Macrophage Inflammatory Markers Are Associated With Subclinical Carotid Artery Disease in Women With Human Immunodeficiency Virus or Hepatitis C Virus Infection

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Objective—Infection with hepatitis C virus (HCV) or human immunodeficiency virus (HIV) may be associated with atherosclerosis and vascular disease. Macrophages are a major component of atherosclerotic plaque, and classically activated (M1) macrophages contribute to plaque instability. Our goal was to identify plasma biomarkers that reflect macrophage inflammation and are associated with subclinical atherosclerosis.

Approach and Results—We tested whether M1 macrophages produce galectin-3–binding protein in vitro. Then, we measured galectin-3–binding protein and the soluble macrophage biomarkers soluble cluster of differentiation 163 and soluble cluster of differentiation 14 in 264 participants in the Women’s Interagency HIV Study. Women were positive for HIV, HCV, both, or neither (66 in each group, matched for age, race/ethnicity, and smoking status). Carotid artery disease was assessed by ultrasound measurement of right distal common carotid artery intima-media thickness, distensibility, and presence of atherosclerotic lesions (IMT>1.5 mm). Plasma galectin-3–binding protein was higher in HCV+ than HCV− women (P<0.01) but did not differ by HIV status. The 3 inflammatory macrophage markers were significantly correlated with each other and negatively correlated with cluster of differentiation (CD)4+ counts in HIV-infected women. We defined a macrophage score as 1, 2, or 3 biomarkers elevated above the median. In models adjusted for traditional risk factors, higher macrophage scores were significantly associated with increased atherosclerotic lesions and lower carotid distensibility. Receiver-operator curve analysis of lesions revealed that the markers added predictive value beyond traditional risk factors and C-reactive protein.

Conclusions—The macrophage inflammatory markers galectin-3–binding protein, soluble cluster of differentiation 163, and soluble cluster of differentiation 14 are significantly associated with carotid artery disease in the setting of HIV and HCV infection. (Arterioscler Thromb Vasc Biol. 2014;34:00-00.)

Key Words: acquired immunodeficiency syndrome ■ atherosclerosis ■ immune system ■ risk factors ■ women

Many risk factors, including dyslipidemia, older age, high blood pressure, diabetes mellitus, and smoking, are established predictors of cardiovascular disease (CVD). The chronic inflammatory process in atherosclerosis is increasingly recognized as a contributing factor. High sensitivity C-reactive protein (hsCRP) has shown some use in atherosclerosis risk assessment as a global marker of inflammation, but other markers may reflect more specific pathways. Atherosclerosis is characterized by infiltration of monocytes into the wall of large and medium-sized arteries, where they form atherosclerotic plaque. These monocytes differentiate to macrophages and foam cells, some of which undergo apoptosis and secondary necrosis, forming a necrotic core that makes plaque vulnerable to rupture and clinical events like myocardial infarctions and stroke. Plaque macrophages show several different phenotypes that can be distinguished by surface markers using flow cytometry and immunofluorescence. Atherosclerotic lesions contain macrophages with phenotypic characteristics of M1 and M2 polarization. In general, M1 macrophages are thought to be associated with inflamed, vulnerable plaques, and M2 macrophages are considered to be with a thick fibrous cap and less with risk of rupture.
In a study of aortas of Ldlr−/− mice, Kadl et al. found that 40% of cluster of differentiation (CD)68+ macrophages showed an M1 phenotype and 25% an M2 phenotype, where M1 was identified by expression of CD86 and M2 by expression of mannose receptor (CD206). In human atherosclerotic lesions, macrophages with both M1 (expressing iNOS, HLA, CD86, and MARCO) and M2 (expressing mannose receptor, CD163, and dectin-1) characteristics have been found, often side by side in the same histological section. M1 macrophages dominated in the rupture-prone shoulder regions of the plaque, M1 and M2 macrophages were equally represented in the fibrous caps, and M2 macrophages dominated in adventitial tissue. Mice lacking the nuclear receptor Nr4a1 showed enhanced atherosclerotic lesions in spite of decreased numbers of circulating monocytes. This was associated with increased expression of the M1 marker IL-12p70 and decreased expression of the M2 marker arginase-1 in peritoneal macrophages and increased mRNA expression for TNF, CD36, and SRA-1 in F4/80+ macrophages sorted from aortae of Apoe−/− Nr4a1−/− mice. Tissue macrophage polarization is a multifactorial cascade, which may be driven by infective agents or endogenously formed TLR2 and TLR4 ligands, NOD-like receptor activation of the NLRP3 inflammasome, and bacterial LPS, expressing CCR7, γ interferon, and T helper cytokines. In addition, the hematopoietic growth factor CSF2 (GM-CSF) promotes M1-like macrophages, and CSF1 (M-CSF) promotes M2-like macrophages in atherosclerosis.

