so-called buffalo hump. While the frequency of lipodystrophy has varied greatly in previous studies (20-80%) the findings in our study, namely 81.7% of subjects reporting loss of peripheral fat while 50% reporting gain of abdominal fat and 25% reporting gain of neck fat, are not unexpected. Again, the fact that these numbers are on the higher end of the range reported in the literature may reflect the nature of the patient population at the St. Paul's HIV lipid clinic.

The relation between lipodystrophy and dyslipidemia in HAART treated patients is not as strong as was initially thought. In fact, Dyslipidemia secondary to HAART may develop in the absence of lipodystrophy although lipodystrophy can exacerbate dyslipidemia [49]. A useful categorization of lipodystrophy proposed by Hadigan [65] who classifies lipodystrophy into lipoatrophy, lipohypertrophy and mixed lipodystrophy, the typical loss of peripheral fat and gain of abdominal fat. When classified this way, 44.7% of subjects in our study observed lipoatrophy, 9.6% lipohypertrophy and 45.7% observed mixed lipodystrophy. A comparison of serum lipids between the groups revealed no significant differences. Again, this finding supports the notion that dyslipidemia in HAART can occur in the absence of changes in fat distribution. While it is possible that fat distribution changes affect serum lipids, this may be overshadowed by the magnitude of the direct effect of HAART on lipid metabolism in this cohort.
4.2 Lipid analysis

Mean serum lipid levels in this cohort were higher than those reported in some previous studies of HIV+ subjects taking anti-retroviral therapy [54, 49]. This reflects the fact that the St. Paul’s lipid clinic is a referral center for HIV associated lipid disorders and thus selects patients with clinically significant lipid disorders. Thirteen subjects had fasting serum glucose levels of at least 7 mmol/L, the cut-off for the diagnosis of diabetes. In that subset, serum TG was significantly elevated while there were no significant differences in all other lipid parameters. The association of increased FBS and serum TG is established in both HIV+ and HIV- subjects.

The mean serum TG and TC/HDL-C ratio were well above the target levels for a low risk population as recommended in the Canadian guidelines for the management and treatment of dyslipidemia [106]. Low risk is defined in the Canadian guidelines as having a ten year risk of CAD below 10% using the Framingham risk tables [98, 107]. According to the Canadian guidelines, patients with serum TG and TC/HDL-C ratio above the limit (3 mmol/L and 7 respectively for the low risk group) should be counseled on lifestyle and diet modification and, failing that, should be treated with lipid lowering medication.

In our cohort, assuming that the average participant is normotensive, does not smoke and does not have diabetes, the mean lipid values estimate the ten year risk of CAD to be 8% based on the Framingham risk tables [98, 107]. This compares to an optimal risk of 4% in the same age group producing a relative risk of CAD in our cohort of 2. Our finding is similar to that of a recently published paper by Hadigan et al. in which they calculated Framingham risk in 91 HIV infected patients with lipodystrophy compared with controls from the Framingham off spring study [194]. In their study they noted a 7.4% average 10-
year risk in HIV patients with lipodystrophy compared with 5.3% average 10-year risk in the control group. This difference was significant. It should be noted that, given the association between anti-retroviral therapy, metabolic syndrome, and insulin resistance, the average cardiovascular risk calculation in this case may underestimate the actual risk. In fact thirteen subjects in this cohort had fasting glucose levels in the diabetic range. That said, previous studies in subjects with HAART associated dyslipidemia have not reported significant increases in blood pressure [41, 195] (And Hadigan 2003) and, while smoking status was not assessed in this cohort, there is no reason to expect increased incidence of smoking compared to the general public.

4.3 Lipoprotein lipase and hepatic lipase analysis

Lipoprotein lipase and hepatic lipase are both important enzymes in the metabolism of lipoproteins. By its role in hydrolyzing TG in the core of VLDL and chylomicrons LPL is essential to clearance of serum TG from the blood, while HL activity has implications for HDL metabolism and, more importantly affects HDL and LDL particle size. Given these roles LPL and HL are likely candidates for a role in HAART associated dyslipidemia and in fact, TG clearance by LPL has been shown to be decreased in PI treated adipocyte cultures [140].

