Identification of Circulating Biomarkers of HIV Disease Progression

by

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Abstract

Understanding the ability of Nonprogressors (NP) to control HIV disease progression may lead to novel therapeutic strategies and vaccines, with the potential to make a significant contribution to the field of public health. A small percentage of HIV infected individuals control HIV disease progression for many years without therapeutic intervention, defined as long-term nonprogressors (NP). We have demonstrated that all professional antigen presenting cells (APC) (B cells, dendritic cells (DC) and macrophages) from NP have the unique inability to mediate HIV trans infection of autologous and heterologous CD4+T cells. We have linked this phenotype to alterations in cholesterol metabolism in APC, specifically an increased activity of the reverse cholesterol transporter ABCA-1, and to depletion of cholesterol in cell membrane lipid rafts. ABCA1 activity could be modulated by transcriptional or post transcriptional factors. Our data show that plasma from NP elicit higher cholesterol efflux from macrophages in males and females than Progressors (PR).

It was also our aim to identify soluble, circulating biomarkers that could modulate ABCA1 activity, thus being associated with control of HIV disease progression. A bioactive fatty acid screen was performed on a subset of NP and PR samples that have been well-characterized by testing for APC mediated trans infection. Analysis by liquid-chromatography-mass spectrophotometry identified metabolites present at a higher level in NP than PR, specifically 5-oxo-eicosatetraenoic acid (5-Oxo-ETE). Preliminary data of 5-oxo show that treatment of APC at
5μM reduces the efficiency of HIV trans infection of CD4+T cells. A second molecule that was tested was 25-Hydroxycholesterol (25HC), which interferes with virus-cell membrane fusion and reduces inflammatory factors and cytokines. Serum from HIV infected individuals was tested for 25HC content by ELISA, and preliminary data show that 25HC levels are slightly higher in NP than PR, but more samples need to be analyzed. A trans infection assay in the presence of 25HC was also performed and it was found that treatment of B cells with 25HC in culture strongly inhibits HIV trans infection.

Genetic factors have been identified that contribute to cholesterol efflux capacity in men and women. SNPs were identified by Villard et.al. that affect the ability of macrophages to efflux cholesterol and were implicated in atherosclerotic development. These SNPs were tested in our HIV NP and PR cohorts. Two SNPs were found to be significantly associated with the male NP, contributing to differences in cholesterol metabolism and homeostasis in male and female NP and PR.
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Preface

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1.0 Introduction

The Human Immunodeficiency virus (HIV) is a positive-sense retrovirus with a double membrane derived from the host cell. The viral envelope contains two major glycoproteins, gp41 and gp120, which mediate viral entry and fusion. Viral RNA is enclosed by a capsid core composed of three structural proteins, p24, p16 and p9 (1). The p24 protein encloses the genomic viral RNA and enzymes required for replication. After entry into the cytoplasm, the capsid disintegrates, and the viral RNA is reverse transcribed into DNA where it is transported to the nucleus. The HIV protein, integrase, then incorporates the viral DNA into the host chromosome. The viral DNA is replicated with the host DNA to make progeny genomes and transcribe viral proteins for assembly into new viral particles at the cellular membrane (1). High content of cellular lipid rafts is desirable for virion assembly (3). A lipid raft is an area of the host membrane that contains high cholesterol and sphingolipid content, and is involved in signal transduction, and endocytosis (2). They are required at multiple points in the viral life cycle and server as docking sites for entry receptors and anchor viral glycoproteins during assembly (2).

HIV primarily infects CD4+ T cells through the CD4 molecule and either the C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) coreceptors. There are two strains of the virus: T-cell-tropic (T-tropic) and macrophage-tropic (M-tropic) (4). T-tropic virus uses the CXCR4 coreceptor more commonly expressed on CD4+ T cells and M-tropic use CCR5 as a coreceptor (1). The virus is also able to infect monocytes, such as dendritic cells, B cells and macrophages though these chemokine receptors as well as DC-SIGN (5).

HIV has infected 75 million people since the epidemic first began and has killed 32 million globally (6). An estimated 38 million are currently living with the disease, and it affects
approximately 0.8% of people age 15-49 globally (7). Since the beginning of the epidemic in the 1980s, new HIV infections have been reduced from 130,000 to 50,000 in 2010 (8). The prevalence of people living with the disease has greatly increased due to treatment advances and the development of antiretroviral therapy (ART), which dramatically reduced the number of deaths from HIV. ART therapy reduces viral load in plasma to undetectable levels inhibiting the virus at different stages of replication, including attachment, fusion, reverse transcription and integration of the virus. Currently there is no cure or vaccine for HIV and the virus cannot be fully eradicated from the body.

1.1 HIV Long-term Nonprogressors

Several years after the emergence of HIV, a small group of infected individuals were found to have slow progression to AIDS and were referred to as Long-Term Nonprogressors (LTNP) (9). Progression from HIV to AIDS can largely be divided into three categories: rapid progression, where AIDS is developed within three years of infection; intermediate progression, where AIDS develops slowly between three and 10 years; and long-term nonprogression (LTNP), where individuals maintain high CD4+ and CD8+ T cell counts for more than 8 years without antiretroviral therapy (10). LTNP comprise a small percentage of HIV infected individuals are able to control disease progression for many years without therapeutic intervention, maintaining high CD4+ cell counts and, in most cases, detectable viral loads (Figure 1). These individuals represent less than 5% of the HIV infected population and can be divided into three categories: (i) long-term nonprogressors, who have high CD4+ cell counts with detectable viral loads; (ii) viremic controllers (VC), who have high CD4+ cell count and high viral loads (>2000 RNA copies/mL),
and (iii) elite controllers (EC), who have no detectable viral load though it is known they have been exposed to the virus. There are several viral, immunological and genetic factors that contribute to HIV-1 nonprogression, but not one of these are present in all NP and are unable to completely explain the phenomenon.

Figure 1. HIV Nonprogressors

HIV nonprogressors maintain high CD4+ counts and have detectable viremia compared to normal progressors and rapid progressors (Poropatich and Sullivan, 2011).

1.1.1 Factors Contributing to Nonprogression

There are host, genetic and viral factors that may influence the rate of progression to AIDS. Below are some common and well-known elements that contribute to HIV nonprogression.
1.1.1 Viral Attenuation

Studies have shown that viruses of HIV nonprogressors have lower diversity than progressors, diversity of quasispecies being hallmark feature of HIV infection. They also show higher replication incompetence of the virus, including stop codons in structural genes and missing long-term repeats (11). Decreased viral evolution correlates with weaker viral fitness and inability to evade the immune system. It has been shown that in many cases viruses from LTNP infect CD4+ monocytes less efficiently and more slowly in culture (12).

