

Subclinical Carotid Artery Atherosclerosis Is Associated With Increased Expression of Peripheral Blood IL-32 Isoforms Among Women Living With HIV

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Background: Persistent inflammation in HIV infection is associated with elevated cardiovascular disease (CVD) risk, even with viral suppression. Identification of novel surrogate biomarkers can enhance CVD risk stratification and suggest novel therapies. We investigated the potential of interleukin 32 (IL-32), a proinflammatory multi-isoform cytokine, as a biomarker for subclinical carotid artery atherosclerosis in virologically suppressed women living with HIV (WLWH).

Methods and Results: Nested within the Women's Interagency HIV Study, we conducted a cross-sectional comparison of IL-32 between 399 WLWH and 100 women without HIV, followed by a case-control study of 72 WLWH (36 carotid artery plaque cases vs. 36 age-matched controls without plaque). Plasma IL-32 protein was measured by ELISA, and mRNA of IL-32 isoforms (IL-32 α , β , γ , δ ,

ϵ , and θ) was quantified by reverse transcription polymerase chain reaction from peripheral blood mononuclear cells. Plasma IL-32 protein levels were higher in WLWH compared with women without HIV ($P = 0.02$). Among WLWH, although plasma IL-32 levels did not differ significantly between plaque cases and controls, expression of IL-32 isoforms α , β , and ϵ mRNA was significantly higher in peripheral blood mononuclear cells from cases ($P = 0.01$, $P = 0.005$, and $P = 0.018$, respectively). Upregulation of IL-32 β and IL-32 ϵ among WLWH with carotid artery plaque persisted after adjustment for age, race/ethnicity, smoking, systolic blood pressure, body mass index, and history of hepatitis C virus ($P = 0.04$ and $P = 0.045$); the adjusted association for IL-32 α was marginally significant ($P = 0.07$).

Conclusions: IL-32 isoforms should be studied further as potential CVD biomarkers. This is of particular interest in WLWH by virtue of altered IL-32 levels in this population.

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INTRODUCTION

Unresolved low-grade inflammation in HIV infection, even with antiretroviral therapy (ART), contributes to cardiovascular disease (CVD).¹ Multiple factors are believed to contribute to this chronic inflammation, including persistent immune stimulation fueled by residual HIV viremia and possibly antigens from other coinfections such as cytomegalovirus or hepatitis C virus (HCV), imbalance of intestinal microbiota composition toward pathogenic bacteria (ie, gut dysbiosis), and bacterial and fungal translocation as a result of compromised gut mucosal barrier integrity.^{2–10} These mediators may sustain overt inflammation by upregulating a large number of inflammatory factors, including tumor necrosis factor alpha, IL-1 β , vascular cell adhesion molecule 1, high-sensitivity C-reactive protein (hsCRP), D-dimer, sCD14, sCD163, and IL-6.^{11–15} Some of these factors, such as hsCRP, are used as biomarkers to enhance risk stratification of CVD and associated mortality.¹⁶ However, the role of hsCRP as an inflammatory marker is limited to prognosis and risk prediction because it does not seem to represent a causal factor for CVD,¹⁷ and moreover, the association of hsCRP with atherosclerosis is blunted in the setting of HIV infection.¹⁸ Other factors, including IL-6, have the potential to be used as both biomarkers and therapeutic targets.¹⁹ However, clinical trials with tocilizumab, a monoclonal antibody targeting the IL-6 receptor, have reported considerable side effects such as increased total and low-density cholesterol levels.²⁰ Thus, the identification of novel inflammatory factors, especially candidates upstream of IL-6 signaling, may provide better alternatives for CVD risk stratification as well as lead to potential treatment targets. In this regard, we have recently reported that IL-32, a proinflammatory cytokine that is expressed in multiple isoforms (α , β , γ , D, ϵ , θ , ζ , η , and small/sm),^{21–23} is upregulated in HIV infection, and its expression is not normalized with ART.²⁴ We have also demonstrated that IL-32 induces a strong inflammatory response in T cells by enhancing the production of IL-6, tumor necrosis factor alpha, and IFN γ and further induces HIV transcription from latently infected cells.^{24,25} These observations suggest that IL-32 may contribute to the persistent immune activation and inflammation that are the major etiologic mediators of atherosclerosis²⁶ and may represent a biomarker of future CVD. Here, we investigated this hypothesis by studying expression of IL-32 and its isoforms in a case–control study of ART-treated, virally suppressed women living with HIV (WLWH), comparing those with and without subclinical carotid artery atherosclerosis.