LPL and HL activity assays were done on post heparin plasma in a subset of our cohort, with more severe dyslipidemia. In this group, LPL activity was significantly decreased (36.03 ± 30.17 nmol/min/ml vs. 86.79 ± 63.96 nmol/min/ml) (p<0.01) while HL was significantly increased (225.15 ± 104.6 vs. 139.01 ± 40.89 nmol/min/ml) (p<0.05) compared to controls. LPL activity was inversely correlated with the log of serum triglycerides and positively correlated with serum HDL cholesterol levels while HL activity
was inversely correlated with serum HDL cholesterol. These correlations have been
demonstrated in previous studies in non HIV populations and are expected given the roles of
HL and LPL [136-138, 196].

Previous studies have explored the relationship between HL and LPL activity and
dyslipidemia in HAART treated subjects. Two studies have reported decreased HL and LPL
activity in a group of PI treated HIV infected men with hypertriglyceridemia [141, 142] and
Purnell et al. have reported a decrease in HL activity in eleven healthy HIV- volunteers after
two weeks of treatment with Ritonavir [143]. Purnell reported no change in the activity of
lipoprotein lipase although serum TG did increase significantly compared to a placebo
control group. To explain the increase in serum TG in the absence of decreased clearance by
LPL, Purnell et al. suggested that increased VLDL secretion by the liver may be the
mechanism. Indeed increased hepatic triglyceride synthesis has been shown to occur in PI
treated HepG2 cells and mice [197] and both C-peptide and central adiposity have been
shown to be better predictors of HIV-dyslipidemia than low LPL activity [142]. None the
less, the results from our study suggest that, in this subgroup with severe
hypertriglyceridemia, decreased LPL activity contributes to the dyslipidemia.

Given the finding of decreased LPL activity and increased HL activity in our cohort, it
is appropriate to consider the implications for risk of CAD. The association between
decreased LPL activity and CAD risk has been previously established, most notably in a
large cohort of 730 male CAD patients from the REGRESS study. In that cohort LPL
activity was significantly lower in CAD patients than in controls. It is likely that the
association of decreased LPL activity with CAD was mediated by the inverse correlation of
LPL with serum TG and positive correlation of LPL with serum HDL-C [196].
The association between HL activity and CAD is less clear. Increased HL activity is associated with decreased serum HDL-C [133] and LDL particle size [198-200], both changes that are strongly linked with increased risk for CAD [83-86]. However, increased HL activity also produces smaller HDL particles that better stimulate cholesterol efflux from peripheral tissue and HL may facilitate uptake of HDL particles by the liver, thus stimulating the reverse transport process. As well, by promoting the conversion VLDL to IDL and IDL to LDL, HL may decrease risk of CAD conferred by atherogenic IDL particles. On the other hand, decreased HL activity is associated with increased HDL and larger more buoyant HDL particles that imply decreased atherogenicity. However, low HL activity may result in decreased clearance of TG rich remnants and may result in slower turnover of the reverse cholesterol transport process, thus implying increased atherogenicity of decreased HL activity (reviewed in [105]). Two large studies by Zambon et al. and Dugi et al. have attempted to resolve the dilemma but have come up with apparently conflicting results.

Zambon et al. provided evidence for a pro-atherogenic role for HL in a study of eighty-eight subjects with CAD treated with cholesterol lowering medication. In their study, changes in HL activity were negatively correlated with changes in LDL buoyancy and positively correlated with changes in coronary stenosis [139]. However, a study, by Dugi et al., of two hundred male coronary angiography patients found that low HL activity was associated with CAD [201]. A mechanism for this finding may be explained by decreased catabolism of atherogenic triglyceride rich remnant particles by low levels of HL activity. In fact, it is possible that HL can be pro or anti-atherogenic depending on the underlying lipid parameters. In a review Jansen et. al. the authors suggest that HL may be "anti-atherogenic in (familial) hypercholesterolemia and pro-atherogenic in hypertriglyceridemia [although] in
normolipidemia, HL seems to have little effect on CAD risk" [105]. This hypothesis relates to the enrichment of LDL and HDL particles with triglycerides in hypertriglyceridemic patients, the result of which is decreased LDL particle size resulting from lipolysis by HL. In this case, increased HL activity is pro-atherogenic. The hypothesis of increased risk with increased HL activity in hypertriglyceridemic patients again raises concern in HAART dyslipidemic patients in whom hypertriglyceridemia is the major lipid abnormality.

