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Association of a 3' Untranslated Region Polymorphism in PCSK9 with HIV Viral Load and CD4+ Levels in HIV/Hepatitis C Virus Co-Infected Women

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Abstract

Objective—To assess variation in genes that regulate cholesterol metabolism in relation to the natural history of HIV infection.

Design—Cross-sectional and longitudinal analysis of the Women's Interagency HIV Study (WIHS).

Methods—We examined 2,050 single nucleotide polymorphisms (SNPs) in 19 genes known to regulate cholesterol metabolism in relation to HIV viral load and CD4⁺ T cell levels in a multiracial cohort of 1,066 antiretroviral therapy (ART) naïve women.

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CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

MHK, MIB and VRP designed and directed the study. MHK, HL and EJP conducted the statistical and bioinformatics analyses. TP and MIB conducted PCSK9 serology. KA, DG, SK, MN, BES, SJG and BEA collected WIHS data. All authors provided critical input on the manuscript and approved the final version.

Results—Six SNPs were associated with both HIV viral load and CD4⁺ T cell levels at a false discovery rate (FDR) of 0.01. Bioinformatics tools did not predict functional activity for five SNPs, located in introns of *NCOR2*, *RXRA* and *TTC39B*. Rs17111557 located in the 3' untranslated region (UTR) of *PCSK9* putatively affects binding of hsa-miR-548t-5p and hsa-miR-4796-3p, which could regulate *PCSK9* expression levels. Interrogation of rs17111557 revealed stronger associations in the subset of women with HIV/hepatitis C virus (HCV) co-infection (n=408, 38% of women). Rs17111557 was also associated with low-density lipoprotein cholesterol (LDL-C) levels in HIV/HCV co-infected (β : -10.4; 95% CI: -17.9, -2.9; $P=0.007$), but not in HIV monoinfected (β : 1.2; 95% CI: -6.3, 8.6; $P=0.76$) women in adjusted analysis.

Conclusions—*PCSK9* polymorphism may affect HIV pathogenesis, particularly in HIV/HCV co-infected women. A likely mechanism for this effect is *PCSK9*-mediated regulation of cholesterol metabolism. Replication in independent cohorts is needed to clarify the generalizability of the observed associations.

Keywords

cholesterol; HIV; African American; hepatitis C virus; *PCSK9*

Cholesterol is used by animal cells to maintain the liquid–solid structure of plasma membranes necessary for cell signaling pathways and other essential functions. Cholesterol is also used by a variety of human pathogens, *Mycobacterium tuberculosis*, HIV, influenza and hepatitis C virus (HCV), to support various aspects of their life cycles: membrane fusion and entry into cells, viral budding, immune evasion and transport through the bloodstream^[1]. Cholesterol use by HIV has been well studied. The HIV Nef protein stimulates cholesterol biosynthesis by HIV-infected cells and also inhibits activity of ATP binding cassette transporter A1 (ABCA1) – a cellular cholesterol transporter – to reduce cholesterol efflux from cells^[2–4]. Excess cellular cholesterol is directed to cholesterol-rich domains of the plasma membrane (lipid rafts) to support assembly and budding of HIV virions^[3–5].

Another role for cholesterol in the HIV lifecycle is to mediate *trans* infection of CD4⁺ T cells by antigen presenting cells (APCs), a process that plays a role in sexually transmitted HIV^[6]. Cholesterol is required for uptake of HIV by dendritic cells (DCs) and subsequent transfer to CD4⁺ T cells^[7], and a recent study demonstrated significantly lower levels of cholesterol in DCs and B cells of HIV-infected (HIV+) non-progressors when compared to HIV+ progressors^[8].

Many groups - including our own^[9] - have sought to identify host genetic factors associated with HIV pathogenesis, as defined primarily by HIV viral load levels, CD4⁺ T cell levels, or time from HIV seroconversion to AIDS/death. Only two genetic regions have consistent associations with HIV pathogenesis in genome-wide association studies (GWAS) – the human leukocyte antigen (HLA) class I region and the chemokine (C-C motif) receptor 5 (CCR5) region^[10]. GWAS can be underpowered to identify associations with moderate effect sizes. GWAS of HIV are additionally limited by small sample sizes (compared to general population samples) and by heavy reliance on European ancestry cohorts^[10], although there are exceptions^[11]. Thus, there remains a place in HIV research for candidate

gene studies where a strong rationale exists for interrogation of certain genes, as is the case for genes that regulate cellular cholesterol levels.

Herein we present a study of 19 candidate genes with roles in cholesterol regulation in relation to two biomarkers of HIV pathogenesis (HIV viral load and CD4⁺ T cell levels) in a multiracial cohort of antiretroviral therapy (ART) naïve women. Our analyses utilized methods that account for correlations between genetic variants involved in a common pathway. Significant associations were interrogated using bioinformatics tools and followed up by statistical and experimental studies.