1.1.1.2 Chemokine Receptor Polymorphism CCR5-Delta32

CCR5 is normally expressed at low levels on CD4+ cells. Studies have shown that some NP have an allele with a mutation in this gene consisting of a 32 base pair deletion that introduces a stop codon that truncates the protein rendering it ineffective. Studies have shown that NP are more frequently heterozygous for this allele than progressors, but there has been conflicting data regarding the significance of this polymorphism with the control of HIV infection (13). Many newer studies have reported it absent in their LTNP cohorts, whereas some older studies reported this to be highly a significant factor contributing to the control of the disease (13).

1.1.1.3 HLA Class I Alleles

HLA Class I Alleles (A, B and C) present antigens to CD8+ T cells to control HIV replication. People who are heterozygous have been shown to slow HIV progression due to presentation of a larger pool of peptides, decreasing viral escape and immune evasion (14). Two HLA-B57 variants have been linked to HIV control, B5701 and B5703, by inducing a strong response against the GAG protein (15). HLA-B27 has also been linked to nonprogression by producing a strong CTL response to the p24 capsid protein, but is found to be ubiquitous in the
human population (16). Between 1 and 10% of humans carry a copy of the HLA-B57 allele and 1.8-8% carries a copy of HLA-B27. They have been found to vary considerably in NP cohorts anywhere from 2-63% (13).

1.1.1.4 APOBEC3G

APOBEC3G is a cytidine deaminase that stops HIV from integrating by introducing G to A base mutations in viral DNA producing premature stop codons, causing the virus to become replication incompetent (14). Higher levels of APOBEC3G have been associated with higher CD4+ cell counts and lower viral loads. People with lower viral loads, who had been infected for three years or more, had higher levels of APOBEC3G than progressors or seronegative controls (17).

1.1.1.5 CD8+ T cells

CD8+ T cells are important in killing HIV infected cells and lowering viremia. It has been shown that NPs have a more broadly effective CTL response against the env, gag and pol proteins compared to PR (18). An inverse correlation has been found between the number of gag epitopes recognized by CTLs and viral load. Regions of the protein have been identified that most strongly correspond with lower viral load and higher CD4+ cell count (19). Studies have also shown that quantitatively, CTL response between NP and PR are largely the same, but differ based on proliferation, cytotoxicity and production of IFN-γ, IL-2 and granzyme B (20). CTLs of NP were also found to be highly functional, having four or five functions compared to two or three in PR (20). CD8+ cells of NP secrete more Granzyme B and perforin on average, required for cytolysis of infected cells (21).
1.1.1.6 CD4+ T Cells

HIV-specific CD4+ T cell counts correlate to higher CD8+ counts and lower viral loads (22). Similar to CD8+ T cells, the CD4+ cells of NP have been found to have a broadly robust defense against the gag protein, and this remains at high levels throughout the duration of their disease, correlating to lower viral load (23). NP have been found to have higher levels of CD4+IL-2+IFN-γ + cells, with larger production of IL-2, skewing the response toward inflammatory Th1 (24). There is some data showing that CD4+ T cells of NP are chronically activated based on their increased HLA-DR expression, increased IFN-γ production and decrease in IL-7a, and this is different from non-specific CD4+ activation in PR (24). IL-7 is important for T cell proliferation and it’s receptor, IL-7R, has been found to be expressed at higher levels in NP compared to PR, helping with thymic replenishment of depleted peripheral CD4+ T cells (25).

1.1.1.7 Pro- and Anti-Inflammatory Cells

HIV-infected individuals have been found to have higher levels regulatory T cells (Treg), a type of CD4+, compared to uninfected controls (26). Tregs are able to downregulate chronic immune activation and lower unspecific immune responses, which can be beneficial to the host. Tregs are found at higher levels in PR compared to NP and uninfected controls, and this may actually be harmful by downregulating the response to the virus too strongly to appropriately fight the infection (27). Th17 cells have an opposing function to Tregs and create an inflammatory response, playing an important role in eliminating extracellular pathogens. Together Tregs and Th17 keep the immune system in check. In normal progressors, this balance is off and Th17 response is weakened, while the Treg response is strengthened. In NP the balance remains intact and is more similar to the uninfected population (28).
1.2 Latency and Reservoirs

Treatment of HIV with antiviral therapy (ART) has dramatically reduced the death rate from HIV/AIDS, but it is not able to completely eradicate the virus from the body. Soon after infection, HIV forms a latent reservoir in a small population of long-lived resting CD4+ memory cells where it is maintained when active cells revert back to their resting state. They can live for many years and replicate at low levels while carrying integrated viral genomes, with little to no viral gene expression occurring (29). HIV also forms a reservoir in cells of the monocyte/macrophage lineage that support low-level replication of the virus. All of these cell types can produce infectious virus when reactivated or during interruptions in ART, and disruptions of treatment causes a rapid rebound in viremia.

1.3 APC-Mediated HIV Trans Infection

HIV-1 infection can occur through two mechanisms: cis and trans. In cis infection, the virus enters through the CD4 molecule and a coreceptor CXCR4 or CCR5, which forms the entry receptor complex for the virus. The complex binds viral glycoproteins gp120 and gp41, causing fusion of the viral envelope and the cell membrane and release of the capsid into the cytoplasm (30). The virus then goes through its normal life cycle of, integration, assembly and budding to form infectious progeny virus. Trans infection most commonly occurs between an APC and CD4+ T cell. In trans infection, the virus uses an alternate receptor, DC-SIGN, and is endocytosed into a non-degrading compartment where it is held for up to 3 days (30). When the APC comes into contact with a CD4+ T cell, the virus causes upregulation of HIV receptors CD4, CXCR4 and
CCR5 on the T cell (31). This forms the infectious synapse, a structure similar to the immunological synapse but orchestrated by the virus. An intact virion is then passed from the APC to CD4+ T cell. This causes a burst of viral production from the CD4+ T cell that is much greater than cis or trans infection between T cells (30).

