ONLINE SUPPLEMENTAL

Increased Circulating Inflammatory Endothelial Cells in African-Americans with Essential Hypertension

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Methods

This study was approved by the Institutional Review Board at the Mayo Clinic in adherence with the Declaration of Helsinki and the Health Insurance Portability and Accountability Act (HIPAA) guidelines. African American (n=19) and Caucasian (n=19) patients with a diagnosis of essential hypertension were enrolled in the study from January 2008 to January 2012. Patients with secondary hypertension were excluded. Hypertension was defined as systolic blood pressure (SBP) ≥140mmHg, or diastolic blood pressure (DBP) ≥90mmHg in supine position, after 20min of rest on 2 separate days. Hypertensive subjects were studied during 150mEq sodium intake during therapy with blockade of the renin-angiotensin system with angiotensin converting enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARBs) at standard recommended daily dose (equivalent: 40mg lisinopril). We administered a single dose of furosemide (20mg IV) to all hypertensive patients one day before renal vein sampling for other protocol studies[1]. The control group consisted of 19 age- and sex-matched healthy volunteers (HV, SBP<130 and DBP<80 mmHg), who were prospectively recruited through the Mayo Clinic Biobank.

Exclusion criteria included uncontrolled hypertension (SBP >180 mmHg, despite antihypertensive therapy), serum creatinine >1.7 mg/dL, diabetes requiring insulin or oral hypoglycemic medications, recent cardiovascular events (myocardial infarction, stroke, congestive heart failure within 6 months), pregnancy, and kidney transplant.

In all patients, clinical and laboratory parameters were collected via the electronic medical records. Clinical parameters included: age, sex, height, weight, body mass index (BMI), SBP, DBP, mean arterial pressure (MAP), and use of concomitant medication. Laboratory parameters included: total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL), triglyceride, C-reactive protein (CRP, R&D systems, Cat# DCRP00, serum creatinine, 24hr urinary protein concentration (Thermo Scientific, Cat#23236, Waltham, MA, USA), and estimated glomerular filtration rate (eGFR) calculated using the chronic kidney disease epidemiology collaboration (CKD-EPI) formula[2].

Inflammatory biomarkers and EPC homing factors

Peripheral blood (HV), renal-vein and inferior-vena-cava (EH and AAEH) samples were collected, centrifuged, and plasma aliquot stored at -80°C. For hypertensive patients, individual kidney (right and left) renal-vein levels were averaged. At the time of the assay, samples were centrifuged at 5000 rpm/5min and samples (25 µl) incubated overnight at 4°C. Renal-vein and circulating levels of soluble E-selectin (sE Selectin), soluble vascular cell adhesion molecule (sVCAM-1), soluble intercellular adhesion molecule (ICAM)-1, myeloperoxidase (MPO), plasminogen activator inhibitor (PAI)-1, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein-(MIP)-1δ, tumor necrosis factor (TNF)-α, interleukin (IL)-6, adiponectin, matrix metalloproteinase (MMP)-9, stromal cell-derived factor (SDF)-1 and stromal cell factor (SCF) were measured by Luminex (Millipore, cat No: MPXHCYTO-60K; MPXHCYP2-62K; HCVD-67AK; and HSCR-32K)[3, 4].

EPC and IEC

Mononuclear cells were isolated from fresh blood samples by the density-gradient method and subsequently characterized for antigen expression of endothelial progenitor markers CD34 (Beckton-Dickinson), KDR (Santa Cruz), and vascular adhesion protein (VAP-1) (Lifespan biosciences). Systemic and renal-vein levels of CD34+/KDR+ (EPC) and VAP-1+ (IEC) were determined by fluorescence-activated cell sorting (FACS, Becton Dickinson, Calibur), as previously described[3]. A total of 150,000 events/sample were counted[5] using CellQuest software (Becton Dickinson). EPC and IEC levels were determined within the lymphocyte gate by using sequential gating strategies, as previously described[6]. In order to eliminate bias secondary to variations attributed to body fluids and total blood cell counts[7], results were expressed as EPC or IEC % (per 100,000 cell counts). To further characterize IEC, VAP-1+ cells were stained with endothelial (CD31), lymphocyte (CD3 and CD45), monocyte/macrophage (CD16 and CD14) and progenitor cell (CD34 and CD133) markers and analyzed using FACS.