METHODS

Study Population

Characteristics of the Women's Interagency HIV Study (WIHS) population have been described previously^[12]. Briefly, HIV-positive (HIV⁺) and HIV-negative (HIV⁻) women were recruited from similar risk settings at six United States (US) sites (Bronx, Brooklyn, Washington D.C., Chicago, San Francisco and Los Angeles) during 1994–1995, 2001–2002, and 2011–2012. Study visits (every six months) include a physical examination, collection of peripheral blood and assessment of self-reported ART use (including querying participants about each antiretroviral agent) and other medications. This nested substudy was approved by the institutional review board (IRB) of the Albert Einstein College of Medicine.

Single Nucleotide Polymorphism (SNP) Typing

SNPs were genotyped using the Illumina HumanOmni2.5-quad beadchip (Illumina, San Diego) for all WIHS women enrolled in 1994–1995 and 2001–2002 who provided consent for genetic testing (n=3,353). Excluded from the dataset were SNPs with a genotype call rate of <95% and SNPs that failed our in-house quality control criteria. Specifically, SNPs with at least 2 discordant genotypes among greater than 20 duplicate samples were excluded.

SNPs within and flanking (i.e., approximately 20 kb surrounding) 19 candidate cholesterol genes (*ABCA1*, *ABCG1*, *ABCA12*, *SCARB1*, *LDLR*, *SLC10A2*, *TTC39B*, *CYP39A1*, *CETP*, *PCSK9*, *MVK*, *RXRA*, *NR1H3*, *NCOR1*, *NCOR2*, *SREBF1*, *NR1H3*, *NR1H2* and *RXRβ*) were used. Ancestry informative marker (AIM) SNPs from the Illumina beadchip were selected from across the genome using Helix Tree (Golden Helix, Bozeman, MT) to calculate principal components (PCs) of genomic race/ethnicity.

Clinical Laboratory Measurements

Plasma HIV RNA levels were originally measured through WIHS visit 6 with a nucleic acid sequence-based amplification method that had 4,000 copies/ml as its lower limit of quantification (LLQ - Organon Teknika Corp., Durham, NC). Similar methods with greater sensitivity were used thereafter as they became clinically available (the LLQ was 400 copies/ml during visits 7 to 9 and 80 copies/ml thereafter). There has been an effort to re-measure HIV RNA levels with more sensitive assays at WIHS visits where assay LLQ was 400 or 4,000 copies/ml and the HIV viral load was at the LLQ. This effort succeeded for most visits but re-measurement of HIV RNA levels was not possible at visits with

insufficient plasma in the WIHS repository. Total CD4+ T cell counts (cells/ μ L) were determined by flow cytometry in laboratories participating in the DAIDS Quality Assurance Program at each study visit^[13].

HCV serostatus was determined at enrollment (defined as WIHS visits 1–3 for hepatitis testing) using a commercial second- or third-generation enzyme immunoassay, and HCV viremia was determined in HCV-seropositive women using either the COBAS Amplicor Monitor 2.0, which has a linear range of 600– 5.0×10^5 IU/ml, as previously described^[14], or the COBAS Taqman assay, which has a linear range of 10– 2.0×10^8 IU/ml (both from Roche Diagnostics, Branchburg, NJ).

The WIHS initiated collection of fasting blood and cholesterol measurements in October 2000. Total cholesterol, high-density lipoprotein cholesterol (HDL-C) and triglycerides were measured on a Roche Modular automated system (Roche Diagnostics Corporation, Indianapolis, IN). Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald equation. LDL-C was measured directly for specimens collected in 2004–2005 and for all women in 2000–2003 with triglycerides >400 mg/dL. Calculated and directly measured LDL-C were comparable (Supplementary Figure 1) – analyses are based on calculated LDL-C data because they are much more complete.

Genetic Epidemiology Methods

The study was restricted to HIV⁺ women who had 1 ART naïve study visit prior to October 1st, 1996 - when prevalence of highly active antiretroviral therapy (HAART) use first exceeded 5% of WIHS subjects. The reason for not including ART naïve visits after this time was because of selective ART use among the sickest individuals^[15], which would have biased our investigation of polymorphisms with untreated HIV viral load. We used this same inclusion criterion in a prior study in which we observed well recognized associations of HLA-B*57:01, B*57:03 and other alleles with HIV viral load and CD4+ T cell levels^[9].

We examined SNPs within and flanking cholesterol genes in relation to two biomarkers: HIV viral load and CD4+ T cell levels. HIV viral load was log₁₀ transformed, while CD4+ T cell levels were square root transformed^[9]. We used elastic-net models implemented using the glmnet package in R^[16].

