

Original Article

Vascular Health Profile Predicts Cardiovascular Outcomes in Patients with Diabetes

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Background: We previously reported the development of a novel high dimensional cytomic assay, the Vascular Health Profile (VHP) based on measurements of angiogenic circulating hematopoietic stem and progenitor cells (CHSPC^{Ang}) and extracellular vesicles (EVs), that discovered a unique signature, differentiating the vascular status of diabetics and normal healthy controls. Here, we present data from a 3-year follow-up to evaluate the power of the VHP to identify individuals at risk for cardiovascular (CV) events.

Methods: The original data were generated as previously described by measuring a broad panel of progenitor cells and EVs and profiled using cytometric fingerprinting. Subjects were classified into groups according to the occurrence of adjudicated CV events including myocardial infarction, stroke, major adverse cardiovascular events, revascularization, and irregular rhythm. Cross-validated Linear Discriminate Analysis (LDA) models were constructed and used to predict the occurrence of events, and were evaluated for predictive accuracy (AUC, area under the curve) using receiver operating characteristic (ROC) analysis.

Results: Over the period of this analysis, follow-up data was obtained on 87 subjects, with 32 events occurring overall, and only in the diabetic group. In all cases, the VHP added significant predictive power, in the form of ROC analysis, for all evaluated outcomes with the exception of irregular rhythm.

Conclusions: The VHP, a relatively simple blood test, can provide sensitive and clinically relevant information on the vascular status of a patient that may be useful for a variety of applications including drug development, clinical risk assessment, and companion diagnostics. © 2015 International Clinical Cytometry Society

Key terms: extracellular vesicle; microparticle; progenitor cell; atherosclerosis; myocardial infarction; stroke; revascularization; diabetes mellitus

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By 2030 it is predicted that over 40% of adults (~116 million people) in the United States of America will have one or more forms of cardiovascular disease (CVD)

(1). On the basis of 2010 death rate data (2), over 2,150 Americans die of CVD each day (2). The development of atherosclerotic coronary artery disease can occur as

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early as the teenage years and may manifest later in life as angina or a myocardial infarction (2). Presently, there is no biomarker reflective of vascular function that is clinically available and predictive of cardiovascular events. Therefore, a precision medicine approach is needed to address this clinical knowledge gap with a diagnostic test that provides a measure of cardiovascular health and an assessment of the efficacy of therapeutic interventions.

The measurement of multiple populations of circulating cells or cell-derived vesicles simultaneously in a sample utilizing high sensitivity flow cytometry is a promising biomarker approach to assess vascular health. Several studies have indicated that progenitor cells, including endothelial progenitor cells (EPCs) and angiogenic circulating hematopoietic stem and progenitor cells (CHSPC^{Ang}) as well as extracellular vesicles (EVs) may be predictive of cardiovascular events (3–5). We previously demonstrated that a cytomics-based assay, the Vascular Health Profile (VHP), that includes CHSPC^{Ang} and EVs, identified subsets of both CHSPC^{Ang} and EVs that were differentially expressed in a high-risk population of patients with atherosclerosis and diabetes mellitus as compared with healthy control subjects (6). In that study, CHSPC^{Ang} were lower, and most EV subsets were higher in the high-risk population compared to the healthy control population. The data were analyzed using a novel bioinformatics approach called cytometric fingerprinting that provides a means to rapidly, comprehensively, and objectively analyze such complex data (7,8).

Diabetes mellitus is associated with high risk of cardiovascular complications including diseases of coronary, peripheral, and carotid arteries (9,10). Thus, we hypothesized that (a) blood samples from patients at high risk for cardiovascular events, those with long-term type 2 diabetes mellitus and with clinically apparent atherosclerosis, will display a unique VHP signature different from healthy controls and (b) that this signature will be predictive of cardiovascular events.

MATERIALS AND METHODS

Patients and Controls

All subjects enrolled in the published baseline study (6) were followed prospectively for cardiovascular events. The population was diagnosed with type 2 diabetes mellitus for [mt]5 years and people with clinically apparent atherosclerosis, history of a myocardial infarction, stroke, claudication, or revascularization procedure were included in this study. Participants were excluded at baseline if there was a history of acute illness, recent myocardial infarction or stroke (within 3 months) or pregnancy. The healthy group included age-similar participants, based on no prior or current history of diabetes mellitus or cardiovascular disease, and lack of major cardiovascular risk factors (smoking, hypertension or elevated LDL cholesterol). Written informed consent was obtained from all study participants and study pro-

ocols were approved by the Institutional Review Board of the University of Pennsylvania.