Specific serum or plasma proteins may act as biomarkers for macrophage activation. CD14, a GPI-linked coreceptor for lipopolysaccharide (LPS) expressed by both monocytes and macrophages, exists in a soluble form that can be detected in human plasma. M1 macrophages, which can be produced in the presence of interferon-γ and bacterial LPS, express CCR7, CD86, and MHCIIN and secrete IL-12p70. M2 macrophages, which can be activated in vitro through several pathways including addition of IL-4, express many lectin-like cell surface receptors, among them the hemoglobin-haptoglobin receptor CD163. CD163 is shed from the cell surface by a proteolytic mechanism involving ADAM-17, and this shed form is soluble and seems in the serum at detectable concentrations.

Because IL-12p70 is usually undetectable in plasma, we sought to find an alternative marker protein for M1 macrophages. Galectin-3–binding protein (Gal-3BP), a secreted 585-amino acid protein and member of the MSR cysteine-rich domain superfamily, is easily detected in plasma; it is a well-established plasma marker of viral infection and cancer; however, its potential role in CVD has not been investigated. Correlating plasma Gal-3BP and CVD can be relevant in light of the pathogen burden hypothesis, which states that cumulative infection burden contributes to atherogenesis.

In the present study, we found that in vitro Gal-3BP is produced by M1, but not M2 or M0 (unpolarized) human macrophages. Then, we tested whether plasma markers of macrophage abundance and activation (soluble cluster of differentiation 163 [sCD163], soluble cluster of differentiation 14 [sCD14], and Gal-3BP) correlated with subclinical carotid atherosclerosis, using epidemiological data from a subset of women participating in the Women’s Interagency HIV Study (WIHS). Previous WIHS studies have established a relationship among known inflammatory markers, human immunodeficiency virus (HIV) infection, antiretroviral therapy, and cardiovascular risk factors with CVD. We selected 4 groups of women with HIV and hepatitis C virus (HCV) infection. Because both HIV and HCV infection have been associated with premature atherosclerosis, we hypothesized they would be also associated with macrophage activation as measured by sCD163, sCD14, and Gal3BP.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**In Vitro Study**

**Expression of Gal-3BP, CD14, and CD163 in M1 and M2 Macrophages**

Human monocytes were isolated from blood of healthy volunteers and differentiated to macrophages (M0) in M-CSF using standard methods under serum-free conditions. These primary macrophages secreted IL-12p70, but not IL-10, into the cell culture supernatant when differentiated to M1 by incubation with LPS and IFN-γ for 24 hours (Figure 1A). M2 macrophages polarized by LPS and IL-4 for 24 hours expressed ample IL-10, but no IL-12 (p70; Figure 1B). LGALS3BP (human gene encoding Gal-3BP) mRNA (Figure 1B) was expressed in M1, but not M2 macrophages, and Gal-3BP protein was secreted by M1, but not M2 macrophages (Figure 1C). The secreted material migrated at 90 kDa as expected and disappeared after knocking down LGALS3BP by antisense (Figure 1D).

As expected, CD14 was expressed on the surface of M0 and M1 macrophages and was reduced on M2 differentiation (Figure 1E). Conversely, CD163 was expressed by M0 and M2 macrophages and disappeared on M1 polarization (Figure 1E).

To confirm the relevance of these in vitro findings to human disease, we analyzed human postmortem coronary arteries from patients with coronary artery disease by immunofluorescence. We found expression of Gal-3BP in CD68+ plaque macrophages (Figure 1F).

**Population-Based Study**

**Study Population and Inflammatory Macrophage Markers**

We measured sCD14, sCD163, and Gal-3BP in plasma obtained from 264 women in the WIHS. Participants were
selected based on their HIV and HCV status and stratified into 4 groups, each containing 66 women: HIV+/HCV+, HIV+/HCV−, HIV−/HCV+, and HIV−/HCV (Table 1). The median age was 45 (interquartile range: 41, 50); 62% of the study population were black, 26% were Hispanic, and 12% were white or of other race. At the time of assessment, 76% were current smokers, with the remaining 24% either past or never smokers. Mean body mass index (BMI) was 29.3 (SD 7.4), with HIV+ individuals on average having a lower BMI (mean 27.6 versus 31.0, \( P=0.02 \)). Among HIV+ women, 77% were on