The elastic-net considers all SNP and phenotype data simultaneously in a single high-dimensional model. The advantage of simultaneous analysis is that correlations and dependencies (multicollinearity) between SNPs are included. Elastic-net models require specification of two tuning parameters: λ_1 and λ_2 . For λ_2 we chose 0.5 as suggested by a recent study that evaluated penalized regression techniques using simulated and real data^[17]. The value of λ_1 was selected by controlling the false discovery rate (FDR) at three commonly used thresholds: 0.1, 0.05 and 0.01.

Because we had access to only a single data set of ART naïve HIV⁺ individuals, we conducted an internal replication step to decrease the likelihood of false positives. We only considered SNPs associated with *both* HIV viral load and CD4+ T cell levels to pass our internal replication criterion. HLA-B*57:01 and B*57:03 met this criterion in our prior

WIHS study^[9]. Nevertheless, our study provides only exploratory data related to host control of HIV. Definitive data (e.g., as generated by the International HIV Controllers Study^[18]) generally require much larger sample sizes, large replication cohorts and *in vitro* mechanistic studies to validate statistical observations.

Bioinformatics Methods

We conducted bioinformatics analyses for associations identified under the most conservative FDR threshold (0.01) that met our internal replication criterion. The software programs were: RegulomeDB^[19], SNPInfo^[20], F-SNP^[21], rSNPBase^[22], Haploreg v4.1^[23], MicroSNiPer^[24] and miRNASNP^[25]. Two programs were used to calculate microRNA binding energies: mrSNP^[26] and MirSNP^[27].

Statistical Methods

We confirmed elastic-net SNP associations using linear regression and generalized estimating equation (GEE) models with exchangeable correlation structures. Longitudinal data analysis using GEE including multiple time-points per participant is a well-established statistical technique, including in the WIHS cohort^[28]. We chose a correlation structure based on comparison of goodness-of-fit statistics using the Quasi-likelihood under the independence model criterion (QIC)^[29]. We also examined associations stratified by HCV status, since HCV co-infection was common in the cohort and use of cholesterol in the HCV lifecycle is documented^[1, 30].

Finally, we determined associations with total cholesterol, HDL-C and LDL-C using adjusted GEE models and stratified by HCV co-infection status. Only data from women contributing a fasting blood sample (self-report of not eating for at least 8 hours) were included. Cholesterol analyses included adjustment for PCs of genomic race/ethnicity, age and self-reported race/ethnicity (to minimize confounding by population substructure) and also other factors known or hypothesized to affect cholesterol levels (to increase statistical power), as described in our prior WIHS study^[31]. The major racial groups in WIHS (African ancestry, European ancestry and Amerindian ancestry) are easily discriminated based on the first two PCs^[32] and adjustment for five PCs is generally sufficient to control for confounding by population substructure. Stratified analyses by self-reported race/ethnicity reduces power and may not have the desired effect in heavily admixed populations such as WIHS where self-report often does not reflect actual ancestry and many participants self-report as ‘Other’ race/ethnicity^[32].

Measurement of PCSK9 Levels in Plasma

PCSK9 levels in plasma were measured by hPCSK9 ELISA (R&D Systems, Minneapolis, USA) according to manufacturer’s protocol.

RESULTS

Study Population Characteristics

The study included n=1,066 HIV⁺ women, all of whom were ART naïve at enrollment into WIHS and for whom genotype data for candidate cholesterol genes was available. WIHS

women who: (i) were HIV-seronegative; (ii) reported any prior ART use (including ART monotherapy) at enrollment, (iii) were enrolled after 1996, or (iv) lacked genotype data were excluded from the analytic dataset. The majority of the studied $n=1,066$ HIV⁺ women were African American and low-income (Table 1). Thirty-two percent of the cohort had a history of acquired immunodeficiency syndrome (AIDS) and 38% were HIV/HCV co-infected.

Because we used ART naïve HIV viral load and CD4⁺ T cell measurements prior to October 1st, 1996 for the analyses (see Methods), measurements from some women were taken from several follow-up visits. The distribution of contributed data was: 1 visit: $n=325$ women; 2 visits: $n=246$ women; 3 visits: $n=295$ women; 4 visits: $n=187$ women; 5 visits: $n=13$ women. In total, $n=2,515$ HIV viral load measurements and $n=2,463$ CD4⁺ T cell measurements were used for genetic analyses. Longitudinal data provide more information regarding genetic effects over time as compared with cross-sectional data.