HIV Nonprogressors have been characterized by the inability of their professional antigen presenting cells (APC) to mediate HIV trans infection to autologous CD4+ T cells (Figure 2). The inability of the NP phenotype to trans infect CD4+ T cells has been linked to altered cholesterol metabolism in APC, increase in reverse cholesterol transporter ATP-binding cassette transporter 1 (ABCA1) activity and depletion of cholesterol in membrane lipid rafts (Figure 3) (32). ABCA1 is a cell membrane protein that transfers cholesterol and phospholipids outside of the cell to lipid poor proteins to be returned to the liver for catabolism (33). Reverse cholesterol transport occurs when free cholesterol inside the cell is transported outside through an ATP-binding cassette, sub-family A protein (ABC1, ABC1, ABCA1) and interacts with APOA1, part of the HDL molecule that transports cholesterol to the liver (34). This directly influences lipid raft content in the membrane by decreasing cholesterol availability in the cell (33). A lipid raft is an area of the host membrane that contains high cholesterol and sphingolipid content, and is involved in signal transduction, and endocytosis (2). They are required at multiple points in the viral life cycle and server as docking sites for entry receptors and anchor viral glycoproteins during assembly (2). HIV trans infection is a major contributor of viral persistence during antiretroviral therapy (ART) and allows the virus to pass through a virological synapse between cells, bypassing antiretroviral drug effects that target receptor binding, integration and membrane fusion (32).

It has previously been shown that ex vivo derived APC from progressors (PR) can still mediate HIV trans infection of CD4+ T cells while under virus suppressive ART, and that NP were still
unable to *trans* infect CD4+ T cells (Figure 4) (32). These data show that the virus can surpass antiviral drug effects and suggest an important role for APC-mediated *trans* infection in the maintenance of viral reservoirs and persistence during ART therapy. Understanding the ability of NP to control HIV disease progression could lead to the development of novel therapeutic strategies and vaccines. The following study aims to further characterize the APC cholesterol pathways involved in the inhibition of *trans* infection in NP through the identification of circulating biomarkers and genotypic characteristics that directly influence cholesterol metabolism and homeostasis.

![Figure 2. APC from NP do not Medicate HIV Trans Infection](image)

*Figure 2. APC from NP do not Medicate HIV Trans Infection*

Trans infection results of autologous CD4+ T cells. APC mediated trans infection: APC from seronegative donors were pulsed with HIVBal at sub-infectious dose, mixed with autologous CD4+T cells at 1:10 ratio and co-cultured for up to 16 days. Co-cultures supernatants were sampled at 4, 8, 12 and 16 days and HIVp24 was measured by ELISA (Rappociolo, G. et al. Mbio 2014, DeLucia D. et al J.Virol 2018).
Images of NP and PR macrophages labeled with CTx-B to detect lipid rafts and analyzed by Millipore Image Stream (DeLucia D. et al J.Virology 2018).

Figure 4 Trans Infection Results of Autologous CD4+ T cells in ART Treated Individuals

B cells and DC derived from participants under suppressive ART were loaded with HIVBal and co-cultured with autologous CD4+ T cells for 12 days. CD4+ T cells were infected with HIV in cis. Supernatants were collected at the indicated time points and tested for HIV Gag p24. Data are mean values ±SE; n=10 experiments (Rappocciolo G. et al. Open Forum Infectious Diseases, 2019).
1.4 Cholesterol Regulation and HIV Progression

Infection with HIV and treatment with ART therapy has been linked to differences in lipid concentrations, including higher serum levels of low density lipoprotein (LDL), triglycerides and total cholesterol and lower levels of high density lipoprotein (HDL) (35). Lower levels of HDL have been linked to greater risk of atherosclerosis. HDL removes atherogenic lipids from atherosclerotic plaques to be cleared by the liver and this process is impaired during chronic inflammation (35). Macrophages in the vascular wall move lipids into HDL or APOA1 for reverse cholesterol efflux through ATP-binding cassette transporters and initiates reverse cholesterol transport through ABCA-1. ABCA-1 in human macrophages are the major cholesterol transporter, interacting with lipid-poor APOA1 to move cholesterol out of the cell (36).

The capacity of HDL to remove cholesterol from macrophages is inversely correlated with coronary heart disease severity, and that increased HDL levels is a protective factor in atherosclerosis (37). It has also been shown that total cholesterol efflux was reduced by 12% in HIV infected individuals and the ability of ABCA-1-dependent cholesterol efflux is reduced by almost 30% (36). HIV infected individuals, in addition to having lower cholesterol efflux rates, are known to have reduced ability of whole plasma to remove cholesterol macrophages compared to the general population (36). This contributes to the lipid dysregulation problems they experience. Because of this need to be on lipid lowering medications even while on ART, as problems still persist while on therapy (38). It is currently not known if HIV NP have higher rates of cholesterol efflux than progressors or the general population.
1.4.1 Single Nucleotide Polymorphism Affecting Cholesterol Efflux

It has been established that genetic factors contribute to variability in plasma HDL levels and account for approximately 40%-60% of that variation (39). Several genetic variants of genes involved in cholesterol biogenesis and maturation have also been identified: ABCA1, APOA1, ABCG1, APOAII, CETP and LIPC. In a study by Villard et.al., the capacity of whole-plasma to mediate cholesterol efflux from macrophages was measured in 846 people (450 men and 496 women). It was determined that rs17231506 (CETP c.-1337 C>T), rs2230806 (ABCA1 p.R219K), rs1799837 (APOA1 c.-75 G>A), rs5086 (APOAII c.-265 T>C), and rs1800588 (LIPC c.-514 C>T) single nucleotide polymorphisms (SNPs) significantly modulate the capacity of plasma to mediate cholesterol efflux from human macrophages and this was sex-dependent. It was estimated that these SNPs represented approximately 6% of total plasma efflux and that genetic plasma efflux capacity was a better predictor of macrophage cholesterol removal than plasma HDL levels.

In women the APOA1 c.-75 G>A and the LIPC c.-514 C>T variants were determined to mediate cholesterol efflux, but in men the ABCA1 p.R219K and the APOAII c.-265 T>C SNPs were more prominent. Other data has shown that these genes are regulated by sex hormones, which may contribute to differences in transcription between genders (39). The rs1800588 (LIPC c.–514 C>T) SNP is located in the hepatic lipase gene promoter and is associated with variation in plasma HDL levels and size, with the T variant associated with higher HDL levels. The rs2230806 (ABCA1 p.R219K) SNP is located on the ABCA1 gene and is associated with higher HDL levels. The rs708272 (CETP TaqIB) SNP is located in the intron of the CETP gene and corresponds to higher HDL levels and particle size. rs5082 (APOAII c.–265 T>C) is a SNP in the APOAII gene that correlates to significantly higher efflux capacity. rs1799837 (APOA1 c.–75 G>A) is a SNP on the APOAI gene and correlates to higher plasma HDL.
1.5 Circulating Factors with Antiviral Properties

1.5.1 Eicosanoids

Eicosanoids are enzymatically generated oxidation products of arachidonic acid and related polyunsaturated fatty acids (PUFAs) produced by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) enzymes, and are known to regulate processes related to infection, inflammation and homeostatic functions (40). They have been found to be implicated in vascular permeability, platelet aggregation and have roles in autoimmunity, HIV, and cancer, as well as having pro-inflammatory and anti-inflammatory roles in immune regulation (41).