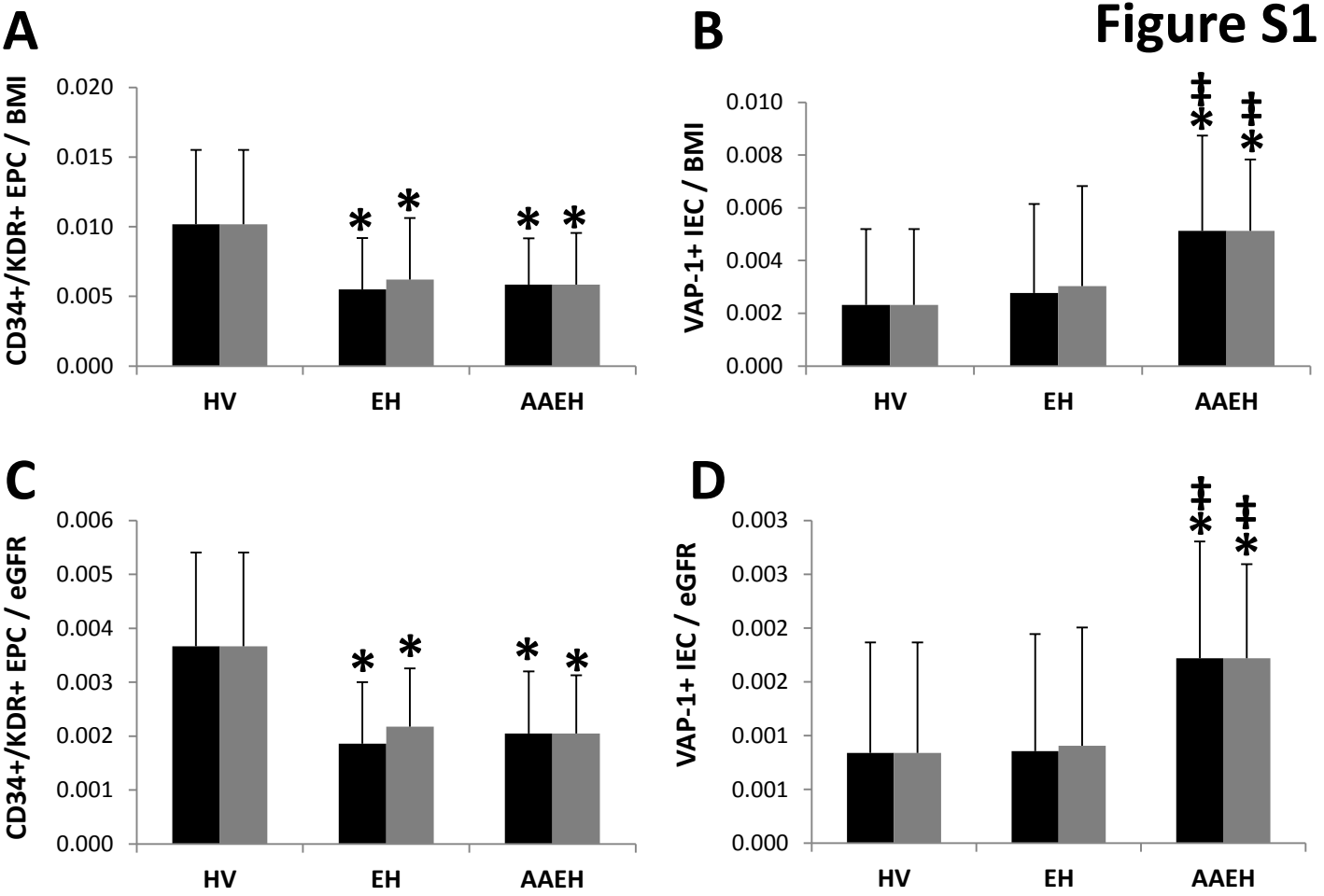
Statistical analysis

Statistical analysis was performed using JMP software package version 9.0.1 (SAS Institute Inc. Cary, NC). The sample size was estimated for detecting differences of 0.1% (per 100,000 cell counts) in VAP-1+ IEC between groups. By assuming an alpha risk of 0.05, a mean of 0.053%, and a standard deviation of 0.041%, at least 10 subjects per group were needed. We used the Shapiro-Wilk test to test for any deviation from normality. Normal distributed variables were expressed as mean±SD. Comparisons within groups were performed using the paired Student t-test/ANOVA or Wilcoxon/ Kruskal Wallis tests were used when appropriate. Regressions were calculated by the least-squares fit. Statistical significance for all tests was accepted for $p \leq 0.05$.