SNP Associations with HIV Viral Load and CD4⁺ T Cell Levels

We considered 2,050 SNPs within and flanking 19 candidate cholesterol genes using elastic-net models with FDR thresholds of 0.1, 0.05 and 0.01. These models identified, respectively, 129, 78 and 43 SNPs significantly associated with HIV viral load and 120, 68 and 25 SNPs significantly associated with CD4⁺ T cell levels (Supplementary Table 1). At FDR thresholds of 0.1, 0.05 and 0.01 - 26, 14 and 6 SNPs, respectively, met our internal replication criterion (associated with *both* HIV viral load and CD4⁺ T cell levels - Supplementary Tables 1 and 2). Elastic net models that included adjustment for age, self-reported race/ethnicity and PCs of genomic race and ethnicity yielded identical results. We confirmed the absence of confounding by age, self-reported race/ethnicity and PCs of genomic race/ethnicity by visually inspecting diagnostic plots (data not shown).

We used linear regression models to confirm SNP associations identified under the most conservative threshold (FDR=0.01) that met our internal replication criterion (Table 2). Five of six SNPs identified by elastic-net had significant associations in linear regression. The only SNP not associated with HIV viral load or CD4⁺ T cell levels in linear regression analysis was rs11610540 (Table 2).

Bioinformatics Evaluation of Significant SNPs

Bioinformatics programs yielded largely conflicting findings regarding potential functional effects of six SNPs identified under the most conservative FDR threshold that met our internal replication criterion (Table 3). However, three bioinformatics programs identified differences in microRNA binding by alleles of rs17111557 – located in the 3′ untranslated region (UTR) of proprotein convertase subtilisin/kexin type 9 (*PCSK9*) – as a putative effect of this SNP (Table 3). A PubMed search on these six SNPs yielded no results for five, and only a search for rs17111557 located a peer-reviewed publication^[33]. We used two bioinformatics programs (mrSNP^[26] and MirSNP^[27]) to calculate microRNA binding energies. Both programs predict binding energy differences for hsa-miR-548t-5p and hsa-miR-4796-3p for individuals with different alleles of rs17111557 (Supplementary Tables 3 and 4). Specifically, microRNA binding energy differences/scores for C→T were: hsa-

miR-548t-5p (mrSNP: 35.0; MirSNP: 142.0) and hsa-miR-4796-3p (mrSNP: 41.8; MirSNP: 153.0).

Statistical Interrogation of rs17111557

Analyses of ART naïve WIHS visits using GEE models confirmed associations of rs17111557 ($n=1,058$ genotyped women, dominant model, TT + TC vs. CC) with HIV viral load (β : 0.3; 95% confidence interval (CI): 0.1, 0.4; $P=0.003$) and CD4+ T cell levels (β : -2.0; 95% CI: -3.5, -0.5; $P=0.008$). In these GEE models rs17111557 was considered with adjustment for age (linear term), self-reported race/ethnicity and the first 5 PCs of genomic race/ethnicity.

Because 38% of the cohort was HIV/HCV co-infected, and use of cholesterol in the HCV lifecycle is documented^[1, 30], we stratified by HCV status. In $n=638$ women with HCV RNA negative status, associations of rs17111557, adjusted for age, self-reported race/ethnicity and the first 5 PCs of genomic race/ethnicity, with HIV viral load (β : 0.2; 95% CI: -0.1, 0.4; $P=0.19$) and CD4+ T cell levels (β : -1.3; 95% CI: -3.2, 0.6; $P=0.17$) were not statistically significant. In contrast, in $n=405$ HIV/HCV co-infected women adjusted associations of rs17111557 with HIV viral load (β : 0.44; 95% CI: 0.2, 0.7; $P<0.001$) and CD4+ T cell levels (β : -3.0; 95% CI: -5.3, -0.6; $P=0.01$) were statistically significant and stronger than in the total study population. Additional adjustment for $\log_{10}$ HCV viral load (measured at the WIHS enrollment visit) did not affect associations of rs17111557 with HIV viral load and CD4+ T cell levels in HIV/HCV co-infected women (data not shown). Rs17111557 was not significantly associated with $\log_{10}$ HCV viral load in HIV/HCV co-infected women in analysis adjusted for age, self-reported race/ethnicity and the first 5 PCs of genomic race/ethnicity (β : 0.2; 95% CI: 0.0, 0.5; $P=0.06$).

To better understand the mechanism(s) through which rs17111557 might affect HIV, we determined associations with total cholesterol, HDL-C and LDL-C measurements using adjusted GEE models. Cholesterol was measured on an annual basis for most WIHS women starting in the year 2000, therefore, the 1994–1996 ART naïve HIV viral load and CD4+ T cell data are not contemporaneous with cholesterol measurements. The analysis was restricted to the subset of the total study population (see Table 1) who remained enrolled in the WIHS cohort through the year 2000 and had ≥ 1 LDL-C measurement.