1.5.1.1 5-oxo-Eicosatetraenoic Acid

Arachidonic acid metabolite 5-oxo-eicosatetraenoic acid (5-oxo-ETE) is an formed by 5-hydroxyeicosanoid dehydrogenase (5-HEDH) oxidation of 5-HETE (5S-hydroxy-eicosatetraenoic acid) (42). This molecule serves as a chemoattractant for eosinophils, neutrophils, monocytes and basophils, and regulates a variety of cellular process including actin polymerization, integrin expression, degranulation, and transport of calcium (43). NADP+ is required to synthesize 5-oxo-ETE and can be stimulated by oxidative stress and the respiratory burst. It is regulated by the OXE receptor, a G-protein receptor, that is expressed at high levels in eosinophils, its primary target; implicating that it may have a role in asthma and allergy development (42). 5-oxo-ETE activates peroxisome proliferator-activated receptor gamma (PPARγ) and is a chemoattractant, acting in a paracrine and autocrine manner as a signaling metabolite. PPARγ activation increases transcription of the cholesterol transporter ABCA1, which in turn increases cholesterol transport out of the cell (44).
1.5.2 Oxysterols

Oxysterols are a family of cholesterol oxidation derivatives that contain a hydroxyl, epoxide or ketone group in the sterol nucleus or a hydroxyl group in the side chain, and are derived from enzymatic or non-enzymatic activity on cholesterol (45). Multiple side-chain oxysterols (24OH, 25OH, 27OH) have been known to activate nuclear receptors such as liver-X-receptors (LXRs), retinoid X receptors (RXRs) and oestrogen receptors that activate transcription factors with key regulatory functions (46, 47). Many of these oxysterols have been known to block infection of many viruses by altering the cellular membrane, inhibiting viral entry, fusion and budding (48).

1.5.2.1 25-Hydroxycholesterol

The metabolite 25-Hydroxycholesterol (25HC) has broad antiviral activity on enveloped and non-enveloped viruses. 25HC is derived from cholesterol by the action of Ch25h, an enzyme that hydroxylates cholesterol at the 25 position, and has been identified as an antiviral interferon-stimulated gene (ISG) (48, 49). Ch25h is expressed strongly in myeloid cells including macrophages and dendritic cells in response to toll-like receptor ligands (TLR) and IFN which causes a decrease of cellular cholesterol through regulation of sterol-responsive element binding proteins (SREBP) and nuclear receptors (47). It has been shown that 25HC inhibits viral entry and fusion of many viruses, including HIV, Ebola, HSV, Dengue, RVF and many more (48). It is known that oxysterols such as 25HC regulate cholesterol homeostasis and make alterations to cholesterol in the cellular membrane affecting viral fusion and entry (50). The effects of this molecule on lipid rafts required for trans infection remain unclear.
2.0 Statement of Project and Specific Aims

The aim of this study is to determine differences in cholesterol biogenesis and metabolism in NP vs PR and whether those differences translate to the inability of NP to trans infect CD4+ T cells. Cholesterol content of the cell is very important to the virus at various stages in its replication cycle and understanding these differences between NP and PR could translate to new therapeutic targets of the HIV reservoir, which is still active during ART therapy. We believe that there are differing cholesterol biomarkers between NP and PR, and these have not been well defined. We aimed to study cholesterol differences and reverse transport outside of the cell. We also aimed to translate these differences into genetic polymorphism between NP and PR that could also account for some of these differences.

2.1 Aim 1: Determination of Cholesterol Efflux Capacity of Plasma From Male and Female NP and PR and Identification of Circulating Biomarkers of HIV Disease Nonprogression that Serve as Natural Ligands that Control ABCA-1 Activity and Link to the Inability of APC to Trans Infect CD4+ T Cells.

Hypothesis: We hypothesized higher rates of cholesterol efflux from plasma of NP in both males and females and propose the identification of circulating biomarkers that serve as natural ligands that control ABCA-1 activity and link to the inability of APC to trans infect autologous CD4+ T cells. It has been shown that APC from NP are incapable of trans infecting CD4+ T cells, and this has been associated with altered cholesterol metabolism, disruption of lipid rafts and lower
cholesterol content in the cell membrane. It is also known that plasma cholesterol efflux capacity from THP-1 macrophages is lower in HIV-infected individuals (36). I predict that cholesterol efflux is higher in NP than PR.

2.2 Aim 2: Correlation of Genetic Polymorphism with NP Phenotype in Male and Female Individuals.

**Hypothesis:** We hypothesize that host genetic factors contribute to cell cholesterol homeostasis and metabolism and that these may differ between male and female NP. In a study by Villard et al. a series of SNPs were examined that have been implicated in cholesterol efflux capacity in male donors: rs17231506 (CETP c.–1337 C>T), rs2230806 (ABCA1 p.R219K), rs1799837 (APOA1 c.–75 G>A), rs5086 (APOAII c.–265 T>C), and rs1800588 (LIPC c.–514 C>T) (3). We have found that the rs5082, located in the APOAII gene, is significantly associated with the NP phenotype (p=0.0003) in male donors. In this study we will extend these findings to an additional 50 male samples from the MACS cohort that have recently been added and to 75 female samples recently received from the WIHS cohort.
3.0 Materials and Methods

3.1 Biosafety

All experiments using live HIV-1 virus were performed in a biosafety level 2 plus (BSL2+) laboratory in the Graduate School of Public Health at the University of Pittsburgh. Protective gowns and double-layer latex gloves were worn when working with live virus under a biosafety level 2 plus hood. All laboratory personnel were trained in sterile technique as well as working with infectious virus prior to being able to work independently with the HIV-1 virus.