**Figure 1.** Galectin-3–binding protein (Gal-3BP), soluble cluster of differentiation 14 (CD14), and soluble cluster of differentiation 163 (CD163) expression in human macrophages. Human blood monocytes (all subsets) were incubated with M-CSF (100 ng/mL) for 6 days to produce monocyte-derived macrophages (M0). These macrophages were incubated with interferon-γ for 1 day to produce M1 or with IL-4 to produce M2 macrophages and then challenged with lipopolysaccharide (10 ng/mL). A, Levels of IL-12p70 and IL-10 in the conditioned media were measured by ELISA. B, Gal-3BP mRNA expression was measured by quantitative RT-PCR, expressed relative to H18S. **\( P=0.0056 \) by paired \( t \) test, \( n=3 \) donors. C, Gal-3BP protein production was measured using ELISA (\( P=0.0008 \)). D, M1 polarized macrophages were treated with cell-permeable antisense (ASO) against LGALS3BP or control sequence (CTL) for 48 hour, conditioned media harvested for immunoblotting with anti–Gal-3BP and anti-ERK2 (loading control) antibodies. E, CD14 and CD163 determined by flow cytometry in M0, M1, and M2 polarized macrophages (representative of 3 healthy blood donors). F, Human postmortem coronary arteries from patients with coronary artery disease were stained with antibodies against Gal-3BP (FITC, green), CD68 (Texas red), and nuclear stain (DAPI, blue) to assess expression of Gal-3BP in plaque macrophages. Bottom shows merge of Gal-3BP and CD68; right-hand side images magnify boxed areas. Top: overview (scale bar, 500 μm), other scale bars, 100 μm.
antiretroviral therapy at the time of their visit. Among HCV+ women, only 2% were currently on interferon treatment, with an additional 8% reporting past interferon use.

Table 1 shows the mean values of Gal-3BP, sCD163, and sCD14 levels in the study population in each of the 4 disease strata. Gal-3BP levels were significantly higher among HCV+ women than those among HCV− women (mean 12.4 μg/mL versus 8.6 μg/mL, \( P < 0.01 \)) but did not differ based on HIV status (mean 10.3 μg/mL versus 10.7 μg/mL, \( P =0.34 \)). Dually infected individuals had the highest mean levels of sCD163 and sCD14, whereas dually uninfected individuals had the lowest levels. Women with either HIV infection only or HCV infection only had intermediate levels of sCD163 and sCD14. Correlations among the 3 macrophage markers were moderate and all positive, suggesting that the biological parameters measured by these biomarkers are partially overlapping (Figure 2).

Gal-3BP levels were not significantly associated with traditional CVD risk factors including age, BMI, race/ethnicity, or smoking status (data not shown). Regarding other CVD risk factors, both sCD163 and sCD14 levels were higher among older women, and sCD163 levels increased with BMI. Inflammatory macrophage biomarker levels were not highly correlated with levels of hsCRP (Figure I in the online-only Data Supplement). Among HIV+ women, CD4+ count was moderately inversely correlated with inflammatory macrophage biomarkers (Figure II in the online-only Data Supplement).

Population-Based Study

**Associations With Subclinical Carotid Artery Disease**

Subclinical carotid artery disease was common in the study population, with 14% of women having ≥1 carotid artery lesion. Mean carotid distensibility was 17.1×10⁻⁶ m²/N (SD 8.5), and the mean carotid artery intima-media thickness (cIMT) was 0.763 mm (SD 0.135).

To reflect the overall levels of macrophage-associated inflammation within an individual, we created a macrophage score that was defined as the number of biomarkers that were found to have levels above the population-wide median (range 0–3). In statistical models accounting for age, race/ethnicity, current smoking status, BMI, hsCRP, HIV, and HCV, higher macrophage score modeled as a continuous variable was significantly associated with both increased odds of lesion (odds ratio, 1.58 per elevated marker; 95% confidence intervals [1.15–2.18]) and lower distensibility (\( \beta \), −1.44 U per marker, 95% confidence intervals [−2.59 to −0.29]; Table 2). Having ≥1 elevated marker was associated with greater subclinical carotid artery disease, with the maximum number (ie, 3) associated with the highest levels of disease.