No significant associations of rs17111557 with total cholesterol or HDL-C were observed for HIV monoinfected or HIV/HCV co-infected women (data not shown). In contrast, we observed a significant association of rs17111557 with LDL-C levels in HIV/HCV co-infected women ($n=205$; 1,533 LDL-C measurements; β : -10.4; 95% CI: -17.9, -2.9; $P=0.007$) but not in HIV monoinfected women ($n=437$; 4,187 LDL-C measurements; β : 1.2; 95% CI: -6.3, 8.6; $P=0.76$) in analyses adjusted for age, self-reported race/ethnicity, the first 5 PCs of genomic race and ethnicity and other factors known or hypothesized to affect cholesterol levels in HIV/HCV co-infected women (Table 4). Nelfinavir use was significantly associated with higher LDL-C levels in both HIV monoinfected and HIV/HCV co-infected women (Table 4). In contrast, lamivudine (3TC) use and the number of protease inhibitors were significantly associated with higher LDL-C levels only in HIV monoinfected women (Table 4).

Plasma Levels of PCSK9 in African American HIV/HCV Co-Infected Women

To clarify associations of PCSK9 pathways with HIV/HCV, we measured PCSK9 in plasma from the subset of HIV/HCV co-infected women who were African American (n=249), to minimize confounding by racial/ethnic differences in PCSK9 levels. One measurement was excluded due to technical issues. Forty-five percent (n=112) of women had an ART-naïve plasma sample prior to October 1st, 1996 available for PCSK9 measurement; for those women without a plasma sample prior to October 1st, 1996 (n=136) we selected the first time point at which fasting blood was collected (Supplementary Figure 2).

Plasma PCSK9 levels were higher among African American HIV/HCV co-infected women with the rs17111557 TC vs. CC genotype (median: 330 ng/ml; IQR: 287–372 vs median: 321; IQR: 277–358 – Supplementary Figure 3), but this difference was not significant in bivariate analysis (p=0.32). None of the women in this subset were TT homozygous. Plasma PCSK9 levels were not associated with contemporaneous log₁₀ HIV viral load in ART naïve women (n=108; adjusted for 5 PCs of genomic race/ethnicity, education, IDU at baseline, income and age: p=0.06; Supplementary Figure 4) but were significantly associated with enrollment log₁₀ HCV viral load (β : 0.003; 95% CI: 0.001, 0.005; $P=0.002$) in similarly adjusted models (Supplementary Figure 5). Additional adjustment for rs17111557 did not change this association (data not shown). Plasma PCSK9 levels were not associated with contemporaneous LDL-C (Supplementary Figure 6) or HDL-C in bivariate analyses and additional adjustment for age, 5 PCs of genomic race/ethnicity, current smoking, education, income, IDU at baseline, receipt of ART and rs17111557 did not change these null associations (data not shown).

DISCUSSION

This study – conducted in a historical US cohort of ART naïve HIV⁺ women – found that a 3' UTR variant of *PCSK9* (rs17111557) is associated with biomarkers of HIV pathogenesis and lipid homeostasis in HIV/HCV co-infected women. Differential binding of hsa-miR-548t-5p and hsa-miR-4796-3p to the *PCSK9* 3'UTR in individuals with different alleles of rs17111557 could represent the mechanism through which rs17111557 affects LDL-C, HIV viral load and CD4⁺ T cell levels. These associations, if replicated, are important because they link *PCSK9*, a gene of clinical and therapeutic importance in the general population, with specific aspects of HIV pathogenesis in HIV/HCV co-infected women.

Genetic variants in or near *NCOR2* (nuclear receptor corepressor 2/silencing mediator of retinoic acid and thyroid hormone receptor (SMRT)), *RXR α* (retinoid X receptor, alpha) and *TTC39B* (tetratricopeptide repeat domain 39B) may also be associated with biomarkers of HIV pathogenesis in WIHS women. However, these SNPs were localized in the introns and our evaluation of these variants using bioinformatics tools yielded conflicting findings. Therefore, we focused on rs17111557 – a putatively functional SNP. Nevertheless, we note that three of the top six SNPs identified in our analyses were in *NCOR2*. *NCOR2* is a member of the nuclear corepressor family of histone deacetylases, which are components of the transcription silencing machinery, and SNPs in *NCOR2* were found to significantly associate with HIV acquisition^[34].

It is not surprising that rs17111557 has not previously been identified as a correlate of HIV pathogenesis. There is little global variation in rs17111557 except in African ancestry populations - for example, in the CEU (Utah Residents with Northern and Western European Ancestry) HapMap population allele frequencies at this locus are 99% C and 1% T. Many if not all genetic studies of HIV have been conducted in European ancestry cohorts^[10, 11].