METHODS

Study Design and Population

We conducted a study nested within the Women's Interagency HIV Study (WIHS), a long-standing prospective multicenter cohort of WLWH and women at risk for HIV infection from the same communities.^{27,28} The WIHS is now

part of the MACS/WIHS Combined Cohort Study.²⁹ To study expression of total IL-32 protein by HIV serostatus, we randomly selected 499 participants (399 WLWH and 100 without HIV) with stored plasma and peripheral blood mononuclear cells (PBMCs) collected during WIHS Visit 34 (April to September 2011). WLWH selected for this study were required to be taking ART and virally suppressed with <100 HIV RNA copies/mL at the time of the visit (COBAS TaqMan v2.0 HIV-1, Roche). Despite good adherence to ART, viral blips <100 copies/mL are relatively common among people with HIV without being associated with clinical variables.³⁰

Next, we conducted a case–control study among WLWH examining expression of IL-32 isoforms based on the presence or absence of subclinical carotid artery atherosclerosis. This study was nested within a vascular substudy of the WIHS. Briefly, starting in 2004, WIHS participants were invited to undergo high-resolution B-mode ultrasound every 2 to 3 years to image the carotid artery.^{31,32} Among participants in the fourth wave (2010–2012) of the vascular substudy, we selected 36 WLWH with subclinical atherosclerosis (cases) and matched them by age with 36 WLWH without subclinical atherosclerosis (controls). WLWH were also required to be taking ART and virologically suppressed (<100 copies/mL) at the time of the most recent scan, as well as free of coronary heart disease (self-report of angina, myocardial infarction, or coronary revascularization) at the baseline vascular substudy visit.

Case Definition

As part of the vascular substudy, 6 locations in the right carotid artery were imaged: the near and far walls of the common carotid artery, carotid bifurcation, and internal carotid artery.^{31,32} A standardized protocol was used at all centers,³³ and measurements were obtained at a centralized reading center (University of Southern California). Cases of subclinical atherosclerosis had plaque, defined as a focal wall protuberance into the lumen of the artery with a minimal diameter of 1.5 mm at its maximum point, measured in at least one of the 6 aforementioned artery locations. Controls were found to not have plaque at any of the imaged locations. Although mean intima–media thickness (IMT) was also assessed from standardized ultrasound images by automated computerized edge detection at the far walls of the common carotid artery and the carotid bifurcation, our previous studies have not found these measurements to be positively associated with HIV serostatus,³¹ and therefore, we did not design our study to examine IL-32 isoforms in relation to mean IMT.

IL-32 Measures

Total IL-32 protein was quantified from stored plasma using the human IL-32 ELISA kit (R&D System, Cat #DY3040-05). Total RNA was isolated from cryopreserved human PBMCs using the RNeasy plus mini kit from Qiagen as per the manufacturer's protocol (Catalog #74134). Quantification of IL-32 isoforms (α , β , γ , D, ϵ ,