Additional analyses examined individual markers for associations with carotid outcomes. Gal-3BP, sCD163, and sCD14 levels were each significantly associated with an increase in the odds of a carotid artery lesion (Table 2). We found that models that contained the 3 markers individually each increased
Higher levels of the 3 markers were correlated with reduced distensibility in bivariate analyses (Figure III in the online-only Data Supplement), but only Gal3BP and sCD14 were significantly associated with distensibility after covariate adjustment (Table 2).

In stratified analyses by HIV and HCV infection status, we found an association between increasing macrophage score and the presence of carotid artery lesions among HIV- and HCV-infected participants, with the effect most pronounced and approaching statistical significance among dually-infected individuals (odds ratio, 2.81; 95% confidence intervals [0.96–8.23]; Table 2). In analyses of individual markers, none of the associations with carotid artery measurements differed significantly across subgroups defined by HIV and HCV after covariate adjustment, despite suggestions of bivariate correlations (eg, Figures IV and V in the online-only Data Supplement). We observed a trend suggesting stronger associations of subclinical carotid artery disease with sCD163 in HIV-infected as compared with HIV-uninfected groups (pinteraction=0.09, Table I in the online-only Data Supplement). Findings did not differ appreciably after taking into account CD4+ count or current antiretroviral therapy use among HIV-infected women, or interferon therapy use among HCV-infected women (data not shown). In general, neither the macrophage biomarker score nor the 3 individual macrophage biomarkers were associated with levels of cIMT, either overall or within subgroups defined by HIV and HCV status (Tables I and II in the online-only Data Supplement).

The prediction of having a carotid artery lesion (C-statistic 0.735–0.743), compared with a model without these markers (C-statistic 0.714). Including all 3 markers simultaneously, either using the macrophage score or as individual covariates, further increased the predictive value (C-statistic 0.746 and 0.762, respectively).

In discussion, we found plasma markers of macrophage abundance, polarization, and activation (Gal-3BP, sCD163, and sCD14) to be associated with 2 measures of subclinical carotid artery disease. Specifically, we found that higher macrophage scores, defined by elevations in multiple macrophage biomarkers, were significantly associated with both increased odds of carotid artery lesions and reduced carotid artery distensibility (inverse of stiffness). Additional analyses suggested that associations of the macrophage markers with carotid artery parameters may have been augmented in HIV- and HCV-infected women, as compared with women free of HIV and HCV. These associations remained after taking into account CD4+ count or adjusting for hsCRP, a known inflammation marker of CVD. We did not observe correlations between macrophage biomarkers and hsCRP levels. Thus, our data suggest that circulating macrophage biomarkers may reflect the inflammatory process contributing to atherosclerosis, above and beyond hsCRP, and therefore may provide additional predictive and diagnostic ability in the future with respect to subclinical CVD. We also found each of the macrophage markers to be more elevated in HIV-infected women with lower CD4+ counts, particularly sCD163 and sCD14, which is consistent with ongoing inflammation and immune activation in these women.

We identified Gal-3BP as a measurable and soluble M1 macrophage marker in our in vitro study, and then found that Gal-3BP was consistently upregulated and positively correlated with subclinical carotid artery disease markers in a clinical study after adjustment for potential confounders. Accumulating data suggest that some plaque macrophages express M1 markers and others M2 markers; although contribution to plaque instability and inflammation is mostly attributed to M1 markers. Recent studies have given rise to interest...
Table 2. Association Between Inflammatory Macrophage Markers and Measures of Subclinical Carotid Artery Disease, Women’s Interagency HIV Study

<table>
<thead>
<tr>
<th>Inflammatory macrophage score, per each elevated marker (range: 0–3)</th>
<th>Any Carotid Artery Lesion</th>
<th>Distensibility (10^−6×N−1×m²)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Odds Ratio</td>
<td>95% CI</td>
</tr>
<tr>
<td>Among entire study population (n=256)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Modeled as continuous</td>
<td>1.58*</td>
<td>1.15, 2.18*</td>
</tr>
<tr>
<td>Modeled as categorical</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>0 (reference group)</td>
<td>4.18</td>
<td>0.84, 20.70</td>
</tr>
<tr>
<td>1</td>
<td>4.97</td>
<td>0.78, 31.64</td>
</tr>
<tr>
<td>2</td>
<td>6.87*</td>
<td>1.40, 33.71*</td>
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<tr>
<td>Stratified analyses</td>
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</tr>
<tr>
<td>In HIV−/HCV− (n=65)</td>
<td>1.18</td>
<td>0.35, 3.98</td>
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<tr>
<td>In HIV−/HCV+ (n=63)</td>
<td>1.24</td>
<td>0.48, 3.21</td>
</tr>
<tr>
<td>In HIV+/HCV− (n=64)</td>
<td>1.58</td>
<td>0.77, 3.24</td>
</tr>
<tr>
<td>In HIV+/HCV+ (n=64)</td>
<td>2.81</td>
<td>0.96, 8.23</td>
</tr>
<tr>
<td>Individual inflammatory macrophage markers, per each standard deviation</td>
<td></td>
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<tr>
<td>Among entire study population (n=256)</td>
<td></td>
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<tr>
<td>Gal-3BP</td>
<td>1.48*</td>
<td>1.01, 2.15*</td>
</tr>
<tr>
<td>sCD163</td>
<td>1.85*</td>
<td>1.21, 2.83*</td>
</tr>
<tr>
<td>sCD14</td>
<td>1.49*</td>
<td>1.04, 2.12*</td>
</tr>
</tbody>
</table>