Sample Collection

Blood was collected from patients, after overnight fasting, as previously described for the VHP and for a variety of serum measures including HbA_{1c}, lipid analysis, high sensitivity C-reactive protein, as well as complete blood count (CBC) (6). For the VHP, 3 ml of blood were collected in sodium citrate vacutainer tubes for EV analysis and 30 ml of peripheral blood were also drawn into a 60 ml heparin-coated syringe for the CHSPC^{Ang} analysis (6).

VHP Assay: Staining and Data Acquisition

The VHP, measuring both CHSPC^{Ang} and EVs, was performed as previously thoroughly described (6). Briefly, for CHSPC^{Ang}, blood was lysed with ammonium chloride, washed with 3% fetal calf serum (FCS) in phosphate buffered saline (PBS), and suspended in this buffer. Following blocking with Mouse Ig for 10 min on ice, cells were stained with following pre-titrated antibodies: FITC-CD31 (BD Cat# 555445, Clone WM59), PE-Cy7-CD34 (BD Cat#348791, Clone 8G12), Percp-Cy5.5-CD3 (BD Cat# 340949, Clone SK7), PerCP-Cy5.5-CD33 (BD Cat# 341650, Clone P67.6), PerCP-Cy5.5-CD19 (BD Cat# 340951, Clone SJ25C1), V450-CD45 (BD Cat#560367 Clone HI30), PE-CD133 (Milenyl Biotec Cat# 130-080-801, Clone AC133), APC-VEGF-R2 (R&D Cat# FAB357A, Clone 89106). Viability was assessed using propidium iodide, and negative regions were assigned using Fluorescence Minus One (FMO) controls (11). BD CompBeads (anti-mouse IgG and negative control, Cat# 552843) were used to set compensation and 8 peak fluorescent calibration beads (Spherotech, cat# RCP-30-SA) were run before and after acquisition each day to normalize for minor instrument response fluctuations over time. All acquisition occurred on a BD FACS Canto A (SORP) using Diva Software version 6.1.2 and stopped after at least 200,000 cells within the small-cell gate were counted. All rare-event flow cytometry was completed in accordance with principles discussed by Khan et al (12).

For isolation and measurement of EVs, a variation of the procedure we previously described was used (13). Platelet-poor plasma (PPP) was prepared by centrifuging whole blood at 2,500g for 15 min at room temperature within 1 h after collection. About 50 µl PPP was labeled with pre-titrated antibodies: FITC-Annexin-V (BD Bioscience Cat# 556570), PE-CD144 (BD Bioscience Cat# 560410, clone 55-7H1), PerCP-Cy5.5-CD64 (BD Bioscience Cat# 561194, Clone 10.1), AF647-CD105 (BD Bioscience Cat# 561439, clone 266), APC-H7-CD41a (BD Bioscience Cat# 561422, clone HIP8), PE-Cy7-CD31 (Biolegend Cat#303118, clone WM59), BV421-CD3 (Biolegend Cat# 300433, Clone UCHT1). After staining, 5 µl of 3.0 µm beads were added to each tube as reference counting beads. Annexin Buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl₂) was added to each

tube to make the total volume 500 μ l. Data acquisition was performed on a BD FACSCanto A (SORP) using Diva Software version 6.1.2. Forward and side scatter threshold, photomultiplier tube voltage, and window extension were optimized to detect sub-micron particles as described (6). For each day samples were analyzed, one tube containing only 0.3, 1, and 3-micron polystyrene size calibration beads was run at a fixed concentration. The acquisition was stopped when a fixed number of 3.0 μ m beads (20,000) were counted. Compensation tubes were also run using PPP (Annexin V) or BD CompBead (remaining markers, BD Bioscience Cat# 552843), and were stained using the same reagents as were used in the sample tubes.

VHP Assay: Data Analysis

The R environment for statistical computing (version 2.13.1, R Development Core Team, Vienna, Austria) and the flowFP (14), flowCore (15), and KernSmooth (16) packages were used for primary data analysis (6). Briefly, the list mode data were read and processed using the flowCore Bioconductor package (15) in untransformed linear coordinates, digital compensation was applied based using FACSDiva acquisition software (Becton Dickinson, San Jose, CA) and stored in the FCS header, and data normalization was performed based on the brightest peak of reference beads (Spherotech, Cat# RCP-30-5A) run each day. This normalized linear fluorescence data were then biexponentially transformed and the scattering data were linearly transformed to put the fluorescence and scattering data on a similar scale. Fully automated gating strategies (designed to eliminate operator bias) were developed for both EPC and EV analysis and are fully described in the previous publication (6). Cytometric fingerprinting was performed to discover signature patterns after gating using the flowFP package (14).