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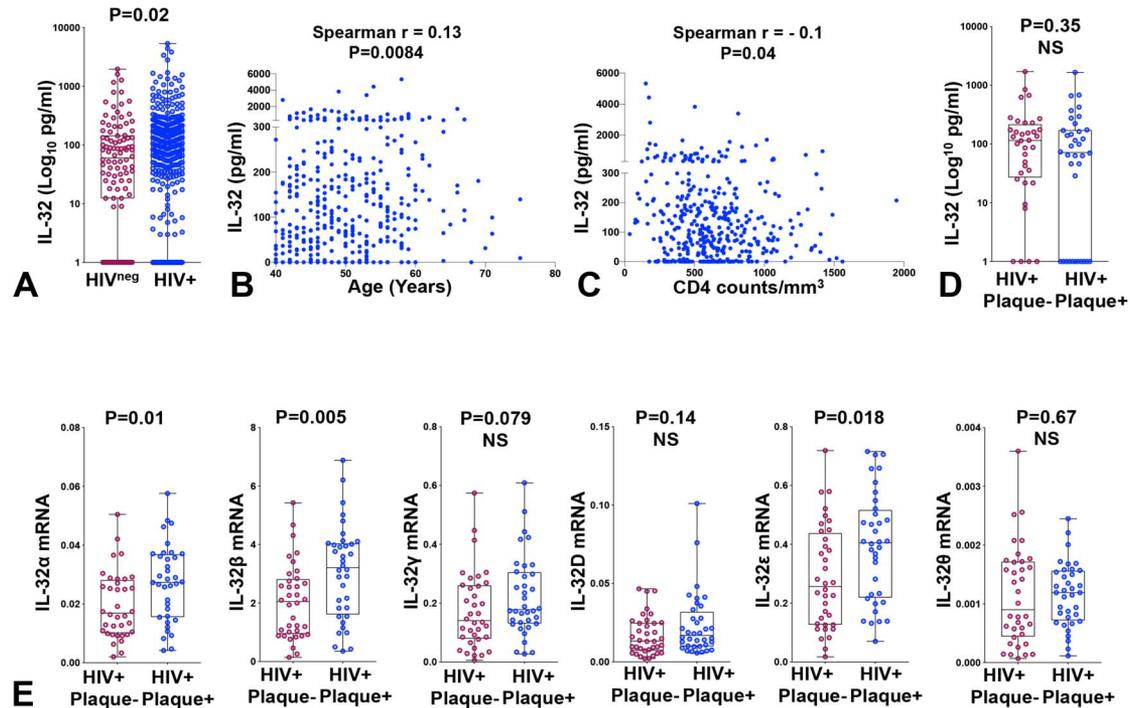


FIGURE 1. Total IL-32 protein levels and isoform mRNA expression in PBMCs of WLWH with or without subclinical atherosclerosis. A, Total IL-32 protein measured by ELISA in plasma from $n = 100$ women without HIV and $n = 399$ WLWH. B, Correlations of IL-32 protein in WLWH ($n = 399$) with participant age. C, Correlations of IL-32 protein in WLWH ($n = 399$) with participant CD4 T-cell count. D, IL-32 protein measured by ELISA in plasma from WLWH with ($n = 36$) or without ($n = 36$) subclinical atherosclerosis (HIV + plaque^{neg} and HIV + plaque⁺, respectively). E, RT-qPCR data for IL-32 isoforms (α , β , γ , D, ϵ , and θ) amplified from total PBMCs of HIV + plaque^{neg} ($n = 36$) compared with HIV + plaque⁺ ($n = 36$). IL-32 mRNA levels were normalized to the housekeeping gene β -glucuronidase. *P* values are calculated with the 2-tailed nonparametric Mann–Whitney test in (A, D, and E) and Spearman correlations in (B and C). NS, nonsignificant.

and θ) was performed using One-step SYBR Green reverse transcription Quantitative reverse transcription polymerase chain reaction (RT-qPCR) performed on LightCycler 480II machine (Roche) with QIAGEN QuantiTect (Catalog #204243). Relative expression of IL-32 RNA was normalized to the housekeeping gene β -glucuronidase. Primer sets to quantify the different IL-32 isoforms and β -glucuronidase as well as conditions for the quantitative PCR and analysis were done as recently reported.²⁴

Potential Confounders

All comparisons in the case–control study accounted for age as part of the matched design. We considered the following variables as additional potential confounders: race/ethnicity; education; current crack/cocaine or alcohol use; history of injection drug use; smoking history; body mass index (BMI); systolic blood pressure; total, low-density lipoprotein, and high-density lipoprotein cholesterol levels (Quest standard lipid panel); history of diabetes mellitus; menopause status; and current use of medications for hypertension, hyperlipidemia, or diabetes. History of HCV

infection was defined by the presence of antibody to HCV by second-generation or third-generation ELISA (Ortho-Diagnostic Systems) or the presence of HCV-RNA by HCV-branched DNA (Quantiplex 2.0, Bayer-Versant Diagnostics) and RT-PCR (COBAS AmpliCor HCV detection kit, Roche). We also considered current and nadir CD4⁺ T-cell count, history of clinical AIDS, CD4:CD8 ratio, and hsCRP levels.