All analyses adjusted for age, race/ethnicity, smoking status, body mass index, high-sensitivity C-reactive protein, and except for stratified analyses, human immunodeficiency virus (HIV) and hepatitis C virus (HCV) infection. CI indicates confidence intervals; m, meters; N, Newtons; sCD14, soluble cluster of differentiation 14; and sCD163, soluble cluster of differentiation 163.

*Significant at P<0.05.

in galectins as potential biomarkers of CVD. In previous work, higher plasma levels of galectin-3 have been associated with increased heart failure risk and higher mortality in those with heart failure. Human Gal-3BP binds specifically to human galectin-3, which is interestingly an M2 marker. To our knowledge, ours is the first study to examine associations between Gal-3BP and measures of subclinical CVD. Plasma Gal-3BP levels were increased in HCV-positive individuals, but did not differ by HIV infection status. The finding relating to HCV is consistent with previous data. The lack of association between HIV and Gal-3BP levels differs from other studies. Underlying differences in study populations, calendar time effects, or availability of newer drugs in our study could have contributed to differences between studies.

Our study also provides further support of a role for sCD163 in CVD, as has been suggested by several recent studies. Although our study focused on women, a cross-sectional analysis of 102 HIV-infected men with undetectable viremia and 41 HIV-uninfected men without a history of CAD found sCD163 to be significantly associated with presence of noncalcified atherosclerotic plaque, even after adjusting for HIV, traditional CVD risk factors, and age and race. Another recent cross-sectional study among 81 HIV-infected individuals using positron emission tomography found sCD163 to be significantly associated with arterial inflammation in the aorta, after adjusting for traditional CV risk factors. However, other small cross-sectional or case–control studies in individuals with HIV have not found associations between sCD163 and plaque or myocardial infarction, and therefore larger, prospective studies may help to further clarify the role of these markers in CVD pathogenesis. Although CD163 is expressed on M2 macrophages, sCD163 may not reflect this, because ADAM-17, the enzyme cleaving CD163 to become soluble, is expressed by M1 macrophages. Higher sCD14 levels have been associated with all-cause mortality in studies of both the general population, as well as in those with HIV. Here, we found that sCD14 level was associated with increased carotid artery lesions and decreased carotid artery distensibility after adjusting for HIV and HCV infection, corroborating other findings in the general population. Another HIV study showed an association between sCD14 levels and yearly rate of change in cIMT. At least one other HIV study failed to find an association between sCD14 and cIMT or lesions, although the authors of that study acknowledged limited power (n=60).

Our study is a first step in understanding the pathophysiology and potential role as risk predictors of these promising new biomarkers in our HIV/HCV population. However, it has limitations. Because the current study is cross-sectional, both macrophage markers and carotid artery outcomes were assessed at the same visit. Therefore, we cannot infer temporality or causality in our study, and thus longitudinal analyses will be important to further elucidate the roles of these markers in predicting the development of CVD, both with and
without the presence of chronic viral infection. Indeed, before these markers can be considered for use as risk predictors in clinical practice, they will need to be validated in other populations as well as in the prediction of major adverse cardiac events. Second, this study was limited to women. Because it is well recognized that the ability to accurately predict CVD risk differs in men compared with women,\(^\text{26}\) it would be important to replicate our findings to assess the role of these markers in men. Finally, we were limited by a relatively small sample size. However, our study is in fact larger than several other studies examining similar subclinical CVD outcomes in the HIV population. Furthermore, because of the excellent characterization of WHIS participants since 1993, we were able to include a substantial number of exceptionally well-matched women both with and without chronic viral infection, suggesting their potential for predictive or diagnostic value with respect to atherosclerosis. The association of plasma macrophage biomarkers with CVD may provide new insights pointing to possible new strategies for prevention or treatment of CVD in patients with and without HIV and HCV.