4.4 Genetic Analysis

Genetic analysis was carried out on a number of candidate polymorphisms. These polymorphisms were selected because of prior evidence for links to dyslipidemia, and hypertriglyceridemia in particular. With the relatively small number of patients available for genetic analysis (seventy eight with white cells for DNA purification) it was not realistic to expect significant relationships between rare alleles and lipid parameters. Rather, the intention was to screen for increases in relative distribution of polymorphisms in this population.

Polymorphisms in LPL (N291S), apo E (E2,E3,E4) and the SstI polymorphism in Apo C-III were all found to be at expected population levels and there was no association with serum lipids in our cohort. This makes it unlikely that any of these particular polymorphisms play a role in development of dyslipidemia in HAART metabolic syndrome. However, the possibility still exists that there is a genetic component to the risk of developing dyslipidemia in response to HAART. Given the relative prevalence of dyslipidemia in HAART treated patients, such a polymorphism would be expected to be very common.
4.5 Effect of Nevirapine on serum lipids

The association between PI use and dyslipidemia is clear, and it appears that NRTI's are associated with lipodystrophy and maybe with dyslipidemia. On the other hand the NNRTI class, Nevirapine in particular, may spare patients of lipid changes and may reverse dyslipidemia if substituted for PI's in HAART regimens [202]. In particular, as evidence from a subset of patients in the Atlantic Trial suggests, nevirapine appears to have a beneficial effect on HDL-cholesterol levels. Patients in this trial were treated with stavudine and didanosine and were randomized to receive either indinivir, lamivudine, or nevirapine [203]. In the nevirapine treated patients, HDL cholesterol increased by forty nine percent. LDL-cholesterol increased significantly in both the nevirapine and indinivir treated groups but, in the nevirapine group this increase was offset by a substantial improvement in total to HDL cholesterol ratio.

As part of a pilot project for a proposed study, a sub-analysis of patients in our study was done to give a preliminary estimate of the effect of nevirapine on HDL cholesterol levels. A comparison was made between subjects on regimens including nevirapine (n=35) and subjects whose regimens did not include nevirapine (n=36). To attempt to control for anti-retroviral regimen all subjects in each group were on regimens including a PI and it was assured that they had been on a stable regimen for three months and were not taking lipid lowering medication. HDL-cholesterol was 16% higher (p<0.05) in the group taking nevirapine (0.98 ± 0.28 mmol/L) as compared with the control group (0.84 ± 0.24). There was no statistically different difference in any other lipid parameter. Although the change in HDL-C is statistically significant, the clinical significance of such a small change is unclear. From a risk factor perspective, even a small increase in what initially is a low HDL-C level
may be beneficial. The VA-HIT and Helsinki Heart studies suggest that every 1% increase in HDL-C results in a 3% decrease in risk of CAD [101, 204]. That said, the fact that there was no change in the ratio of total cholesterol to HDL-C suggests that the gains seen in improved HDL-C may be negated by a concomitant increase in LDL-C. In fact, the ratio of total to HDL cholesterol is a well established marker of CAD risk. However, one must also consider that all subjects were treated with a PI. Given that PI use demonstrably affects lipid metabolism and lowers HDL-C it is likely that the effect of nevirapine is masked somewhat by an overwhelming influence of PI’s.