Definition and Adjudication of Cardiovascular Events

The prospectively defined primary endpoints included myocardial infarction, stroke, death, major adverse cardiovascular event (MACE, defined as the combination of MI, stroke and/or death) and revascularization procedure (coronary artery, carotid or peripheral). The endpoints were then confirmed with a chart review. Myocardial infarction was defined as per the universal definition of this event (17). Stroke was defined as the presence of a new focal neurologic deficit, with signs or symptoms lasting >24 h, or resulting in death (in <24 h). The diagnosis of stroke was confirmed with neuroimaging and arrhythmia via chart review.

Statistical Analysis

For each event group, Linear Discriminant Analysis (LDA) was performed using Leave-One-Out cross validation (LOOCV) to determine linear combinations of VHP subsets that best separated the event group from the non-event group. The leave-one-out strategy was chosen to provide an out-of-sample performance estimate in the context of the small sample size available in this study.

Confounding variables (age, gender, race, exercise, history of MI/Stroke/PAD, blood pressure, cholesterol, body mass index) were included independently and in conjunction with various combinations of the VHP measurement subsets. The VHP measurement subsets, as reported in Ref. 6, consisted of CHSPC^{Ang} and 8 distinct EV phenotypes as follows: CD31⁺/CD41⁺ dim (double positive, "DP_1"), CD31⁺/CD41⁺ bright (double positive, "DP_2"), annexin V positive (AV⁺), AV⁺/CD31⁺/CD41⁺ (triple positive, "TP"), CD3⁺, CD105⁺, CD31⁺, and CD41⁺. In order to exclude information-dilutive effects of VHP subsets that may not have been related to specific outcomes, all combinations of the nine VHP subsets were exhaustively modeled for each outcome separately, with and without the confounding variables included. For each condition defined by adjudicated events, the LOOCV strategy entailed that each instance in the comparison data set was set aside, an LDA model was trained on the remaining instances, and finally the resulting LDA model was used to predict the class of the set-aside instance, where the class was either "experienced the event" or "did not experience the event." This procedure was repeated for each individual instance. Receiver operating characteristic (ROC) curves were used to evaluate the ability of each combination of VHP subsets, with and without the confounding variables, to blindly predict subjects who experienced an adjudicated event versus those that did not. The combination of VHP subsets yielding the highest cross-validated area under the ROC curve (AUC) was selected as the best predictive profile for each of the event categories (myocardial infarction, stroke, death, MACE, irregular rhythm or revascularization procedure). The 95% confidence limits of the AUC (C-statistic) values were calculated using Monte Carlo estimation. The LDA model posterior probabilities for each outcome were determined to be approximately log-normally distributed using the Shapiro-Wilk test. Consequently, the posteriors were perturbed by 2 standard deviations (corresponding to 95% confidence interval) in a log-transformed space 100 times, generating a distribution of AUC values. The standard deviation of this distribution is reported as the 95% confidence interval of the AUC. All analyses were completed using the R Statistical Programming Environment (18).

RESULTS

As previously published, assay data for the combined VHP assay (both CHSPC^{Ang} and EVs) were available for 90 subjects ($n = 47$ diabetes mellitus, $n = 43$ healthy control) (6). The participants in the study were prospectively followed from Spring 2011 until July 2014 and assessed for adverse events. Among the 90 subjects for whom combined VHP assay data were available, follow-up data were successfully collected for a total of 87 of these subjects. All patients with diabetes mellitus had a history of a cardiovascular disease secondary to atherosclerosis and none of the healthy participants had a history of cardiovascular disease (Table 1). Gender and

Table 1
Characteristics of the Study Population

	Diabetic	Control
Average age (years)	65.65	61.24
Female (%)	36.96	63.41
Black or African-American (%)	52.17	21.95
Current smoker (%)	21.74	0.00
Former smoker (%)	63.04	36.59
Never smoker (%)	15.22	63.41
Average BMI	31.98	24.70
High blood pressure (%)	89.13	0.00
Statin use (%)	80.43	0.00
Average LDL (mg/dL)	105.72	129.34

LDL indicates low density lipoprotein cholesterol; BMI, body mass index.

race distribution differed slightly between the two groups with more females and fewer African Americans in healthy control group compared to the diabetes mellitus group and the mean age of the diabetes mellitus group was also slightly older. However, low density lipoprotein levels were lower in those with diabetes mellitus compared to controls as patients with diabetes mellitus were taking HMG-CoA reductase inhibitors (statins).