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Disclosures

None.

References


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METHODS

In vitro study

For generation of human blood-derived macrophages, human PBMCs were isolated from freshly heparinized blood of 3 healthy donors by density gradient centrifugation with Histopaque®-1077 (Sigma, USA). Human monocytes were isolated by negative selection using the EasySep® Human Monocyte Enrichment Kit (Stemcell Technologies, Vancouver, Canada). Cells were cultured for seven days in Macrophage-SFM serum free media (SFM) (Gibco) supplemented with Nutridoma-SP (Roche, USA), 100 units/ml of penicillin, 100 µg/ml streptomycin and 100 ng/ml human M-CSF (PeproTech, Rocky Hill, NJ, USA), in plastic tissue culture dishes (Corning Inc.). The culture media was replaced with fresh media every 3 days. Cells were then treated with 2.5 µM morpholino antisense oligonucleotides (ASO) targeting GALS3BP or Control ASO (Vivo-Morpholino standard control oligo: CCTCTTACCTCAGTTACAATTTATA) (GenTools.LLC, USA) for 2 days followed by stimulation with 50 units/ml interferon-γ (PeproTech, Rocky Hill, NJ) 1 day to produce M1 or with 20 units/ml IL-4 (PeproTech, Rocky Hill, NJ) to produce M2 macrophages and then challenged with Lipopolysaccharide (LPS, from Escherichia coli 0111:B4, Sigma, USA) (10 ng/ml) for 12 hours. Level of IL-12p70 and IL-10 as well as Gal-3BP in the conditioned media were measured by ELISA (eBioscience, San Diego, CA) according to manufacturers’ recommendations.

Immunohistochemistry

Immunostaining of PPFE post mortem coronary arteries was performed as described previously 1. Human coronary arteries were obtained from the University of
Virginia Department of Pathology/Tissue bank, Charlottesville, VA. Antibodies and staining reagents used for immunohistochemistry were anti-CD68 (rabbit IgG polyclonal, Santa Cruz, Santa Cruz, CA); anti-rabbit (goat IgG, polyclonal Texas red, Santa Cruz, Santa Cruz, CA); anti-Gal-3BP (90K) (mouse IgG1, clone SP-2, FITC, eBioscience, San Diego, CA); and DAPI Milipore, Billerica, MA

Population-based study

Source population: The WIHS is a longitudinal study that enrolled over 4,000 HIV-infected and -uninfected women from HIV primary care clinics, hospital-based programs, community outreach sites, women’s support groups, and other locations at six U.S. sites during 3 recruitment waves (1994-1995, 2001-2002, and 2010-2012). Women are followed at 6-month intervals, with detailed examinations, specimen collection (blood collection as in Bacon et al. 2), and structured interviews assessing health behaviors, medical history, and medication use 3. In contrast to clinic-based cohorts that collect data through routine care, the WIHS is an interval-based cohort, meaning that visits occur independently of clinical care. The demographic composition of study participants in the WIHS is representative of the U.S. female HIV-infected population 4.

Cardiovascular assessment and study design: During 2004-2005, we obtained B-mode ultrasound carotid artery images from 1,827 participants initially enrolled in the WIHS during the first 2 waves of recruitment (1994-1995 and 2001-2002). Standardized images were centrally measured by automated computerized edge detection software 5. From this source population of 1,827 participants, we used a 2x2 factorial design based on the prevalence of HIV infection and HCV infection to create 4 strata containing 66 women each: HIV+/HCV+, HIV+/HCV-, HIV-/HCV+, and HIV-/HCV-. HCV infection was defined on the basis of a detectable hepatitis C viral load at study entry. Briefly, we selected all eligible HIV-/HCV+ participants from the source population, and for each
selected HIV-/HCV+ participant we randomly selected (without replacement) a participant in each of the other three strata (i.e., HIV-/HCV-, HIV+/HCV+, HIV+/HCV-) who matched by age (within 5 years), race/ethnicity, and smoking status (current versus past or never). Matching was done to account by design for potential confounding by these characteristics.

