MATERIALS AND METHODS

Study Population

Participants (n=345, >18 years of age) with moderate to high CVD risk were recruited between October 2009 and May 2013 from the University of Louisville Hospital and affiliated clinic system. These studies were approved by the Institutional Review Board at the University of Louisville (IRB 09.0174 and 10.0350), and all individuals gave written informed consent. Persons unwilling or unable to provide informed consent or with significant and/or severe comorbidities were excluded. Exclusion criteria included: significant chronic lung, liver, kidney, or hematological disease; chronic neurological or psychiatric illness; chronic infectious diseases such as HIV or hepatitis; severe coagulopathies; drug/substance abuse; and chronic cachexia. Pregnant women, prisoners, and other vulnerable populations were also excluded from the studies. Patients who met the enrollment criteria were consented and administered a questionnaire which included demographic information; residential address; smoking status and history; secondhand smoke exposure; alcohol consumption; physical activity status; medication usage; and CVD history including heart attack, heart failure, angina, hypertension, hypercholesterolemia, diabetes, stroke, revascularization, arrhythmia, peripheral artery disease, aortic aneurism, and bleeding disorders. Medical records were reviewed to verify data obtained from subject interviews.

We recruited participants through advertisements in the University of Louisville daily staff email newsletter and flyers posted at University of Louisville Health Sciences Campus buildings. In addition, participants were recruited at the University of Louisville cardiology, preventive cardiology, endocrinology, and outpatient clinics. All accessible patients visiting the respective clinic on the day of recruitment (up to 1600 individuals total) were pre-screened through medical records review prior to recruitment. The patients were screened prior to recruitment to exclude recruitment of anyone that did not meet the enrollment criteria. Therefore, there was minimal selection bias. Due to IRB constraints, we cannot account for the individuals that were screened and did not enroll. In total, there was 1 individual that withdrew from the study after successful enrollment and was subsequently excluded from the analyses. This information has been updated in the Materials and Methods Section.

Biological Sample Collection and Processing

Blood and urine were obtained from each participant. Urine collected at the time of the visit was used to measure the levels of cotinine and creatinine. The blood was used for measuring circulating angiogenic cell populations, and high-sensitivity C-reactive protein (VITROS kit).

Circulating Angiogenic Cell Quantification

Specific circulating angiogenic cell levels in blood were characterized using a 7-color flow cytometry procedure with established cell surface markers indicative of endothelial and stem/progenitor cells: CD31⁺, CD34⁺, CD45+dim, and AC133⁺ as described before.¹,² A total of 15 circulating angiogenic cell populations were measured.

Within 24 h of the draw, blood was separated in a CPT mononuclear separator tube by centrifugation at 1700xg for 30 min. Mononuclear cells were separated from serum by centrifugation at 400xg for 10 min. The pelleted cells were washed twice with 2% FBS in PBS and then incubated with 2%FBS/PBS and FcR Blocking Reagent (Miltenyi Biotec) for 10 min. on
ice in the dark. The cells were incubated in the dark for 30 min. on ice with a panel of fluorescently-conjugated antibodies including: PE-labeled anti-CD34 (Becton Dickinson), APC-labeled anti-AC133 (Miltenyi Biotec), PE-Cy5.5-labeled anti-CD14 (Abcam), APC-AlexaFluor 750-labeled anti-CD45 (Invitrogen), PE-Cy7-labeled anti-CD16 (Becton Dickinson), FITC-labeled anti-CD31 (Becton Dickinson), anti-CD41a (Becton Dickinson) and anti-CD235a (Becton Dickinson), Pacific Blue (Pacific Blue monoclonal antibody labeling kit; Invitrogen), and a marker for dead cells (LIVE/DEAD fixable dead cell stain; Invitrogen). The cells were then pelleted and washed once in 2%FBS/PBS and resuspended in 1% FACS formaldehyde.1

Following re-suspension, 500,000 events were collected using the LSR II flow cytometer (Becton Dickinson). Positive/negative boundaries for all gating were established using unstained controls. The lymphocyte population was selected in the initial gating scheme by measuring the population that was negative for CD235a, CD41a, and the dead cell marker (pacific blue staining). From that population, the CD14 and CD16 negative population was selected. Cells positive for both CD34+ (stem cells) and CD31+ (endothelial cells) were selected for the final population. This population was further subdivided into mononcytic/non-mononcytic (CD45+/dim) and early/mature progenitors (AC133+). FlowJo software was used to analyze the collected events and circulating angiogenic cell counts were normalized to the sample volume used in analysis.1,2 The gating scheme is shown in Supplemental Figure I.