Patients were classified into the following groups: no adjudicated cardiovascular event, myocardial infarction, stroke, revascularization (either cardiac, carotid, or peripheral) or irregular rhythm. All of the adverse events occurred among the diabetes mellitus population. Thus, for purposes of evaluating the ability of the VHP to stratify risk in an already at-risk population, only diabetes mellitus subjects were included in the no-event group.

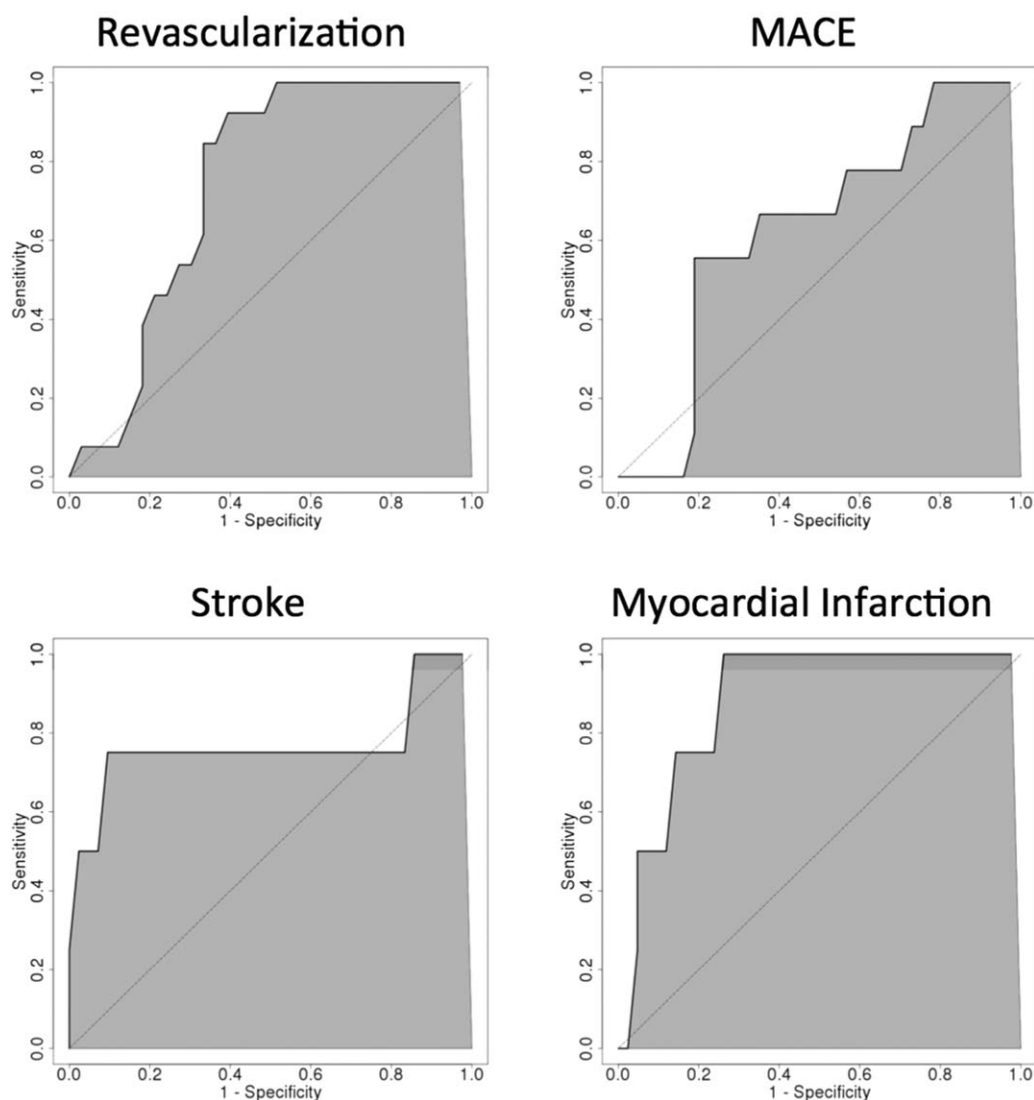


FIG. 1. The prognostic power of the VHP. Linear Discriminant Analysis models were constructed using Leave-One-Out cross validation to prevent over-fitting. ROC curves shown were computed from the models with confounders + VHP. AUC values were 0.734 (revascularization), 0.625 (MACE), 0.756 (stroke), and 0.875 (MI).