Statistical Analysis

Data were analyzed using GraphPad Prism 8 (GraphPad Software, San Diego, CA) and SAS 9.4 (SAS Institute, Cary, NC). Differences by HIV serostatus or case status were assessed by the Mann–Whitney *U* test or χ^2 test, as appropriate. Correlations were assessed with the nonparametric Spearman test. Multivariable logistic regression analyses controlled for potential confounders, including those significantly associated with carotid artery plaque in bivariate analyses ($P < 0.10$), as well as age and smoking status based on a priori knowledge.³⁴ Levels of IL-32 isoforms were log transformed when not normally distributed and scaled to

TABLE 1. Demographic and Clinical Parameters of ART-Treated Virally Suppressed Case–Control Study Participants

	Controls (No Plaque, N = 36) N (%) or Median (IQR)	Cases (Plaque, N = 36) N (%) or Median (IQR)	P
Demographic characteristics			
Age, yr	55 (51–58.5)	55 (51–58.5)	0.92
Race/ethnicity			
Black, non-Hispanic	15 (42)	21 (58)	0.08
Hispanic	19 (53)	10 (28)	
White, non-Hispanic, or other	2 (6)	5 (14)	
Education			
Did not complete high school	17 (47)	18 (50)	0.99
Completed high school	11 (31)	8 (22)	
At least some college	8 (22)	10 (28)	
Behavior-related characteristics			
Current crack/cocaine use	0 (0)	3 (8)	0.24
Current alcohol use	8 (22)	8 (22)	0.99
History of injection drug use	12 (33)	18 (50)	0.23
History of HCV infection	12 (33)	20 (56)	0.10
History of smoking	21 (58)	27 (75)	0.21
Cardiometabolic risk factors			
BMI, kg/m ²	30.1 (26.6–38.9)	26.9 (24.3–31.0)	0.01
Systolic blood pressure, mm Hg	116 (108–125.5)	123.5 (113.5–137)	0.049
Current use of antihypertensive medications	16 (44)	22 (61)	0.24
Total cholesterol, mg/dL	184.5 (164.5–224)	185 (150–221)	0.59
LDL cholesterol, mg/dL	103 (77–125)	96.5 (78–124)	0.79
HDL cholesterol, mg/dL	59 (47–70.5)	53 (42–68)	0.30
Current use of lipid-lowering medications	12 (33)	13 (36)	0.99
History of diabetes mellitus	9 (25)	12 (33)	0.60
Current use of diabetes medications	7 (19)	5 (14)	0.75
Menopausal (includes surgical)	25 (69)	27 (75)	0.79
Current use of anti-inflammatory medications	7 (19)	7 (19)	0.99
IMT, common carotid artery, mm	0.733 (0.662–0.803)	0.830 (0.738–0.954)	0.0003
IMT, bifurcation, mm	0.816 (0.739–0.888)	0.884 (0.788–1.003)	0.02
HIV-specific characteristics and biomarkers			
CD4 ⁺ count, cells/uL	627 (520.5–779)	600.5 (452.5–796)	0.74
Current ART regimen			
Integrase inhibitor based	6 (17)	7 (19)	0.67
NNRTI based	15 (42)	11 (31)	
Protease inhibitor based	13 (36)	17 (47)	
Other	2 (6)	1 (3)	
History of clinical AIDS	20 (56)	17 (47)	0.64
Nadir CD4 ⁺ count, cells/μL	194.5 (131.5–353)	241.5 (105–348.5)	0.82
CD4:CD8 ratio	0.82 (0.49–1.03)	0.67 (0.49–0.91)	0.32
hsCRP, μg/mL	1.9 (1.0–4.2)	1.9 (1.0–6.8)	0.82

HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Z-score before model fitting. We used alpha <0.05 to determine statistical significance.

Research Center (approval #CE.11.063) and each participating WIHS center.

Ethical Considerations

Participants provided written informed consent, and all analyses were performed in accordance with the guidelines and regulations approved by the Institutional Review Boards of the “Centre Hospitalier de l’Université de Montréal”

RESULTS

Total IL-32 protein was quantified in plasma from n = 399 ART-treated, virologically suppressed WLWH and n = 100 women without HIV. Although characteristics between WLWH and women without HIV were generally

similar, WLWH were slightly older (median age 50 vs. 47.5), had higher total cholesterol levels (median 185.5 vs. 177.5 mg/dL), lower systolic blood pressure (median 119 vs. 124 mm Hg), lower CD4 counts (median 598 vs. 989 cells/mm³), and had fewer behavioral risk factors like smoking history (66% vs. 80%), current crack/cocaine use (4% vs. 10%), and current alcohol use (33% vs. 59%). Plasma samples were blinded to presence of subclinical atherosclerosis to confirm the upregulation of IL-32 in HIV infection as we previously reported,²⁴ replicated here in an independent sample.