Residential Proximity to Major Roadway

Residential addresses of study participants were obtained during the patient interview questionnaire or through the review of medical records. Distance to roadway was determined using the Geographic Information System (GIS) ArcMap 9.3+ software. Addresses were geocoded using data obtained from the Louisville/Jefferson County Information Consortium (LOJIC) composite locator using the GIS software. Subject addresses were corrected for flaws including spelling errors, invalid characters, and invalid formats. Addresses that could not be automatically geocoded were cross-referenced with other known addresses and manually placed at the accurate location, when possible. Aerial imagery was used to identify geocoded points that did not match actual residential locations (e.g., mobile home communities) and were subsequently located when possible. Road vehicle counts were provided by the Kentucky Transportation Cabinet. A major roadway was defined as a road carrying an annual mean of 5,000 or more vehicles/day.

To measure proximity to major roadways, straight-line distance to the nearest major roadway was measured for each subject residential location. In addition, buffer areas of 50 and 300m from the residential locations of study subjects were created. Major roadways were overlaid on buffer areas, where the cumulative distance of major roadway segments within the buffer area was calculated. In addition, total roadway distance for all roads within a 50m residential buffer was measured and compared with total major roadway distance (Supplemental Figure II). Traffic intensity was calculated by multiplying the length of individual major roadway segments by the number of vehicles travelling on those segments. The sum of all segments within a 50m buffer of each individual subject was calculated to determine residential traffic intensity, or total distance traveled by all vehicles within the 50m buffer area. Vehicle traffic and distance-weighted roadway density at a maximum distance of 300m from major roadways was calculated using the ArcMap kernel density tool, with roadway traffic used as the weight field. Density values were generated on a raster surface at 10m resolution and extracted by address points for statistical analysis. The maximum distance of 300m was selected because it is the point at which most pollutants reach background levels,3 and it is a more distant major roadway exposure metric to investigate adverse cardiovascular outcomes.4
**PM$_{2.5}$ Estimation**

Ambient levels of fine particulate matter (particulate matter with an aerodynamic diameter < 2.5µm; PM$_{2.5}$) were obtained by calculating the daily average of all regional EPA-validated monitoring stations within 30 kilometers of Jefferson County, KY that report daily PM$_{2.5}$ levels. These values were determined for the 24 h period prior to the day of study visit for each study participant. Variations in PM$_{2.5}$ between monitors was limited and remained rather uniform over large distances. This was supported by the close correlation of data obtained from different monitors.

**Statistical Analyses**

Population demographics, CVD risk factors, and hsCRP were compared across roadway proximity strata using independent sample t-tests and Chi-squared analyses. Demographic variables that were not normally distributed were transformed to their natural logarithms. Roadway proximity was entered as a dichotomous variable indicating whether or not the individual was living within 50m of a major roadway. The factors significantly associated with distance to roadway in the bivariate analysis were later used in the adjusted regression model. Independent sample t-tests were used to test for bivariate associations between roadway proximity strata and circulating angiogenic cell levels before adjustment for potential confounders. Circulating angiogenic cell levels were normalized to the sample volume, transformed to their natural logarithm.

Generalized Linear Modeling (GLM) techniques were used to examine whether the circulating angiogenic cell levels were associated with distance to a major roadway, adjusting for age, gender, ethnicity, body mass index (BMI), cigarette smoking, median household income, and 24h PM$_{2.5}$ level. The variable “median household income” was used to approximate income level of the study participants and was designated at the U.S. Census Bureau block group geographic level. PM$_{2.5}$ was included as an adjustment factor in order to understand the association between proximity to a major roadway and circulating angiogenic cell levels independent of PM$_{2.5}$ exposure. Circulating angiogenic cell levels appeared to follow the gamma distribution; therefore, GLM models that assessed circulating angiogenic cells as the dependent variable utilized the gamma probability distribution and the log link function.

GLMs that assessed the sum of CVD risk factors (age, male gender, hypertension, hyperlipidemia, diabetes, and current smoking) as the dependent variable utilized the normal probability distribution and the identity link function. Roadway proximity was initially entered into the statistical model as dichotomous data indicating living within 50m of a major roadway. Major roadway density within the buffer areas was entered individually into the statistical model as a continuous variable. The more rigorous models only included the population that had a residential duration at their current home of at least 6 months, these results were only presented when significant associations were found. Percent change in cells presented in Tables 3-7 represents the interpreted β value where the original β coefficient is exponentiated, subtracted by 1, and multiplied by 100 to present a percent change in each cell population.
Traditional model-fit statistics (log-likelihood) were used to develop the most parsimonious model. In addition, we tested whether higher order modeling (e.g., exponential, cubic) improved model-fit using traditional model-fit statistics (AIC, log-likelihood, etc.). Data management and statistical analyses were performed using IBM SPSS Statistics version 21.0 for Windows (Armonk, NY, USA). Additionally, p-value adjustments for multiple comparisons were completed using the two-stage Benjamini and Hochberg (2006) step up FDR controlling method. The adjusted P-values were implemented using the R/Bioconductor package multtest.
REFERENCES


5. EPA. Download daily data. 2011-2013