Table 2
Results of Cross-Validated Linear Discriminant Modeling

	Nb	AUCa			VHP subset(s)
		VHP	Confounders	VHP+confounders	
Myocardial infarction	4	0.881	0.077	0.875	CHSPC ^{Ang} , CD31/CD41 ^{dim} , CD31/CD41 ^{bright} , Annexin V, Annexin V/CD31/CD41
Stroke	4	0.565	0.404	0.756	CHSPC ^{Ang} , CD31/CD41 ^{dim} , CD31/CD41 ^{bright} , Annexin V/CD31/CD41, CD31, CD41
MACE	9	0.499	0.306	0.625	CD31/CD41 ^{dim} , CD31/CD41 ^{bright} , Annexin V/CD31/CD41, CD41
Revascularization	13	0.601	0.548	0.734	Annexin V, CD3, CD105, CD31, CD41
Irregular Rhythm	10	0.350	0.312	0.383	Annexin V

^aArea under the receiver operating characteristic curve.

^bNumber of subjects in the diabetes mellitus group with the condition.

High predictive power in the form of AUC was found for all outcomes (Fig. 1, Table 2) except for irregular rhythm. The 95% confidence limits of the AUC (c-statistic) for events and including confounders were for MI 0.875 ± 0.139 , stroke 0.756 ± 0.133 , MACE 0.625 ± 0.127 , revascularization 0.734 ± 0.143 , and irregular rhythm 0.383 ± 0.143 . In all cases, the VHP added significant power to the prediction of events. Strikingly, in the case of myocardial infarction, confounders alone were unable to predict outcome, whereas the VHP (either with or without confounders) had very high combined sensitivity and specificity.

LDA loading coefficients provide insight into the relative importance of features included in the model. Figure 2 shows LDA loadings for the four conditions (revascularization, MACE, stroke, and MI) for which the VHP demonstrated significant predictive power. Note that using LOOCV, n (the number of instances in the set) individual LDA models are computed using all but one of the subjects to calculate the model, and then the resulting model is used to predict the class of the left-out instance. The "rays" shown in Figure 2 each correspond to the coefficients in one of these LDA models. Black rays correspond to instances in the group with no event, and red rays correspond to instances in the event group. Tightly clustered coefficients suggest a high degree of consistency across the individually modeled instances. In general, the VHP coefficients tend to be more tightly grouped than the confounder coefficients, suggesting that the VHP is more homogeneous, and therefore more tightly correlated with outcome, than several of the factors often used to assess cardiovascular risk.

DISCUSSION

The aim of this study was to determine whether a cytomic signature of high-risk individuals predicted cardiovascular events. The results show that the VHP is predictive of major cardiovascular events and revascularization. Other studies have shown that EPCs (19), CHSPC^{Ang}(5), and EVs (20) are correlated with cardiovascular events. However, this study is unique in that both CHSPC^{Ang} and EVs were measured together and subjects were then followed prospectively for cardiovas-

cular events. The analysis of confounding variables showed that the VHP is an independent predictor indicating a robust biomarker of vascular health.

Comparison to Stress Testing to Predict Cardiovascular Events

Routine stress testing for asymptomatic patients with or without a history of myocardial infarction is not recommended given the poor positive predictive value for cardiovascular events (21–23). Patients with type 2 diabetes mellitus, especially with heightened risk for cardiovascular events, are a population well-studied with stress testing. The Detection of Ischemia in Asymptomatic Diabetics (DIAD) study involved randomization of 1,123 patient with diabetes mellitus to systematic baseline screening with stress myocardial perfusion imaging or no screening (22). The positive predictive value for cardiovascular events in the DIAD study was only 12%. The likely reason for this relatively poor prognostication is that the presence of obstructive coronary artery disease does not identify the patient with a vulnerable plaque as it is well known that a patient may have a myocardial infarction with a history of a normal stress test. A cytomic biomarker approach as shown in the present study is an assessment of vascular health, which may be a more powerful prognosticator than stress testing.