PCSK9 plays a central role in lipid homeostasis by enhancing degradation of hepatic low-density lipoprotein receptor (LDLR), resulting in increased serum LDL-C^[35]. PCSK9 is also expressed in extra-hepatic tissues (e.g., small intestine) where it can affect vascular biology through LDL-dependent and/or LDL-independent mechanisms, including mechanisms that regulate inflammation, blood pressure and glucose homeostasis^[36, 37]. PCSK9 also downregulates CD81 and very-low-density lipoprotein receptors (VLDLR) on hepatocytes^[38, 39]. CD81 and VLDLR are used by HCV to gain entry into cells^[40] and PCSK9 levels modulate HCV infectivity *in vitro*^[39]. The role of PCSK9 in inflammation, immunologic processes and infection is an area of active investigation^[41, 42].

Surprisingly, despite higher median levels of plasma PCSK9 in HIV/HCV co-infected women with rs17111557 TC vs. CC genotype, no significant association of rs17111557 was observed. This finding suggests that rs17111557 may exert only a modest effect on plasma PCSK9 levels, and a larger sample size could show statistical significance. It is also possible that 3' UTR polymorphism in *PCSK9* may alter temporal regulation of *PCSK9* expression, or it may affect *PCSK9* expression only in certain cell populations (e.g., those expressing high levels of hsa-miR-548t-5p and hsa-miR-4796-3p). It is also possible that rs17111557 affects lipid and viral load levels through PCSK9-independent mechanisms, such as altering the levels of inflammatory cytokines or other pathways^[43]. Both plasma PCSK9 and IL-6 levels were elevated in HIV/HCV co-infected as compared to HIV monoinfected patients in a majority male Caucasian population^[44].

In addition to rs17111557, several associations with LDL-C were observed in multivariate analyses. Particularly striking was the inverse association between age and LDL-C levels in HCV/HIV co-infected women. These data are consistent with those of a study that showed progressive declines of LDL-C over time among HCV monoinfected veterans with known dates of HCV seroconversion^[45]. The association of older age with lower LDL-C in that study was independent of FIB-4, a marker of liver fibrosis^[46], as was observed in WIHS women. Use of lipid lowering medications was inversely associated with LDL-C levels in both HCV/HIV co-infected and HIV monoinfected women, highlighting the effectiveness of these medications despite dysregulated lipid homeostasis. Furthermore, a recent study observed that statin use was associated with lower incidence of liver cirrhosis and hepatocellular carcinoma in HCV⁺ veterans^[47]. A future research priority will therefore be to define effects of recently-approved PCSK9 inhibitors – which have favorable toxicity and drug-drug interaction profiles as compared to statins - in HIV monoinfected and HCV/HIV co-infected women.

Limitations must be considered in the interpretation of these data. First, WIHS did not collect fasting blood or measure lipids during the pre-HAART era (1994–1996). Second, we identified a SNP in the seed of hsa-miR-548t-5p (rs73872515) with variation in African

ancestry populations but were unable to test this SNP for epistatic interaction with rs17111557 because it was not available in WIHS. Third, our study only included women. Plasma PCSK9 levels were significantly higher in women as compared to men in a large (n=3,138) ethnically diverse US population, and sex differences persisted after adjustment for age, ethnicity, body mass index, systolic blood pressure, menopausal status, and fasting levels of glucose, LDL-C, HDL-C, triglycerides, and c-reactive protein^[48]. Thus, it is possible that interactions of HIV, HCV and PCSK9 also differ by sex. Fourth, the primary phenotypes considered in our study were HIV viral load and CD4 T cell levels, but other phenotypes not considered under the current investigation may also be important. For example, recent studies in the general population have considered host variation at PCSK9 and 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR; the target of statins) loci in relation to cardiovascular disease and cognitive function phenotypes^[49, 50]. The relation of cholesterol pathway gene variation in relation to other HCV-related phenotypes such as spontaneous HCV clearance and liver disease progression may also warrant further investigation. Fifth, unlike some cohorts (e.g., the Multicenter AIDS Cohort Study (MACS)), the WIHS did not have a long period in the pre-HAART era and thus our capacity to study CD4 decline in WIHS or clinical outcomes associated with untreated HIV infection is limited. Finally, associations of rs17111557 need to be replicated. Nevertheless, we report the discovery of these associations because rs17111557 was associated with multiple distinct phenotypes in WIHS women, has supporting bioinformatics data, and is potentially of interest to HIV and/or HCV clinicians considering use of PCSK9 inhibitors in their patients. Cholesterol plays a critically important role in the lifecycle of HIV and HCV. Candidate gene study designs may provide useful insights regarding which specific cholesterol-related pathways may be targeted for the development of new strategies to prevent or control HIV and other human pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Baseline characteristics of ART naïve HIV⁺ WIHS women, enrolled from October 1994 to November 1995
(n=1,066)^a