4.6 Effect of lipid lowering medications on serum lipids

A second sub-analysis done in this cohort of patients was that of the effect of lipid lowering medications on serum lipid levels. Analysis compared lipid levels before and during treatment with fibrates alone, statin alone and statin/fibrate combination. Treatment with fibrates alone resulted in a significant decrease in serum triglycerides (37%, p=0.028) while treatment with a combination of statins and fibrates resulted in a significant decrease in both serum triglycerides and total cholesterol (52%, p=0.002 and 20%, p=0.007 respectively).

It should be made clear that this was not a comparative analysis in the sense that the goal was not to compare therapy with fibrates against statins. Rather, the analysis was retrospective based on clinically prescribed medications. Nonetheless, the results demonstrate a clear benefit to the use of fibrates to lower serum triglycerides and statins to lower total cholesterol in the HAART dyslipidemia population. Although a small number of patients were followed in this sub-study we feel that we have demonstrated the efficacy of
lipid lowering medication in clinical practice based on normal prescribing habits and patient compliance.

The efficacy of statins and fibrates was assessed in HAART dyslipidemia by Calza et al. [205]. One hundred and six subjects received either bezafibrate, gemfibrozil, fenofibrate, pravastatin, or fluvastatin for 12 months. As a group the fibrates reduced serum TG by 41% and LDL-C by 23% while statins decreased serum TG by 35% and LDL-C by 26%. HDL-C increased by 20% and 24% in the fibrate and statin groups respectively. All changes were highly significant. Similar findings were reported in a second study statin use [206]. In our study no significant change was seen in HDL-C or in TC/HDL-C ratio, although trends to increased HDL-C and decreased TC/HDL-C were noted in each treatment sub-group. In the fibrate group HDL-C increased by 8%. In clinical trials of fibrates, HDL-C is generally increased by about 10% to 20% [169]. In our study it is possible that the lack of significance was due either to a small sample size, short follow up, or that fibrates are not as effective at raising HDL in the HIV metabolic syndrome patient.

Because HAART metabolic syndrome often includes dyslipidemia, insulin resistance and lipodystrophy, combination therapy with Fibrates and thiazolidinediones (TZDs) appears a reasonable choice. TZDs have previously been shown to improve insulin resistance in HIV+ populations [207]. Unpublished data from our center shows that combination therapy with fibrates and TZDs paradoxically lowers HDL-C. In nine HIV+ patients treated with a combination of micronized fenofibrate and rosiglitazone HDL-C decreased by an average of 33% (0.80 ± 0.19 mmol/L to 0.56 ± 0.27 mmol/L) while the triglyceride levels increased by 48% (5.10 ± 2.22 mmol/L to 7.25 ± 4.16 mmol/L). It is not yet known whether the decrease in HDL represents an increase in risk of CAD because it is possible that HDL-C decreases
secondary to increased flux of the reverse cholesterol transport process. Because both fibrates and TZDs work by activating PPARs (PPARγ for TZDs and PPARα for fibrates) it is possible that adverse effect of TZDs and Fibrates is mediated by a PPAR related mechanism.

4.7 Risk of coronary artery disease with HAART

HAART metabolic syndrome is associated with a multitude of known CAD risk factors. These include lipid risk factors such as elevated serum TG and decreased serum HDL-C, as well as insulin resistance and a tendency towards a pro-atherogenic central body fat distribution. As well, there is a report of significant increases in atherogenic lipoprotein (a) Lp (a) [68] and evidence of endothelial dysfunction [208] in HAART metabolic syndrome. Both Lp (a) and endothelial dysfunction are known to confer risk for CAD in the HIV- population [209, 210]. Add to these the findings of this study, namely that LPL is decreased and HL is increased in a subset of our cohort, and it seems clear that HAART metabolic syndrome would confer a significant risk of CAD. Indeed this is a question that has been of great concern, particularly considering the significant improvement in morbidity and mortality provided by HAART treatment.