References

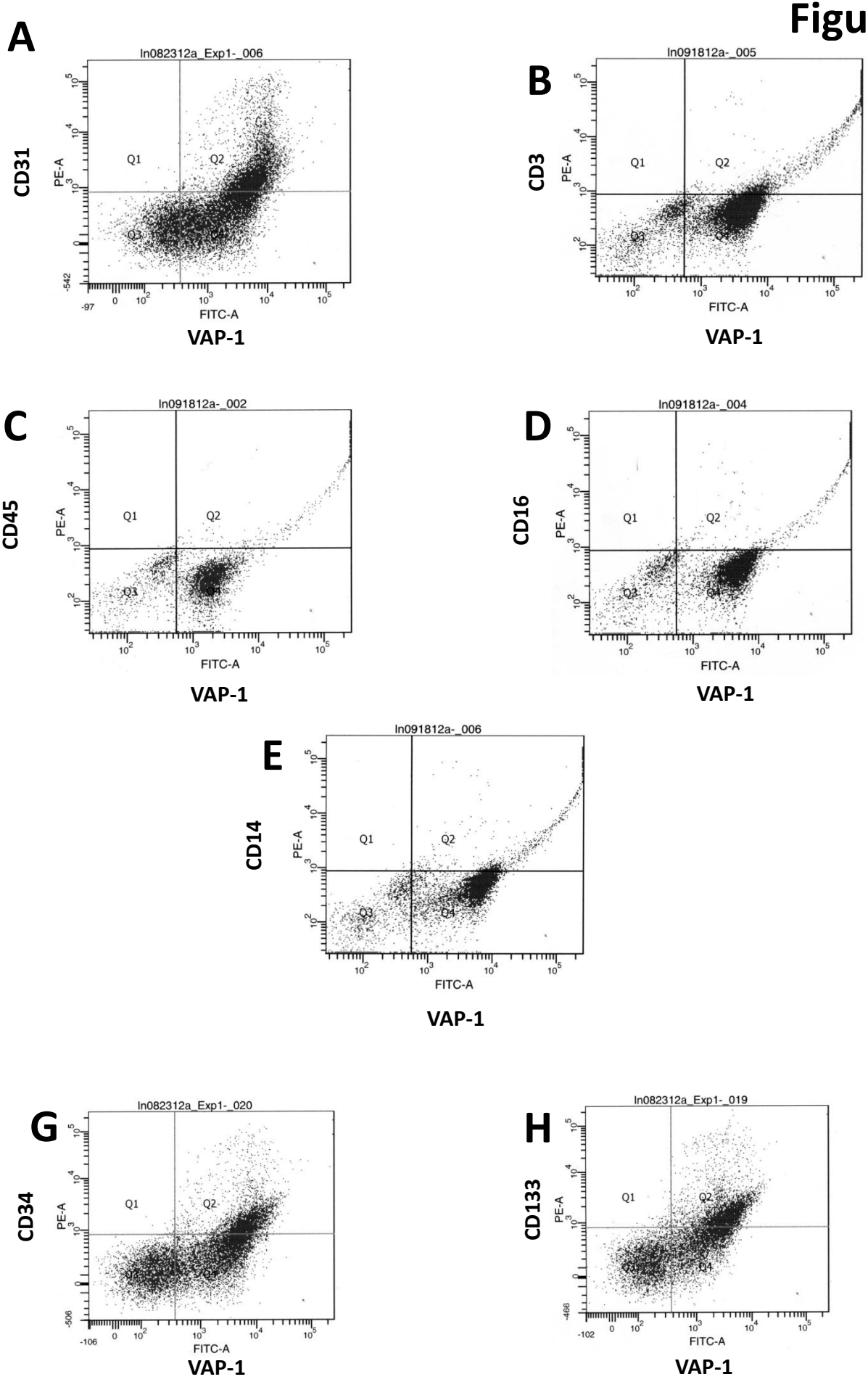
1. Gloviczki ML, Glockner JF, Crane JA, McKusick MA, Misra S, Grande JP, Lerman LO, Textor SC. Blood oxygen level-dependent magnetic resonance imaging identifies cortical hypoxia in severe renovascular disease. *Hypertension*. 2011;58:1066-1072.
2. Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, 3rd, Feldman HI, Kusek JW, Eggers P, Van Lente F, Greene T, Coresh J. A new equation to estimate glomerular filtration rate. *Ann Intern Med*. 2009;150:604-612.
3. Eirin A, Gloviczki ML, Tang H, Gossl M, Jordan KL, Woollard JR, Lerman A, Grande JP, Textor SC, Lerman LO. Inflammatory and injury signals released from the post-stenotic human kidney. *Eur Heart J*. 2013; 34: 540-548a.
4. Eirin A, Gloviczki ML, Tang H, Rule AD, Woollard JR, Lerman A, Textor SC, Lerman LO. Chronic renovascular hypertension is associated with elevated levels of neutrophil gelatinase-associated lipocalin. *Nephrol Dial Transplant*. 2012;27:4153-4161.
5. Chiang CH, Huang PH, Chung FP, Chen ZY, Leu HB, Huang CC, Wu TC, Chen JW, Lin SJ. Decreased circulating endothelial progenitor cell levels and function in patients with nonalcoholic fatty liver disease. *PLoS One*. 2012;7:e31799.
6. Boilson BA, Kiernan TJ, Harbuzariu A, Nelson RE, Lerman A, Simari RD. Circulating cd34+ cell subsets in patients with coronary endothelial dysfunction. *Nat Clin Pract Cardiovasc Med*. 2008;5:489-496.
7. Fadini GP, Baesso I, Albiero M, Sartore S, Agostini C, Avogaro A. Technical notes on endothelial progenitor cells: Ways to escape from the knowledge plateau. *Atherosclerosis*. 2008;197:496-503.

Figure S1



Quantification of circulating EPC and IEC levels adjusted by body mass index (BMI, A-B) and estimated glomerular filtration rate (eGFR, C-D) in healthy volunteers (HV), Caucasian essential hypertensive (EH) and African American essential hypertensive (AAEH) patients. *p≤0.05 vs. HV, ‡ p≤0.05 vs.EH.

Figure S2



Representative flow cytometric plots showing double staining for VAP-1+ IEC cells and CD31 (A), CD3 (B), CD45 (C), CD16 (D), CD14 (E), CD34 (G), and CD133 (H).