EPCs and Vascular Homeostasis

One physiological function of EPCs is to preserve vascular homeostasis, which is crucial to prevent the pathogenesis of various vascular diseases as reviewed previously (24). EPCs are mobilized from bone marrow and migrate to areas of ischemia and or damaged endothelium, participating in angiogenesis (25). Thus, EPCs may engage in a reparative function, involved in overcoming vascular damage and reducing cardiovascular risk. Levels of EPCs are increased in conditions such as unstable angina compared to stable angina (26) and decreased in patients undergoing in-stent restenosis compared to those not undergoing such injury, emphasizing the potentially protective effect of these cells. In the present study, CHSPC^{Ang} were overall lower in the high-risk

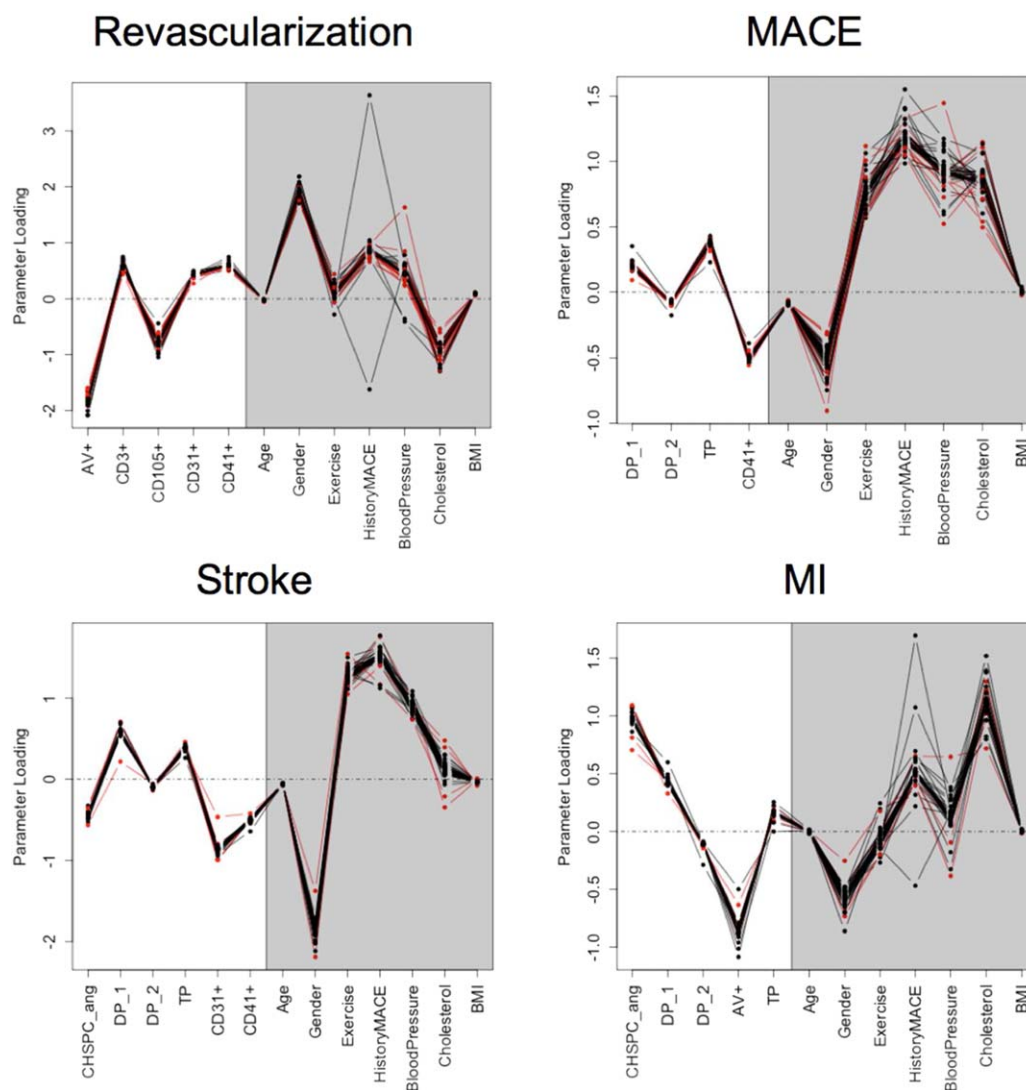


FIG. 2. Linear Discriminant Analysis model loadings. Loading coefficients for each condition are shown for each of the 46 LOOCV models. Traces in black represent the no-event group. Traces in red represent the "event" group. Gray regions denote the confounding variables. Key to the coefficients: AV = Annexin V, DP_1 = CD31/CD41^{dim} double-positive, DP_2 = CD31/CD41^{bright} double positive, TP = Annexin V/CD31/CD41 triple positive, and CD3, CD31, CD41, and CD105 refer to the respective single-positive populations. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

population compared with healthy controls, consistent with the concept of EPCs as beneficent.