Subclinical carotid artery disease outcomes examined in this study included right distal common carotid artery (CCA) intima-media thickness (cIMT), carotid arterial distensibility, and presence of atherosclerotic lesions (yes versus no). Distensibility is a direct measure of carotid arterial stiffness and was quantified as a function of the right CCA diameter at systole and at diastole, and pulse pressure at the brachial artery. Distensibility was standardized to the units reported by Lage, et al. (10^6 * Newtons^-1 * meters^2), with lower values reflecting a stiffer carotid artery. Atherosclerotic lesions were assessed at the near and far walls of the right CCA, right carotid artery bifurcation, and right internal carotid artery, and a lesion was defined as the presence of focal intimal-media thickening of 1.5 mm or greater.

**Laboratory values:** Blood samples were drawn, processed and stored as described. Enzyme-linked immunosorbent assay methods were used to measure Gal-3BP (MAC-2BP; eBioscience, dynamic range 0.2-2.3 OD at 450nm, which corresponds to 12.5-200 ng/ml protein concentration, samples diluted 1:100-1:200), soluble (s) CD163 (Macro 163; Trillium Diagnostics, dynamic range 0.6-32 ng/ml, samples diluted 1:500), and sCD14 (DC140; R&D Systems, dynamic range 16-250 ng/ml, samples diluted 1:200-1:500) according to manufacturers’ recommendations. Among other laboratory measures, HIV infection was determined via serologic testing using ELISA and confirmed using Western blot assays, HIV viral load was assessed by the NucliSens HIV-1 assay (1 mL input), bioMérieux (Durham, NC). HCV infection was assessed as
detectable plasma HCV RNA by the COBAS Amplicor Monitor 2.0 assay (Roche Diagnostics, USA) or the COBAS Taqman assay (Roche Diagnostics, USA)\textsuperscript{10}. High-sensitivity C-reactive protein (hsCRP) levels were measured using a nephelometric immunoassay (Dade-Behring BN II)\textsuperscript{11}.

We examined correlations among the three macrophage biomarkers and with other characteristics of the study population, after log-transformation as appropriate. To reflect the overall levels of macrophage-associated inflammation within an individual, we created a macrophage score which was defined as the number of biomarkers that were found to have levels above the population-wide median (range 0-3), and analyzed it both as a continuous variable and categorically in order to explore non-linear trends.

**Epidemiologic methods:** We assessed the cross-sectional association between inflammatory macrophage markers (macrophage score) and subclinical carotid artery disease outcomes. We performed linear and logistic regression analyses using generalized estimating equations to take into account the matched design, scaling each of the biomarkers by z-score transformation to be able to report results based on a common unit. We ran successive regression models controlling for HIV infection, HCV infection, both HIV and HCV infection, and potential confounders including body-mass index (BMI) and hsCRP. hsCRP was missing for 8 participants, who were dropped from adjusted models that included this variable. We additionally adjusted for age, race/ethnicity, and smoking status in these analyses. To assess the impact on subclinical carotid artery disease, we used C-statistics to examine the additional predictive value of the macrophage markers individually and collectively. We performed stratified analyses in each of the four strata (HIV+/HCV+, HIV+/HCV-, HIV-/HCV+, and HIV-/HCV-) to examine potential effect modification of the biomarker-subclinical CAD associations by HIV and HCV infection. SAS 9.3 and R 3.0.2 were used for all analyses.
References:


**Supplemental Table I. Association between individual inflammatory macrophage markers and measures of subclinical carotid artery disease, by HIV and HCV status, Women’s Interagency HIV Study.**

<table>
<thead>
<tr>
<th></th>
<th>Any carotid artery lesion</th>
<th>Distensibility ($10^{-6} \times N^{-1} \times m^2$)</th>
<th>cIMT (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Odds ratio</td>
<td>95% CI</td>
</tr>
<tr>
<td>Gal-3BP (per SD)</td>
<td>HIV- /HCV-</td>
<td>65</td>
<td>1.60</td>
</tr>
<tr>
<td></td>
<td>HIV- /HCV+</td>
<td>63</td>
<td>2.07</td>
</tr>
<tr>
<td></td>
<td>HIV+ /HCV-</td>
<td>64</td>
<td>1.48</td>
</tr>
<tr>
<td></td>
<td>HIV+ /HCV+</td>
<td>64</td>
<td>1.72</td>
</tr>
<tr>
<td>sCD163 (per SD)</td>
<td>HIV- /HCV-</td>
<td>65</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>HIV- /HCV+</td>
<td>63</td>
<td>1.29</td>
</tr>
<tr>
<td></td>
<td>HIV+ /HCV-</td>
<td>64</td>
<td>4.12</td>
</tr>
<tr>
<td></td>
<td>HIV+ /HCV+</td>
<td>64</td>
<td>2.68</td>
</tr>
<tr>
<td>sCD14 (per SD)</td>
<td>HIV- /HCV-</td>
<td>65</td>
<td>1.89</td>
</tr>
<tr>
<td></td>
<td>HIV- /HCV+</td>
<td>63</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>HIV+ /HCV-</td>
<td>64</td>
<td>1.37</td>
</tr>
<tr>
<td></td>
<td>HIV+ /HCV+</td>
<td>64</td>
<td>1.78</td>
</tr>
</tbody>
</table>