Using Framingham tables in our cohort we estimate a ten year risk of 8%, assuming no hypertension, smoking, or diabetes in our participants, an assumption that will result in an underestimation of risk. Similar calculations have been done in other cohorts of HIV+ patients. Law et al. estimated the three year “worst case scenario” risk in 17,600 participants enrolled in the DAD study (Data Collection on Adverse Events of Anti-HIV Drugs Study) to be 2% in patients taking both NNRTI and PIs. They conclude that the “modest risk” in their cohort was outweighed by the substantial decrease in HIV associated morbidity and mortality [211]. A similar study using the Framingham risk tables in a ninety one HIV+ subjects with
lipodystrophy found significantly elevated ten year risk in patients with fat redistribution compared to 273 matched control subjects from the Framingham offspring study (7.4% vs. 5.3%, \( p=0.002 \)). The risk estimate was greatest in subjects with primary lipoatrophy vs. mixed or lipo-hypertrophy [194].

The bulk of the evidence to support increased incidence of CAD is based on case reports in HAART treated patients [72-78] (total of twenty reports of CAD) as well as a report of acceleration of CAD in HAART treated patients with previously known CAD [212]. More recently, Matetzky et al. compared the course of twenty four HIV+ patients who were admitted to hospital for acute myocardial infarctions with a group of appropriately matched HIV- controls. All the patients had benign hospital stays but the HIV+ group had a higher rate of re-infarction 20% vs 4% (\( p=0.07 \)) once discharged [213]. Holmberg et al. provided some epidemiological support when they noted that in 5672 American HIV+ outpatients followed between 1993 and 2002, the frequency of MIs increased after introduction of PIs in 1996. In interpreting these results however, one must consider that the significant decline in HIV related mortality attributable to PI use may give the appearance that mortality from other causes has increased [214].

Recently an evaluation of four hundred and twenty three HIV+ patients revealed that intima media thickness, a surrogate for CAD severity, was related only to traditionally known CAD risk factors in multivariate analysis, although in univariate analysis both presence of lipodystrophy and HAART treatment were significant variables [215]. The authors suggest that HAART therapy does not, on its own, increase CAD progression beyond the effect of traditional risk factors.
In the past year, two large studies have been published in the New England Journal of Medicine. The first study, by Bozzette et al., retrospectively followed 36,766 patients in the US Veterans Affairs health network between 1993 and 2001 [81]. The results show a significant reduction in all cause mortality associated with PI use and do not show any increased incidence of cardiovascular or cerebrovascular events in patients treated for HIV. Similar findings have been noted in another cohort and in retrospective analysis of clinical trials data [79, 80]. A second large study prospectively followed 23,468 patients in twenty one countries in Europe, Australia and the United States as part of the DAD study group [82]. Their results show a 1.26 (p<0.001) adjusted relative rate of MI per year of exposure to combination antiretroviral therapy despite a low absolute risk of MI, only 126 patients had an MI in 36,199 patient years. Classical risk factors including age (RR=1.38 p<0.001), smoking (current and prior) (RR=2.17 p<0.007), previous cardiovascular disease (RR=5.84 p<0.001), male sex (RR 1.99 p<0.04), elevated serum cholesterol (RR=1.20 per 1 mmol/L increase, p<0.007), elevated serum triglycerides (RR=1.16 per doubling, p<0.03), presence of diabetes (RR=1.25 p<0.001) and hypertension (RR=1.25 p<0.001) were also all associated with increased incidence of MI.

In general the incidence studies conducted thus far have not stratified patients based on the presence of the metabolic syndrome but instead have calculated incidence based solely on treatment. Because metabolic syndrome likely does not develop in all subjects, and because the severity of metabolic syndrome varies from person to person and from drug regimen to drug regimen the possibility of increased incidence of CAD remains significant. Indeed the DAD study did show significant contributions to risk of MI from traditional risk factors including hyperlipidemia and diabetes, both of which are markers of HIV metabolic
syndrome. This may be particularly true for a subset of severely affected patients. It remains possible that the relative risk of MI in the subset of HIV patients that develop metabolic syndrome is significantly elevated.