3.2 Cohorts and Samples

Samples were received from the Multicenter AIDS Cohort (MACS) participants (men who have sex with men) who are HIV-infected nonprogressors (NP), progressors (PR) and seronegative (SN). Samples were also received from the Women’s Interagency HIV Study (WIHS). In both of these cohorts, infected samples were matched to controls by age and race in sets of 3: 1 PR, 1 NP and 1 SN. Samples were all ART naïve and not on lipid lowering medications. The Multicenter AIDS Cohort Study (MACS), is an ongoing prospective cohort study of the natural and treated history of HIV/AIDS in men who report sex with men (51, 52) Participants were recruited from four U.S. metropolitan areas (Baltimore/Washington, Chicago, Los Angeles, and Pittsburgh), and study visits have been held at 6-month intervals beginning in 1983 (www.statepi.jhsph.edu/macs/macs.html). A total of 7,350 men have been enrolled in the MACS.
The WIHS cohort sites were established in 1993 in Brooklyn, NY; the Bronx/Manhattan, NY; Washington, DC; Chicago, IL; San Francisco, CA; and Los Angeles, CA. Four southern sites were added in 2013: Chapel Hill, NC; Atlanta, GA; Birmingham, AL/Jackson, MS; and Miami, FL. The cohort enrolled 4982 women (53).

At each semi-annual study visit, participants underwent standardized interviews, physical examinations and a blood draw. The institutional review boards of each center approved the study protocol and all participants gave informed consent.

3.3 In vitro Assays

3.3.1 Cell Culture and Activation

3.3.1.1 B Lymphocytes

CD19+ B Lymphocytes were separated from PBMC from SN donors using MACS microbead magnetic separation (Miltenyi Biotech) and activated for 2 days with IL-4 (R&D Systems) and CD-40 ligand (Enzo Life Sciences) in R10 medium at 1 million cells/mL prior to trans infection assay. R10 medium consisted of RPMI, 10% FBS and 25mM HEPES.

3.3.1.2 CD4+ T lymphocytes

CD4+ T cells were separated from PBMC from a SN donor using MACs magnetic bead separation (Miltenyi Biotech) and activated for 2 days with phytohemagglutinin P (PHA) (Sigma) and IL-2 (Roche) at 2 million cells/mL in RPMI/20% FBS/25mM HEPES prior to trans infection assay.
3.3.2 APC Mediated Trans Infection Assay

R-tropic HIV$_{Bal}$ purified from PM1 cells (obtained through the Aids Reagent Program, Division of AIDS, NIAID, NIH Dr. Marvin Reitz) was used for cis and trans infection experiments. Virus stock titration and experimental HIV-1- Gag p24 measurements were acquired by ELISA using the HIV-1 p24 Antigen Capture Immunoassay kit (SAIC-Frederick) per the manufacturer’s instructions (54). Activated B lymphocytes (1x10$^6$) were incubated with HIV-1 at a sub infectious dose (m.o.i. of 10$^{-3}$) at 37°C for 2 h. HIV-1-infected B cells were washed three times with R10 medium and incubated with activated, autologous CD4$^+$ T cells at a 1:10 ratio. As a control, CD4$^+$ were cis infected with HIV-1 at an m.o.i. of 10$^{-1}$. Supernatants from the trans and cis cultures were sampled every 4 days and tested for HIV-1 Gag p24 by ELISA.

3.3.3 Bioactive Fatty Acid Screen

Bioactive fatty acid analysis was conducted on plasma from HIV infected nonprogressors (NP), progressors (PR) and seronegatives (SN) in collaboration with the University of Pittsburgh lipidomic Core by LC-MS/MS. Plasma samples were mixed with 2 parts chloroform and 1-part methanol and centrifuged at 2800xg for 10 minutes. The organic phase was transferred to a new vial and the internal standards 5-oxoETE-d7 (2 µg/mL) and 15-HETE-d6 (1 µg/mL) were added before drying under N$_2$. Samples were reconstituted in 100 µL MeOH and 10 µL was injected onto a Luna C18(2) reversed phase column (Phenomenex, Torrence, CA) for separation. Bioactive fatty acids were separated and analyzed using a Sciex Exion UHPLC coupled to a 6500+ QTrap mass spectrometer in negative ion mode. Bioactive fatty acids were reported as a ratio of peak area of analyte/peak area of the internal standard.
3.3.4 Cholesterol Efflux Assay

Plasma was assessed for cholesterol efflux capacity using a commercially available kit: Cholesterol Efflux Fluorometric Assay Kit (BioVision). Macrophage cell line THP-1 cells were loaded with labelled cholesterol and exposed to plasma of NP or PR for four hours. Efflux was measured by fluorescence intensity according to manufacturer’s instructions.

3.3.5 25-Hydroxycholesterol ELISA

PR and NP were measured for serum concentration of metabolite 25HC using a manufactured 25-HC ELISA kit (MyBioSource). The assay uses the competitive enzyme immunoassay containing a polyclonal anti-20S-PSM antibody and an 20S-PSM-HRP conjugate. The assay sample and buffer are incubated together with conjugate in pre-coated plate for one hour. After the incubation period, the wells are washed five times and incubated with a substrate for HRP enzyme. Intensity was measured at 450nm.

3.3.6 DNA Extraction Assay

DNA of NP, PR and SN was extracted from PBMC pellets using the Qiagen DNA Easy Core Blood Kit A per manufacturer’s instructions.
3.3.7 TaqMan SNP Detection Assay

TaqMan assays were performed for SNPs identified by Villard et.al. Probes were designed to amplify 100-150 base pair regions containing the SNP and the wild type for SNPs identified by Villard et.al. with a different fluorochrome attached to each probe. Fluorescence was analyzed using PCR genotyping assays to determine hetero and homozygous samples in NP and PR cohorts.

3.3.8 Millipore Image Cytometry

B cells were activated for two days in charcoal-stripped FBS with IL-4 and CD-40L, treated for 4 and 24 hours with or without 25HC and subsequently stained using the Vybrant Alexa Fluor 488 lipid raft labeling kit (Molecular Probes) and analyzed by Millipore Image Cytometry for membrane intensity.
4.0 Results

4.1 Aim 1: Determination of Cholesterol Efflux Capacity of Plasma from Male and Female NP and PR, and Identification of Circulating Biomarkers of HIV Disease Nonprogression that Serve as Natural Ligands that Control ABCA-1 activity and Link to the Inability of APC to \textit{trans} infect CD4+ T cells.