As shown in Figure 1A, plasma IL-32 protein levels were significantly higher in WLWH compared with women without HIV ($P = 0.02$). Among WLWH, IL-32 showed a positive correlation with participant age ($r = 0.13$, $P = 0.0084$, Fig. 1B) and, similar to our previous findings,²⁵ was negatively correlated with CD4 count ($r = -0.1$, $P = 0.04$, Fig. 1C), albeit weakly. After restricting the analysis to the 36 atherosclerosis cases and 36 age-matched controls (Table 1), total IL-32 plasma protein did not differ significantly between the 2 groups ($P = 0.35$) (Fig. 1D).

Given the multitude of IL-32 isoforms and their differential functions as we and others have previously shown,^{22,24} we aimed to investigate whether these isoforms are differentially expressed based on atherosclerosis status. As shown in Figure 1E, levels of the IL-32 α , β , and ϵ isoforms were significantly higher in PBMCs isolated from WLWH with subclinical atherosclerosis ($P = 0.01$, $P = 0.005$, and $P = 0.018$, respectively) compared with age-matched controls without atherosclerosis. Upregulation of the IL-32 β and ϵ isoforms persisted after additional adjustment for age, race/ethnicity, smoking status, systolic blood pressure, BMI, and history of HCV ($P = 0.04$ and $P = 0.045$, respectively). After adjustment, every Z-score increase in IL-32 β was associated with a 96% higher odds of plaque [adjusted odds ratio (aOR) 1.96, 95% confidence interval (CI) 1.05 to 3.68]. Similarly, every Z-score increase in IL-32 ϵ was associated with a 92% higher odds of plaque (aOR 1.92, 95% CI: 1.01 to 3.63). The adjusted association for IL-32 α was marginally significant (aOR 1.80, 95% CI: 0.96 to 3.37, $P = 0.07$). Levels of IL-32 γ and IL-32D were higher in plaque cases compared with controls, but these differences were not statistically significant.

DISCUSSION

Our study provides evidence for a potential role of the proinflammatory cytokine IL-32 as a biomarker for subclinical atherosclerosis in well-controlled WLWH. This role was restricted to cell-associated IL-32 mRNA that distinguished differentially expressed isoforms, rather than circulating total IL-32 proteins detected by a common set of antibodies. Of note, we previously showed in a separate sample that mRNA levels of IL-32 isoforms positively correlate with cell-associated IL-32 proteins but not with plasma IL-32.²⁴ These data suggest that differences in mRNA expression observed in the current study between WLWH with or without subclinical atherosclerosis are likely to be translated into functional proteins at the cellular level.

We showed here that the IL-32 α , β , and ϵ isoforms were highly expressed (and IL-32 β and ϵ significantly so) in WLWH with subclinical atherosclerosis, independent of age, smoking status, and other CVD risk factors including BMI. Of note, higher BMI is known to be associated with low-grade inflammation, due in part to the production and secretion of proinflammatory cytokines in adipose tissue.³⁵ However, in the current study, average BMI was lower among cases with subclinical atherosclerosis compared with controls (Table 1). Despite this, expression of IL-32 isoforms was significantly higher among cases, bolstering the link between IL-32 and subclinical atherosclerosis and suggesting the potential for use of these isoforms as biomarkers of CVD. However, we acknowledge the limitation of the relatively small sample size used in the current study. Thus, replication of these observations in larger cohorts of both men and WLWH is warranted.

Moreover, the functional consequence for the simultaneous coexpression of the 3 IL-32 isoforms (IL-32 α , β and ϵ) remains to be determined. Although IL-32 β plays a proinflammatory role by inducing IL-6 and IFN γ in activated T cells²⁴ (likely inducing a Th1 phenotype) and similarly IL-32 ϵ induces a distinct form of caspase-independent apoptosis,³⁶ IL-32 α shows anti-inflammatory potential because it induces IL-10 expression but not IL-6, as we have previously shown.²⁴ However, IL-32 β and IL-32 ϵ are expressed at a relatively higher ratio compared with IL-32 α (100- and 10-fold more, respectively²⁴), and therefore, the overall dominant function of IL-32 expression is likely to be inflammatory, which favors atherogenesis with plaque development and growth.

In conclusion, our observations align with mounting evidence for a potential role of IL-32 as a key player in vascular inflammation and CVD^{37–39} and warrant further investigations to build the case for this novel proinflammatory cytokine as a CVD biomarker and therapeutic target.

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