EVs and Vascular Damage

EVs are released as buds from the cell membrane and contain cargo of miRNA and proteins from their parent cell and are often pro-coagulative and pro-inflammatory (27–29). Several studies demonstrated elevated cell-specific EVs in conditions of vascular dysfunction (28). EVs are present in atherosclerotic plaque and given their pro-coagulative and pro-inflammatory capacity are now considered an important part of the pathophysiology of atherosclerosis, especially in patients with diabetes mellitus where oxidative cell stress induces elevated EV numbers (13,30,31). In the present study, various subsets of EVs seem to play disparate roles in different path-

ophysiological conditions. In particular, EV subsets that are likely to be related to platelet origin are significant contributors to the prediction of myocardial infarction, whereas, in addition to platelet-derived EVs, those of T-cell and endothelial origin appear to play significant roles in stroke and vascular disease leading to revascularization procedures.

Although not evaluated in the present study, EVs are significantly elevated in patients with acute coronary syndromes compared to patients with stable anginal symptoms (32), and are a significant predictor of secondary myocardial infarction or death (33). EVs are also elevated following acute ischemic stroke/cerebrovascular accident (34). Patients with diabetes mellitus are particularly unique in elevation of EVs (6,35,36). A study of patients with diabetes mellitus showed elevated EVs robustly

predicted the presence of coronary lesions, and was a more significant independent risk factor than lipid concentrations, presence of hypertension, or length of diabetic disease (37). Our study and others suggest that EV presence, number, and type could be a component of a sensitive and specific VHP assay with clinical utility in predicting cardiovascular events.

EV Subsets and Atherosclerotic Disease

Given that all eukaryotic cells generate EVs it is not surprising that various EV subsets may reflect vascular health. For example, endothelial EVs, like platelet EVs, are elevated in patients with diabetes mellitus, are a sign of cellular apoptosis and are associated with vascular dysfunction (38). Vascular ultrasound studies show endothelial EVs negatively correlate with flow-mediated dilation indicating that they are associated with endothelial dysfunction (39,40). Other studies showed that endothelial EVs are significantly higher in patients with coronary artery disease (32) and AV⁺ endothelial EVs are elevated in patients with cardiac allograft vasculopathy (41). Interestingly, they found that combining cellular and EV measures was more informative with respect to cardiac allograft vasculopathy than either measure alone, consistent with the present findings. Other EV subsets such as T-lymphocyte EVs are pro-inflammatory and contribute to vascular dysfunction by decreasing nitric oxide production via a reduction of the level of endothelial nitric oxide synthase expression and increasing oxidative stress in endothelial cells (42). Additionally, T-lymphocyte EVs induce endothelial dysfunction as evidenced from impaired acetylcholine-induced relaxation of aortic rings at concentrations similar to those found in human blood (42). In the present study, T-lymphocyte EVs were predictive of cardiovascular events perhaps due to proinflammatory effect with an adverse effect of vascular homeostasis.

Study Limitations

This study is prospective observational follow-up of a cohort of patients recruited to develop the VHP assay and as such consists of a relatively small number of observed events. The results need to be replicated in a larger study in order to confirm the findings reported in this study. Also, the results of this study indicate an association of the VHP blood test with cardiovascular events but given the observational nature of the study data they cannot indicate causality. Finally, the flow cytometry data on EVs, upon which this study depends, were acquired before the EV international community recognized and developed improved methods of detection and enumeration of EVs using flow cytometry, including for example the exclusion of coincidence events, improved centrifugation protocols, and the use of pan-EV dyes. Nevertheless, even with this early methodology, we were able to show a very powerful predictive result. Replication of these results in a larger study will provide the opportunity to incorporate the latest developments in EV detection methodology, offering an even

greater sensitivity and specificity for the EV portion of our assay.

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