4.1.1 Determination of Cholesterol Efflux Capacity of Plasma from Male and Female NP and PR

It has been shown that APC from NP are incapable of \textit{trans} infecting CD4+ T cells, and this has been associated with altered cholesterol metabolism and disruption of lipid rafts and lower cholesterol content in the cell membrane (55). It has also known that plasma cholesterol efflux capacity from THP-1 macrophages is lower in HIV-infected individuals (36). We hypothesized that cholesterol efflux could be higher in NP than PR. Plasma was tested from 60 male NP and matched PR and SN controls to determine the rate of cholesterol efflux from macrophage cell line THP-1. Our data show that plasma from NP had higher cholesterol efflux than from PR (unpublished). In this study plasma from female NPs will show a similar pattern. As shown in Figure 5, plasma from NP participants induce higher cholesterol efflux compared to PR and SN donors. Our preliminary data shows cholesterol efflux in NP and PR by gender in our MACS and WIHS cohorts follows a similar trend, with NP having a significant increase in cholesterol efflux compared to PR in both cohorts (p = 0.0001).
Figure 5. Plasma from NP Induces Higher Cholesterol than PR

Macrophage cell line THP-1 were loaded with labelled cholesterol and exposed to plasma of NP or PR for four hours, using a commercially available kit (Biovision) per manufacturer’s instructions. Efflux was measured by fluorescence intensity at 450 nm. MACS N= 25 NP, PR, SN; WIHS NP = 20, PR = 22, SN = 24. Significance was calculated using a two-sided T test. Bars represent mean±SE.

4.1.2 Bioactive Fatty Acid Screen

To determine if circulating factors were responsible for higher efflux activity, a bioactive fatty acid screen was performed with the Health Sciences and Metabolomics Lipidomic core at the University of Pittsburgh on a subset of NP and PR samples. Analysis by liquid-chromatography-mass spectrometry identified metabolites present at a higher level in a number of NP than PR, specifically 5-oxo-eicosatetraenoic acid (5-oxo-ETE) (Figure 6). 5-oxo-ETE is derived from lipoxygenase activity on arachidonic acid from membrane sphingolipids produced by DC and B lymphocytes, macrophages and eosinophils (43). The first three cell types mediate CD4+ trans infection. 5-oxo-ETE is a chemoattractant and acts in a paracrine and autocrine manner as a signaling metabolite, binding and activating nuclear receptor PPARγ and inducing cholesterol efflux by increasing transcription of the cholesterol transporter ABCA1. Although the overall
increase in 5-oxo-ETE levels were significantly increased, not all of the NP samples show a difference compared to PR. This increase seems to be a trait that only a percentage of NP will have.

![Figure 6. Fatty Acid Analysis of Plasma from NP Contains Higher Levels of 5-Oxo-ETD than PR](image)

Bioactive Fatty Acid screen analysis was conducted on plasma from HIV infected NP and progressors (PR) in collaboration with the lipidomic Core by LC-MS. Plasma from NP was found to have increased levels of cholesterol metabolite 5-oxo-ETE compared to PR. PR N = 14; NP N = 23. Significance was calculated using a two-sided T test. Bars represent mean±SE.
4.1.3 APC Mediated Trans Infection in Presence of 5-oxo-Eicosatetraenoic acid (5-oxo-ETE)

To determine if treatment with 5-Oxo-ETE would affect trans infection, a trans infection assay was performed. B and T cells were treated with 5µM 5-Oxo-ETE and supernatants were taken at days 4, 8 and 12. HIV-1 p24 capsid protein concentration in cell cultures supernatants was measured by ELISA. Data show that 5-oxo-ETE treatment of APC reduces the efficiency of HIV trans infection of CD4+T cells, but was not statistically significant (Figure 7). We hypothesize that this molecule is limiting levels of trans infection of APC to CD4+ T cells by altering rate of cholesterol efflux from APCs.

![Trans Infection with 5-OXO-ETE](image)

**Figure 7. APC Mediated Trans Infection in Presence of 5-oxo-ETE**

APC from seronegative donors were pulsed with HIVBal at sub-infectious dose, mixed with autologous CD4+T cells at 1:10 ratio and co-cultured for 12 days with or without 5-oxo-ETE at 5µM. Co-culture supernatants were sampled at 4, 8, 12 days and HIVp24 was measured by ELISA. N=3 Bars represent mean±SE.
4.1.4 Detection of 25-Hydroxycholesterol in Serum of NP vs PR

Another signaling metabolite known to modulate immune cells activity is 25-Hydroxycholesterol (25HC) (56), which has been shown to have broad antiviral activities against other viruses. It plays multiple roles in lipid biosynthesis and immunity and is able to modulate cholesterol metabolism by promoting ABCA1 expression, resulting in reduced cellular-associate cholesterol. It also interferes with virus-cell membrane fusion and reduces inflammatory factors and cytokines (56). We have tested the effects of 25HC in plasma of NP and PR using an Elisa kit purchased from Biosource (Figure 8) Preliminary data show that NP have higher levels of 25HC than PR and SN, but given the limited number of participant tested further testing of samples is needed to determine its significance.

![25HC in Serum of NP and PR](image)

Figure 8. Preliminary Data pf 25HC Elisa Assay

Plasma of NP, PR and SN were tested using an anti-25HC Elisa kit purchased from Biosource. N=24.
### 4.1.5 APC-Mediated Trans Infection in the Presence of 25-Hydroxycholesterol

In order to determine the effect of 25HC on HIV trans infection, we performed a trans infection assay (Figure 9) in the presence of this bioactive molecule. Treatment of B cells with 25HC at 0.3µg/mL in culture strongly inhibited HIV trans infection compared to untreated cells. *Trans* infection efficiency was also decreased when cells were pre-treated with 25HC 12 hours prior to infection at 0.3µg/mL. In the pre-treatment, 25HC was removed from culture prior to infection with HIV-1.

![Graph showing treatment with 25HC inhibits B-cell mediated trans infection](image)

**Figure 9. Treatment with 25HC Inhibits B-cell Mediated Trans Infection**

APC from seronegative donors were pulsed with HIVBal at sub-infectious dose, mixed with autologous CD4+T cells at 1:10 ratio and co-cultured for up to 12 days with or without 25HC at .3µg/m. Co-culture supernatants were sampled at 4, 8 and 12 days and HIVp24 was measured by ELISA. Significance was calculated using a two-way Anova test **, p < .008, ***, p < .0005. n = 3. Bars represent mean±SE.
4.1.6 Dose Response to Pre-Treatment of 25HC in B Cell Mediated *trans* Infection.

In order to determine the effect of different concentrations of 25HC on B cell mediated *trans* infection, we performed a dose response experiment. B cells were treated with various concentrations of 25HC 12 hours prior to infection and then removed from the media prior to infection. Our data show that pre-treatment with 25HC does not significantly affect *trans* infection (Figure 10).