The exposure to PI's in the paper by Bozzette et al. was eighteen months while the median exposure to combination antiretroviral therapy in the DAD study was 1.9 years. Because of this the authors caution that ongoing follow-up is important to determine the long term risk. Particularly in light of findings of endothelial dysfunction [208] and IMT thickening [215] which are signs of the early stages of atherosclerosis. Findings such as these, and those of the DAD study will likely be of particular importance as the HIV+ population, which tends to be younger than the CAD population, ages.

At present recommendations point out that the benefits of combination antiretroviral therapy are well established, but that clinicians remain aware of the potential for increased incidence of CAD. It is recommended that patients with the HAART metabolic syndrome population be treated as aggressively as in HIV- populations with respect to risk factor and lifestyle modification [65]. Considering the evidence of other studies and results from this study, namely that LPL is decreased and HL is increased and that these subjects have a proportionally greater waist circumference than is desirable it is prudent to encourage an aggressive approach to minimizing CAD risk in HAART treated patients.

4.8 Speculations on a mechanism for HAART metabolic syndrome

As yet no satisfactory mechanism has been proposed that can account for the spectrum of symptoms involved in HIV metabolic syndrome. Rather, there have been a number of
hypotheses suggesting that the multitude of metabolic effects of PI use may result by a number of mechanisms, all playing a role and contributing to the milieu of symptoms.

Hyperlipidemia may result either from hepatic overproduction of VLDL and LDL, or from reduced clearance from circulation. While we, and others, have demonstrated decreased clearance by diminished LPL activity in humans and in cultured adipocytes [140] [141], there is good evidence from in-vitro studies and studies in animals to suggest that lipid synthesis is increased as well. In hepatocyte cultures, PI treatment has been shown to inhibit degradation of apo B which results in increased VLDL secretion [216]. Indeed PI treatment has been shown to increase hepatic triglyceride synthesis [197] as well as apo B synthesis and VLDL secretion in cell culture and in animal models [217]. In summary, hyperlipidemia results from a number of changes involving both hepatic overproduction and clearance from circulation. However, hyperlipidemia is only one component of the HAART metabolic syndrome.

PIs, and to a lesser extent NRTIs, have been shown to inhibit adipocyte differentiation and in some cases lead to adipocyte death in culture [64, 218]. PIs have also been shown to inhibit the activity of GLUT-4, the insulin-responsive glucose transporter [219], a reaction that may contribute to insulin resistance. Complicating the matter, NRTIs have been implicated in the development of peripheral lipodystrophy and lactic acidosis through inhibition of mitochondrial DNA polymerase gamma [56, 57] which leads to impairment and death of the mitochondria [61, 220]. This has been postulated as the underlying mechanism for peripheral fat wasting in HAART treated patients [58-62]. The sum of all this proves that there are several disruptions in several aspects of lipid metabolism, glucose metabolism and
fat distribution but clearly none of these individual insults can be responsible for the full range of symptoms.

At this point a single, unifying hypothesis has neither been tested nor proven but there are a few candidates in the literature. Both of which involve transcription factor regulation which allows for wide ranging downstream effects. The initial hypothesis was proposed by Carr et al. when they noted homology between the catalytic site of HIV-1 protease and the lipid binding domain in the low density lipoprotein receptor related binding protein (LRP) and a C-terminal region of the cytoplasmic retinoic-acid binding protein type 1 (CRABP-1) [221]. Their proposal was that protease inhibitors bind to these domains and inhibit their normal function. CRABP-1 binds to retinoic acid and presents it to the cytochrome p-450 system which converts it to cis-9-retinoic acid which is a ligand for retinoid X receptor (RXR) which functions in a heterodimer with PPARγ. In this way, PIs, by binding to CRABP-1 would lead to a deficiency of cis-9-retinoic acid which may result in increased adipocyte apoptosis, decreased adipocyte differentiation and proliferation and possibly hyperlipidemia and insulin resistance, all endpoints of regulation by RXR-PPARγ. Unfortunately, evidence from animal studies suggests that this mechanism does not adequately account for the hyperlipidemia (reviewed by Mooser and Carr [222]).