Demographic & behavioral characteristics	
Age	37 (31, 42)
Race/ethnicity	
African American	606 (57%)
Hispanic	227 (21%)
White	200 (19%)
Other	33 (3%)
Education	
< High school	396 (37%)
High school	338 (32%)
> High school	332 (31%)
Household income	
\$12,000	671 (63%)
\$12,001– \$30,000	254 (24%)
> \$30,000	98 (9%)
Unknown	43 (4%)
Ever injection drug use	477 (45%)
Laboratory & clinical characteristics	
Log ₁₀ HIV copies/mL	4.3 (3.6, 5.0)
CD4 ⁺ cells/mL	413 (217, 608)
AIDS	337 (32%)
HCV RNA ⁺	408 (38%)

ART: antiretroviral therapy; AIDS: acquired immunodeficiency syndrome; HCV: hepatitis C virus

^aContinuous variables expressed as median (interquartile range)

Table 2

SNPs identified in elastic-net models with FDR=0.01 that met our internal replication criterion^a

SNP name	Gene	Position	Genotype	N ^b	HIV viral load		CD4+ count	
					β	P-value	β	P-value
rs17111557	PCSK9	3' UTR	CC	2075	Ref	Ref	Ref	
			TC	270	0.49	<0.01	-1.84	<0.01
			TT	8	-0.03	0.97	-6.28	0.02
rs3933785	TTC39B	intron	AA	1831	Ref	Ref	Ref	
			AG	501	-0.25	0.03	0.17	0.66
			GG	21	2.25	<0.01	-6.00	<0.01
rs12003265	RXRA	intron	GG	1926	Ref	Ref	Ref	
			AG	297	0.84	<0.01	-3.65	<0.01
			AA	130	0.48	0.02	-0.85	0.22
rs1263991	NCOR2	intron	TT	1590	Ref	Ref	Ref	
			TC	656	0.41	<0.01	-1.47	<0.01
			CC	107	-0.39	0.10	1.34	0.08
rs11610540	NCOR2	intron	GG	1874	Ref	Ref	Ref	
			AG	431	0.32	0.15	-1.10	0.13
			AA	48	-1.29	0.07	3.32	0.16
rs10846682	NCOR2	intron	TT	1725	Ref	Ref	Ref	
			TC	565	-0.45	0.03	1.80	0.01
			CC	63	-0.32	0.61	1.90	0.37

SNP: single nucleotide polymorphism; FDR: false discovery rate; UTR: untranslated region

^a SNPs identified in elastic-net models were considered together in multivariable linear regression models (one model for log₁₀HIV RNA levels and one model for CD4+ count)

^b N represents the number of HIV RNA and CD4 measurements used for these analyses – most WHHS women contributed more than one measurement to the analysis (see Results)

Predicted function of SNPs identified in elastic-net models with FDR=0.01 that met our internal replication criterion^a

Table 3

SNP name	Gene	Position	PubMed articles	Regulome DB ^b	SNPInfo ^c		F-SNP ^c		rSNPBase	Haploreg v4.1 ^d	MicroSNIPe ^e	miRNA-SNP
					Score	Function	Score	Function	Regulatory SNP	eQTL evidence	eQTL evidence	
rs17111557	PCSK9	3' UTR	1	4	0.29	miR-binding	0.11	TR	Yes	Yes	No	miR-binding
rs3933785	TTC39B	intron	0	4	0.00	None	0.00	TR	Yes	Yes	Yes	None
rs12003265	RXRA	intron	0	2b	ND	None	0.18	TR	Yes	No	No	None
rs1263991	NCOR2	intron	0	4	0.35	None	0.50	TR	Yes	No	No	None
rs11610540	NCOR2	intron	0	4	0.00	None	ND	None	Yes	No	Yes	None
rs10846682	NCOR2	intron	0	4	0.08	None	ND	None	Yes	No	No	None

SNP: single nucleotide polymorphism; FDR: false discovery rate; ND: not determined by the software; TR: transcriptional regulation; eQTL: expression quantitative trait loci; miR: microRNA

^a SNPs associated with *both* HIV viral load and CD4+ T cell levels in elastic-net models with FDR=0.01

^b Regulome scores are from 1a to 6. Lower scores reflect greater evidence for regulation of gene expression

^c SNPInfo and F-SNP scores are from 0 to 1. Higher scores reflect greater evidence for regulation of gene expression

^d AFR 1000G Phase 1 population was used for LD calculation

^e Specification of 7bp “seed length” to increase specificity

Table 4

Associations of rs17111557 with low-density lipoprotein cholesterol (LDL-C) in HIV monoinfected and HCV/HIV co-infected women, 10/02/2000–09/30/2014^{a,b,c}