cIMT = right distal common carotid artery intima-medial thickness, CI = confidence interval, HCV = hepatitis C virus, HIV = human immunodeficiency virus, m = meters, mm = millimeters, N = Newtons, SD = standard deviation.

All analyses adjusted for age, race/ethnicity, smoking status, body mass index, high-sensitivity C-reactive protein, and HIV and HCV infection. Bold indicates significant at p<0.05.
Supplemental Table II. Association between inflammatory macrophage markers and right distal common carotid artery intima-media thickness, Women’s Interagency HIV Study.

<table>
<thead>
<tr>
<th>Inflammatory macrophage score, per each elevated marker (range: 0-3)</th>
<th>β</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among entire study population (N=256)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Modeled as continuous</td>
<td>-0.009</td>
<td>-0.03, 0.01</td>
<td>0.34</td>
</tr>
<tr>
<td>Modeled as categorical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (reference group)</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-0.03</td>
<td>-0.07, 0.01</td>
<td>0.11</td>
</tr>
<tr>
<td>2</td>
<td>-0.03</td>
<td>-0.07, 0.02</td>
<td>0.21</td>
</tr>
<tr>
<td>3</td>
<td>-0.03</td>
<td>-0.09, 0.02</td>
<td>0.25</td>
</tr>
<tr>
<td>Stratified analyses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Modeled as continuous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In HIV-/HCV- (N=65)</td>
<td>0.01</td>
<td>-0.01, 0.04</td>
<td>0.30</td>
</tr>
<tr>
<td>In HIV-/HCV+ (N=63)</td>
<td>-0.04</td>
<td>-0.08, 0.01</td>
<td>0.11</td>
</tr>
<tr>
<td>In HIV+/HCV- (N=64)</td>
<td><strong>-0.03</strong></td>
<td><strong>-0.06, -0.001</strong></td>
<td><strong>0.04</strong></td>
</tr>
<tr>
<td>In HIV+/HCV+ (N=64)</td>
<td>-0.005</td>
<td>-0.03, 0.03</td>
<td>0.75</td>
</tr>
<tr>
<td>Individual inflammatory macrophage markers, per each standard deviation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among entire study population (N=256)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gal-3BP</td>
<td>-0.01</td>
<td>-0.03, 0.01</td>
<td>0.29</td>
</tr>
<tr>
<td>sCD163</td>
<td>-0.02</td>
<td>-0.04, 0.01</td>
<td>0.15</td>
</tr>
<tr>
<td>sCD14</td>
<td>0.004</td>
<td>-0.02, 0.03</td>
<td>0.79</td>
</tr>
</tbody>
</table>

cIMT = right distal common carotid artery intima-media thickness, CI = confidence interval, HCV = hepatitis C virus, HIV = human immunodeficiency virus, mm = millimeters.
All analyses adjusted for age, race/ethnicity, smoking status, body mass index, high-sensitivity C-reactive protein, and except for stratified analyses, HIV and HCV infection. Bold indicates significant at p<0.05.
Supplemental Figure I. Spearman correlation, comparing high sensitivity C-reactive protein (hsCRP) levels to (A) Gal-3BP, (B) sCD163, and (C) sCD14, across four HIV/HCV strata.

hsCRP log-transformed after adding one to original value. Line represents regression line fit to observed data.
Supplemental Figure II. Spearman correlation, comparing CD4+ count to (A) Gal-3BP, (B) sCD163, and (C) sCD14, among HIV+ women, stratified by HCV status.

Line represents regression line fit to observed data.
Supplemental Figure III. Spearman correlations comparing distensibility index with (A) Gal-3BP, (B) sCD14, and (C) sCD163, across four HIV/HCV strata.

Line represents regression fit to observed data.
Supplemental Figure IV. Spearman correlations between Gal-3BP and distensibility index, by HIV/HCV strata.
Supplemental Figure V. Spearman correlations between sCD14 and distensibility index, by HIV/HCV strata.