Another interesting mechanism, recently reviewed by David Hui [223], implicates PI inhibition of proteasome mediated degradation of nuclear sterol regulatory element binding proteins 1 and 2 (SREBP-1 & SREBP-2). In animal models PIs have been shown to significantly increase the levels of activated SREBP-1&2 in the liver and in adipose tissue, despite no change in mRNA levels [224]. SREBP-1 stimulates fatty acid synthase and decreases levels of leptin while SREBP-2 increases serum TG and cholesterol by increasing
lipid biosynthesis in the liver. In transgenic mouse models, increases in SREBP-1 have been shown to result in increased expression of genes responsible for fatty acid synthesis as well as in generalized lipodystrophy, insulin resistance and elevated serum TG and cholesterol levels [225]. SREBP-2 transgenic mice show increased expression of cholesterol biosynthesis genes [226, 227]. Given the evidence for decreased apo B degradation by PIs [216], which increases the level of apo B available for VLDL production, SREBP related increases in lipid biosynthesis would provide a good explanation for hyperlipidemia in HAART treated patients.

Because of the far reaching downstream effects of both CRABP-1 and SREBP both theories are appealing as candidates for a single unified mechanism. However, neither has been convincingly proved or disproved and indeed it is likely that the spectrum of symptoms is in fact the result of several insults on the body’s regulatory mechanisms.

4.9 Limitations

We chose a small number of polymorphisms to screen for based on their effect in previous studies in both HIV- and HIV+ populations. Given the relatively small size of our cohort, the hope of the genetic analysis was to describe a polymorphism that was in significantly higher proportion and had a significant effect on lipid levels in our cohort. This is understandably a long shot, particularly given the rarity of some of the polymorphisms.

We have compared LPL and HL levels in our cohort to an age matched male control group. While we have shown significant differences between the groups it must be acknowledged that this control group is not ideal. In order to conclusively state that HAART affects LPL and HL levels it would be desirable to compare enzyme activity in patients with
HAART dyslipidemia with HIV+ patients, on equivalent drug regimes, who do not present with dyslipidemia. Given the complexities of HAART regimens however, such a control group would be difficult to establish. As well, the nature of the laboratory procedure for the lipase activity in our lab limits the size of the study and limits the ability to compare results between studies. The large inter-assay variability in the procedure requires samples to be batched and run with a single substrate, therefore there is a reasonable limit to the sample size. For this reason the lipase levels were only assayed in a limited number of subjects and controls.

The study of the effect of n-3 FAs showed that diet counselling and fish oil supplementation may decrease serum triglycerides in this group. Again, the significance of this effect was limited by the small number of patients enrolled in the trial. In retrospect the burden of taking nine pills daily was likely a significant deterrent in a population which already has a substantial pill burden. That said, fish oil supplementation is an appealing treatment alternative and this study has provided some indication of a positive effect. With the availability of improved and more concentrated n-3 FA products it may be possible to conduct a larger study with less difficulty.

4.10 Conclusion and implications

We have shown that our cohort of dyslipidemic HIV+ patients has elevations in serum lipids that put them at risk of CAD. On average, our cohort has twice the relative risk developing CAD in the next ten years than an optimal control. We have also shown that 12% of our cohort has FBS above the cut-off for diabetes. This finding would substantially increase the risk of CAD in those subjects and increase the average risk in the cohort in general. We have demonstrated that LPL is significantly decreased and HL is significantly
increased in a subset of patients with severe or persistent dyslipidemia. While there is clear evidence that increased lipid production by the liver does occur in HAART dyslipidemia, our results show that changes in the catalytic activities of LPL and HL contribute to the lipid profile, and to the risk of CAD in our cohort.

While we have not demonstrated an association between any of the genetic polymorphisms examined and dyslipidemia in our cohort, the possibility remains that there is a genetic component to the development of dyslipidemia with HAART treatment. This is likely the case because it appears that only a proportion of patients on HAART develop dyslipidemia and there is significant variability in severity of dyslipidemia in those patients. We have examined only a few polymorphisms in this work but there is a wealth of polymorphisms on many genes that affect serum lipids in HIV- populations which may play a role in HIV metabolic syndrome.

