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 [5-7]. 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 [8]. Distensibility was standardized to the units reported by Lage, et al. (10^{-6} * $Newtons^{-1}$ * $meters^{2}$),[9] 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 [2]. 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) [10]. High-sensitivity C-reactive protein (hsCRP) levels were measured using a nephelometric immunoassay (Dade-Behring BN II) [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:

1. Gleissner CA, Sanders JM, Nadler J, Ley K. Upregulation of aldose reductase during foam cell formation as possible link among diabetes, hyperlipidemia, and atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2008;28:1137-1143
2. Bacon MC, von Wyl V, Alden C, Sharp G, Robison E, Hessol N, Gange S, Barranday Y, Holman S, Weber K, Young MA. The Women's Interagency HIV Study: an observational cohort brings clinical sciences to the bench. *Clin Diagn Lab Immunol*. 2005;12:1013-1019
3. Barkan SE, Melnick SL, Preston-Martin S, Weber K, Kalish LA, Miotti P, Young M, Greenblatt R, Sacks H, Feldman J. The Women's Interagency HIV Study. WIHS Collaborative Study Group. *Epidemiology*. 1998;9:117-125
4. Centers for Disease Control and Prevention. HIV among women. http://www.cdc.gov/hiv/topics/women/index.html. Accessed February 21, 2014.
5. Selzer RH, Hodis HN, Kwong-Fu H, Mack WJ, Lee PL, Liu CR, Liu CH. Evaluation of computerized edge tracking for quantifying intima-media thickness of the common carotid artery from B-mode ultrasound images. *Atherosclerosis*. 1994;111:1-11
6. Hodis HN, Mack WJ, Lobo RA, Shoupe D, Sevanian A, Mahrer PR, Selzer RH, Liu Cr CR, Liu Ch CH, Azen SP. Estrogen in the prevention of atherosclerosis. A randomized, double-blind, placebo-controlled trial. *Ann Intern Med*. 2001;135:939-953
7. Selzer RH, Mack WJ, Lee PL, Kwong-Fu H, Hodis HN. Improved common carotid elasticity and intima-media thickness measurements from computer analysis of sequential ultrasound frames. *Atherosclerosis*. 2001;154:185-193
8. Seaberg EC, Benning L, Sharrett AR, Lazar JM, Hodis HN, Mack WJ, Siedner MJ, Phair JP, Kingsley LA, Kaplan RC. Association between human immunodeficiency virus infection and stiffness of the common carotid artery. *Stroke*. 2010;41:2163-2170
9. Lage SG, Kopel L, Monachini MC, Medeiros CJ, Pileggi F, Polak JF, Creager MA. Carotid arterial compliance in patients with congestive heart failure secondary to idiopathic dilated cardiomyopathy. *Am J Cardiol*. 1994;74:691-695
10. Al-Harthi L, Voris J, Du W, Wright D, Nowicki M, Frederick T, Landay A, Kovacs A. Evaluating the impact of hepatitis C virus (HCV) on highly active antiretroviral therapy-mediated immune responses in HCV/HIV-coinfected women: role of HCV on expression of primed/memory T cells. *J Infect Dis*. 2006;193:1202-1210
11. Palella FJ, Jr., Gange SJ, Benning L, Jacobson L, Kaplan RC, Landay AL, Tracy RP, Elion R. Inflammatory biomarkers and abacavir use in the Women's Interagency HIV Study and the Multicenter AIDS Cohort Study. *AIDS*. 2010;24:1657-1665