![Graph showing dose response to pre-treatment with 25HC](image)

**Figure 10. Dose Response to Pre-Treatment with 25HC**

APC from seronegative donors were treated for 12 hours before infection with 25HC at varying concentrations, pulsed with HIVBal at sub-infectious dose, mixed with autologous CD4+T cells at 1:10 ratio and co-cultured for up to 12 days. Co-culture supernatants were sampled at 4, 8 and 12 days and HIVp24 was measured by ELISA.
4.1.7 25HC Reduces Membrane Associated Lipid Rafts

The signaling metabolite 25HC has been shown to inhibit viral entry and membrane fusion by altering cholesterol in cellular membrane. To determine the effect of this molecule on cellular membrane lipid rafts, B cells were treated for 4 and 24 hours at 0.3 ug/mL and were exposed to CTx-B for lipid raft labeling and analyzed by flow cytometry using Millipore Image Stream. TO901317 was used as a control because it is a Liver X Receptor agonist that plays a key role in reverse cholesterol transport by increasing cholesterol efflux and reverse cholesterol transport through HDL. LXR activation does not interfere with CD40L and IL-4 mediated B cell activation and proliferation (55). Our data show that treatment with 25HC reduces membrane intensity, lowering lipid raft content in B cells (Figure 11). Images are representative of treated and untreated populations.
Figure 11. Treatment with 25HC Reduces Membrane Associated Lipid Rafts

B cells were treated with 25HC for 4 and 24 hours in charcoal-stripped R10, stained for Ctx-B lipid rafts and flow cytometry was performed using Millipore Image Stream. N = 3. Bars represent mean±SE. Representative images of B cells labeled with CTx-B to detect lipid rafts and analyzed by Millipore Image Stream. Significance was calculated using a two-sided T test. Bars represent mean±SE.
4.2 AIM 2: Identification of genetic factors contributing to the NP genotype and phenotype.

In a study by Villard et al. a series of SNPs were examined that have been implicated in cholesterol efflux capacity in male donors: rs17231506 (CETP c.–1337 C>T), rs2230806 (ABCA1 p.R219K), rs1799837 (APOA1 c.–75 G>A), rs5086 (APOAII c.–265 T>C), and rs1800588 (LIPC c.–514 C>T) (39). Previously we have found that the SNP rs5082, located in the APOAII gene, is significantly associated with the NP phenotype (p=0.0003) in male donors from the MACS. In this study we extended this analysis to 50 additional male samples from the MACS cohort and 75 female samples received from the WIHS cohort. DNA was extracted from PBMC cell pellets using the Qiagen DNA Easy Core Blood Kit A. SNP genotyping assays were then performed to identify the presence of SNPs identified by Villard et al. as modulators of cholesterol efflux using PCR.

We found SNP rs1800588 to be significantly associated with the NP cohort (p = 0.0003), which had not previously been significant when tested in a smaller cohort. This SNP is a mutation in the Hepatic Lipase (LIPC) gene promoter. LIPC plays a key role in reverse cholesterol transport, metabolism of several lipoproteins and is associated with higher serum high density lipoprotein (HDL) levels. It was shown to be a major determinant of cholesterol efflux in women by Villard et.al, but we found it to be very significant in the male NP cohort samples. The rs5082 SNP was still significantly associated with the NP phenotype, although at a higher p-value (p = 0.02). As stated above, this is a mutation in the promoter of the APOAII gene which decreases its transcription and was identified to be associated with significantly higher cholesterol efflux in men by Villard et.al (39). The remaining SNPs were not found to be significant in our cohort. SNPs and corresponding P-values are listed in Figure 12.
Figure 12. Single Nucleotide Polymorphism in NP Cohort

DNA was extracted from PBMC cell pellets using Qiagen Puregene Blood Core Kit B according to manufacturer instructions. PCR Genotyping Assays were then performed to determine heterozygous and homozygous allele frequencing. P-values were calculated using an odds ratio. N=50.

<table>
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<tr>
<th>SNP</th>
<th>P-VALUE</th>
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<td>RS1800588</td>
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<tr>
<td>RS5082</td>
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</table>
5.0 Discussion

Currently there are 1.1 million people living with HIV in the U.S., and there are approximately 38,500 new infections each year (8). Deaths from the virus have been greatly reduced with the invention of ART therapy, but this has been associated with metabolic side effects, including dyslipidemia, hypertriglyceridemia, hypercholesterolemia and diabetes (57). For this reason levels of HIV patients need to be closely monitored and many people have to take lipid lowering medications to protect against coronary heart disease and other conditions. In this study, we aimed to identify circulating biomarkers of HIV nonprogression that modulate cholesterol metabolism for alternative therapeutics and vaccines.

HIV nonprogressors comprise a small percentage of individuals who are able to control HIV infection for many years without therapeutic intervention. They have been characterized based on their HLA class I alleles, CCR5-Delta32 mutation, viral attenuation and many other factors, but these do not explain all of the known NPs. We have found that the APC of NP are unable to trans infect CD4+ T cells and this has been linked to differences in cholesterol metabolism between NP and PR (32). In our current cohort, the inability of APC of NP to trans infect CD4+ T cells is present in every NP we have studied. Previously it has been shown that HIV infected individuals have lower cholesterol efflux than the normal population and have lower levels of HDL and increased risk of atherosclerosis (35, 36). In our study, we found that plasma of NP had higher cholesterol efflux from macrophages than PR in both the MACS and WIHS cohorts (Figure 5) (p = 0.0001). This implies that plasma of male and female NP have higher levels of reverse cholesterol transport out of the cell.
We performed a bioactive fatty acid screen was on plasma of NP and PR to identify potential metabolites involved in cholesterol metabolism in NP compared to PR by LC-MS/MS (Figure 6). A molecule 5-oxo-eicosatetraenoic acid (5-oxo-ETE) was found to be significantly increased in NP and PR. 5-oxo-ETE is derived from lipoxygenase activity on arachidonic acid from membrane sphingolipids produced by DC and B lymphocytes, macrophages and eosinophils. The first three cell types mediate CD4+ trans infection. This molecule serves as a chemoattractant for eosinophils, neutrophils, monocytes and basophils, and regulates a variety of cellular processes including actin polymerization, integrin expression, degranulation, and transport of calcium (43). 5-oxo-ETE is a chemoattractant and acts in a paracrine and autocrine manner as a signaling metabolite, binding and activating nuclear receptor PPAR and inducing cholesterol efflux by increasing transcription of the cholesterol transporter ABCA1.

When B and T cells were treated with 5µM 5-oxo-ETE and a trans infection assay was performed, efficiency was reduced but this was not statistically significant (Figure 7). We plan to perform this assay at higher concentrations with the drug to determine toxicity levels. If non-toxic to at higher concentrations, more studies should be performed to understand it’s potential as a therapeutic target in the control of HIV. In the lipidomic screen we did notice that only some NP had higher concentrations of this metabolite (Figure 6). This could be related to nonprogression in only some individuals in our cohort and since it is not ubiquitously found at higher levels in all NP.