As well, we have shown that treatment with diet counselling and with n-3 FAs decreases hypertriglyceridemia in this cohort. Although the changes were not statistically significant, there was a trend to improved lipids which supports the initiation of a larger and more carefully controlled trial.

Although there is conflicting evidence regarding the incidence of CAD, we feel that there remains a significant long term risk of CAD in HAART treated patients. Given the cluster of risk factors described in our study, and in previous studies, and the association of many of these risk factors with CAD in HIV- populations it is very likely that the rates of CAD among HIV+ patients will begin to climb over the next several years.
REFERENCES


6 FIGURES

Figure 1. The exogenous, endogenous and reverse cholesterol transport pathways of lipid metabolism.
Frequency distribution of weight

Frequency distribution of waist circumference

Figure 2. Frequency distribution of body weight at the time of recruitment

Figure 3. Frequency distribution of waist circumference at the time of recruitment
Frequency distribution of body mass index (BMI)

![Frequency distribution of body mass index](image)

**Figure 4.** Frequency distribution of body mass index at the time of recruitment

Frequency distribution of serum total cholesterol

![Frequency distribution of serum total cholesterol](image)

**Figure 5.** Frequency distribution of serum total cholesterol prior the use of lipid lowering medication
Figure 6. Frequency distribution of serum HDL cholesterol prior to the use of any lipid lowering medication

Figure 7. Frequency distribution of serum triglycerides prior the use of lipid lowering medication
Figure 8. Frequency of serum fasting blood sugar prior the use of any lipid lowering medication.

Figure 9. Frequency distribution of serum total to HDL cholesterol ratio prior the use of lipid lowering medication.
Figure 10. Lipoprotein lipase activity in HIV metabolic patients vs. healthy controls

Figure 11. Correlation of lipoprotein lipase activity with serum triglycerides
Hepatic Lipase Activity

Figure 13. Hepatic lipase activity in patients with HIV metabolic syndrome vs. healthy controls

Healthy: 139.02±40.89
HIV+: 225.15±104.6

Kruskal-Wallis: p<0.05

Figure 12. Correlation of lipoprotein lipase activity with HDL cholesterol

HDL cholesterol vs. LPL activity

Figure 13. Hepatic lipase activity in patients with HIV metabolic syndrome vs. healthy controls
Figure 14. Correlation of hepatic lipase activity with HDL cholesterol

Figure 15. Comparison of serum HDL cholesterol in HAART treated patients taking PIs, whose regimens do, and do not include nevirapine.
Change in serum HDL-cholesterol following dietary counselling alone (n=24)

![Graph showing change in serum HDL-cholesterol](image)

**Figure 16. Effect of diet counseling alone on serum HDL cholesterol**

Change in serum LDL-cholesterol following dietary counselling alone (n=24)

![Graph showing change in serum LDL-cholesterol](image)

**Figure 17. Effect of diet counseling alone on serum LDL cholesterol**
Change in serum total cholesterol following dietary counselling alone (n=24)

Figure 18. The effect of dietary counselling alone on serum total cholesterol

Change in serum triglycerides following diet counselling alone (n=24)

Figure 19. Effect of diet counseling alone of serum triglycerides
Change in serum HDL-cholesterol over 16 weeks of diet counseling and n-3 or placebo

Figure 20. Effect of diet counseling and n-3 or placebo treatment on serum HDL cholesterol

Change in serum LDL-cholesterol over 16 weeks of diet counseling and n-3 or placebo

Figure 21. Effect of diet counseling and n-3 or placebo on serum LDL cholesterol
Change in serum triglycerides over 16 weeks of diet counseling and n-3 or placebo

Figure 22. Effect of diet counseling and n-3 or placebo on serum triglycerides

Change in serum total cholesterol over 16 weeks of diet counseling and n-3 or placebo

Figure 23. Effect of diet counseling and n-3 or placebo treatment on serum total cholesterol.