	HIV monoinfected women (n=437, 4,187 LDL-C measures)				HIV/HCV co-infected women (n=205, 1,533 LDL-C measures)			
	β	95% CI	P		β	95% CI	P	
rs17111557	1.2	-6.3, 8.6	0.76		-10.4	-17.9, -2.9	0.007	
Age								
< 30 years	Ref				Ref			
30–40 years	1.9	-2.4, 6.2	0.38		-11.7	-14.2, -9.1	<0.0001	
40–50 years	1.0	-4.2, 6.1	0.71		-17.6	-25.4, -9.8	<0.0001	
50 years	0.3	-5.6, 6.2	0.93		-20.8	-29.1, -12.4	<0.0001	
Log ₁₀ HIV viral load	0.2	-0.9, 1.4	0.71		-1.8	-3.2, -0.4	0.01	
CD4+ T cell count	0.4	0.1, 0.6	0.002		0.7	0.4, 1.0	<0.0001	
FIB-4 ^d	-1.8	-3.3, -0.2	0.03		-1.5	-2.3, -0.7	0.0004	
Lipid lowering agents ^e	-18.4	-23.3, -13.5	<0.0001		-11.2	-20.9, -1.5	0.02	
BMI	0.7	0.4, 1.0	<0.0001		0.2	-0.2, 0.6	0.29	
Smoking								
Never	Ref				Ref			
Current	-2.7	-8.3, 2.9	0.34		-14.6	-28.7, -0.4	0.04	
Former	-1.9	-7.4, 3.5	0.49		-10.0	-24.6, 4.5	0.18	
Antiretroviral therapies ^f								
Lamivudine (3TC)	3.8	0.9, 6.6	0.01		1.3	-2.2, 4.7	0.47	
Ritonavir	-4.8	-12.2, 2.6	0.20		3.2	-4.4, 10.8	0.41	
Nelfinavir	12.0	5.1, 18.8	0.0006		7.8	0.6, 15.0	0.03	
# Protease inhibitors	4.1	0.4, 7.9	0.03		0.2	-3.2, 3.6	0.90	
Principal components of ancestry								
PC1	-99.1	-451.6, 253.4	0.58		265.6	-119.4, 650.7	0.18	
PC2	-297.3	-523.2, -71.4	0.01		75.4	-266.9, 417.7	0.67	
PC3	163.4	22.2, 304.6	0.02		-47.3	-227.9, 133.3	0.61	
PC4	28.7	-125.7, 183.1	0.72		-1.7	-214.4, 211.1	0.99	

HIV monoinfected women (n=437, 4,187 LDL-C measures)			HIV/HCV co-infected women (n=205, 1,533 LDL-C measures)		
	β	95% CI	P	β	95% CI
PC5	76.0	-63.8, 215.8	0.29	-99.3	-278.6, 79.9
Race/ethnicity					
African American	Ref			Ref	
Hispanic	-6.3	-18.3, 5.8	0.31	-5.6	-16.8, 5.7
White	1.9	-14.3, 18.0	0.82	18.2	1.8, 34.7
Other	2.0	-13.0, 16.9	0.80	-4.0	-25.7, 17.7

LDL-C: low-density lipoprotein cholesterol; HCV: hepatitis C virus; PC: principal component of genomic race/ethnicity; BMI: body mass index

^ars17111557 was considered in a dominant genetic model (TT + TC vs. CC) because few women were TT homozygous

^bHCV RNA status was defined at baseline (first three WIHS visits). Women with indeterminate HCV RNA statuses at baseline were excluded.

^cThe analysis was restricted to the subset of the total study population (see Table 1) who remained enrolled in the WIHS cohort through the year 2000 and had 1 LDL-C measurement.

^dFIB-4 is a non-invasive marker of liver fibrosis, as defined in [46]

^eUse of lipid lowering agents was self-reported: Lescol (fluvastatin), Lipitor (atorvastatin), Mevacor, Altacor (lovastatin), Pravachol (pravastatin), Zocor (simvastatin), Crestor (rosuvastatin), Lopid (gemfibrozil), TriCor (fenofibrate), Colestid (colestipol), Questran (cholestyramine), Welchol (colesevelam), Niaspan (niacin), Zetia (ezetimibe), Caduet (amlodipine + atorvastatin), Vytorin (ezetimibe + simvastatin), Advicor (lovastatin + niacin), Pravigard (pravastatin + buffered aspirin) or Omacor.

^fAntiretroviral therapies significantly associated with LDL-C levels in our prior study of HIV⁺ WIHS women [31] were included in the analysis.