25-Hydroxycholesterol is a potent molecule that had been featured recently in scientific literature as an antiviral, and we wanted to investigate the effect of it on APC-mediated trans infection. It is known to play multiple roles in lipid biosynthesis and immunity, and modulates cholesterol metabolism by altering cholesterol content in the cellular membrane, interfering with
virus-cell membrane fusion and reduces inflammatory factors and cytokines (48). We tested the content of 25HC in NP and PR using an Elisa kit purchased from Biosource. More samples need to be tested to determine significance, but our preliminary data show that NP may have higher levels of 25HC which may alter cholesterol metabolism and protect against viral infection by interfering with membrane fusion during formation of the infectious.

To determine the effect of 25HC on trans infection, B cells were treated with .3 ug/mL and in culture for up to 12 days or pre-treated for 12 hours and removed from the culture media prior to infection. When 25HC was left in culture, it strongly inhibited trans infection (Figure 9). When 25HC was pre-treated and then removed from culture, it reduced efficiency, but this was not significant. We hypothesize that 25HC is continuously altering cholesterol in the cellular membrane and interfering with membrane fusion and the ability of APC to trans infect CD4+ T cells. We speculate that it is likely that when left in culture, 25HC is modulating cholesterol in the cellular membrane and severely limiting the ability to form the infectious synapse in trans infection. Because this molecule was so effective, we believe it may be useful as a potential addition to ART therapy.

We were curious to see if altering the concentration of the pre-treatment in the trans infection assay would create a dose response to the drug, so three concentrations were tested. Cells were treated with varying concentrations of the drug for 12 hours prior to infection. The drug was then washed from the media and a trans infection assay was performed. The results of the experiment were not significant. A benefit of this may have been to use this effectively in a PREP treatment for HIV. We speculate that 25HC is most effective when left in culture, because it is continuously making changes to the cellular membrane. The membrane is very fluid, and removing 25HC may inhibit it from making alterations to new cholesterol being added.
In other viruses, 25HC alters lipid raft formation in the cellular membrane. To confirm this in HIV, image cytometry was performed. B cells were treated with 25HC for 4 and 24 hours (Figure 10) and analyzed via Millipore Image Stream. Cells that were treated with 25HC for 24 hours had lower, more diffuse florescence than cells that were untreated or treated with the TO901317 control. Our data show that 25HC is significantly limiting lipid raft formation, and this could interfere with the ability of the virus to orchestrate trans infection and maintain the HIV reservoir under ART therapy.

In a study by Villard et al. a series of SNPs were examined that have been implicated in cholesterol efflux capacity in male and female donors. It has been established that genetic factors contribute to variability in plasma HDL levels and account for approximately 40%-60% of that variation (39). Several genetic variants of genes involved in cholesterol biogenesis and maturation have also been identified: ABCA1, APOA1, ABCG1, APOAII, CETP and LIPC. Previously in our cohort we found one SNP to be significantly associated with being an NP. 50 new male samples from the MACS study were added, and we found two SNPs to be significantly associated with the NP cohort, rs1800588 (p = 0.0003) and rs5082 (p = 0.02) (Figure 11). Our lab recently received 75 new samples from the WIHS cohort, and plan to carry out the same analysis with these using Taq-Man Genotyping assays.

The rs1800588 SNP is a mutation in the Hepatic Lipase (LIPC) gene promoter that plays a key role in reverse cholesterol transport, metabolism of several lipoproteins and is associated with higher serum high density lipoprotein (HDL) levels. It was shown to be a major determinant of cholesterol efflux in women by Villard et.al, but it was found to be very significant in our male NP cohort samples. Because this SNP is decreasing transcription of the LIPC gene, it is also decreasing the ability of hepatic lipase to bind HL, part of the HDL molecule, and this inhibits the
function of HDL. Higher serum HDL levels is a protective factor of atherosclerosis and is also likely to be protective many viruses by decreasing cholesterol availability in the cellular membrane. This SNP could be beneficial in HIV infection because of the increased transfer of cellular cholesterol to HDL for processing by the liver instead of remaining inside of the cell.

The rs5082 is a mutation in the promoter of the APOAII gene which decreases its transcription and was identified to be associated with significantly higher cholesterol efflux in men by Villard et.al (39). APOAII is associated with higher serum LDL levels, a contributing factor to atherosclerosis, and higher cellular associated cholesterol. This SNP is decreasing cellular associated cholesterol and ability of the virus to spread from cell to cell by limiting lipid raft formation. Both of the SNPs we found likely contribute a small amount to the overall capacity of NP and PR ability to trans infect.

In this study we dove deeper into the differences in cholesterol metabolism between NP and PR, and found differing biomarkers and genetic polymorphism between the two groups. Even though we found some characteristics of the NP cohort that can help explain their ability to control HIV infection, more studies are needed. Possible next steps in the project would be to expand the analysis of cholesterol and bioactive metabolites in NP vs PR through the lipidomic core. The test we performed was not designed to detect oxysterols, and this was a type of metabolite we were interested in exploring deeper. We also wanted to try different APC, such as dendritic cells and macrophages, for lipid rafts content and to measure total cellular cholesterol as well in the presence of bioactive molecules identified through lipidomic analysis. Hopefully we will be able to understand the complete picture of what differentiates NP and PR, and this will lead us to new therapeutics that target the ability of HIV to spread, and to maintain the latent reservoir.
6.0 Supplementary Data

6.1 Cellular Toxicity to 25-Hydroxycholesterol

To determine cellular toxicity levels to 25HC, T and B cells were cultured separately with or without 25HC at .3µg/mL and viability was measured on day 4 using a hemocytometer. These data show that there is not a difference in viability of cells treated at this concentration (Figure 13).

Figure 13. Cellular Toxicity of 25 Hydroxycholesterol

B and T cells were cultured with 25HC at 0.3µg/mL and counted using a hemocytometer to measure cellular viability on day 4. Significance was calculated using a Chi-Square test. n = 3. Bars represent mean±SE.
5. Giovanna Rappocciolo PP, Craig L Fuller, Todd A Reinhart, Simon C Watkins, David T Rowe, Mariel Jais, Phalguni Gupta, Charles R Rinaldo Jr. 2006. DC-SIGN on B Lymphocytes Is Required For Transmission of HIV-1 to T Lymphocytes. PLOS Pathogens doi:https://doi.org/10.1371/journal.ppat.0020070.


