Circulating Apoptotic Endothelial Cells and Apoptotic Endothelial Microparticles Independently Predict the Presence of Cardiac Allograft Vasculopathy

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Objectives
Maintenance of endothelial homeostasis may prevent the development of cardiac allograft vasculopathy (CAV). This study investigated whether biomarkers related to endothelial injury and endothelial repair discriminate between CAV-negative and CAV-positive heart transplant recipients.

Background
CAV is the most important determinant of cardiac allograft survival and a major cause of death after heart transplantation.

Methods
Fifty-two patients undergoing coronary angiography between 5 and 15 years after heart transplantation were recruited in this study. Flow cytometry was applied to quantify endothelial progenitor cells (EPCs), circulating endothelial cells (CECs), and apoptotic endothelial microparticles. Cell culture was used for quantification of circulating EPC number and hematopoietic progenitor cell number and for analysis of EPC function.

Results
The EPC number and function did not differ between CAV-negative and CAV-positive patients. In univariable models, age, creatinine, steroid dose, granulocyte colony-forming units, apoptotic CECs, and apoptotic endothelial microparticles discriminated between CAV-positive and CAV-negative patients. The logistic regression model containing apoptotic CECs and apoptotic endothelial microparticles as independent predictors provided high discrimination between CAV-positive and CAV-negative patients (C-statistic 0.812; 95% confidence interval: 0.692 to 0.932). In a logistic regression model with age and creatinine as covariates, apoptotic CECs (p = 0.0112) and apoptotic endothelial microparticles (p = 0.0141) were independent predictors (C-statistic 0.855; 95% confidence interval: 0.756 to 0.953). These 2 biomarkers remained independent predictors when steroid dose was introduced in the model.

Conclusions
The high discriminative ability of apoptotic CECs and apoptotic endothelial microparticles is a solid foundation for the development of clinical prediction models of CAV. (J Am Coll Cardiol 2012;60:324–31) © 2012 by the American College of Cardiology Foundation

Cardiac allograft vasculopathy (CAV) in heart transplant recipients is characterized by the coexistence of diffuse fibromuscular intimal hyperplasia and focal atherosclerosis (1,2). It is largely an immunologic phenomenon that is modified by nonimmunologic factors. Diffuse concentric lesions of fibromuscular intimal hyperplasia develop in the epicardial and the smaller intramyocardial arteries, whereas focal, eccentric atherosclerotic plaques are observed in the larger epicardial arteries (1,2). CAV may lead to late graft failure and is, in addition to malignancy, the most important cause of death in heart transplant recipients after the first year (1,3,4).

According to the response to injury concept of CAV, vascular lesions are considered to be the result of cumulative endothelial injury by both alloimmune responses and nonallo-
immune insults (1,5). T-cell alloimmunity, antibody-mediated immune attack, and nonimmune factors induce endothelial activation or endothelial cell death. This may initiate a cascade of events, including platelet activation and inflammation with infiltration of predominantly macrophages and T cells in the vessel wall followed by smooth muscle cell activation, migration, and proliferation.

To maintain endothelial homeostasis, endothelial cell death should be balanced by endothelial repair mechanisms. After endothelial cell detachment induced by prolonged activation of endothelial cells or immunologic injury, endothelial cells can be detected in the peripheral blood as viable circulating endothelial cells (CECs) or as apoptotic CECs (6,7). In addition to CECs, endothelial microparticles constitute another biomarker of endothelial injury. Endothelial microparticles arise from exocytic budding after endothelial cell activation or apoptosis (8–10). The process of endothelial injury is counteracted by endothelial repair mechanisms. Increased endothelial progenitor cell (EPC) number and function may enhance endothelial repair in allografts directly via increased EPC incorporation (“building block” role) or indirectly via production of growth factors (paracrine role). EPCs that promote reendothelialization in a paracrine way have been named proangiogenic progenitor cells (11) and are in fact hematopoietic lineage cells. Cells of the hematopoietic lineage may be mobilized from the bone marrow and are entrapped in peripheral tissues, where they release angiogenic signals (12). A comprehensive analysis of endothelial repair mechanisms should therefore not be restricted to classic EPC quantifications but also entail enumeration of hematopoietic progenitor cells (HPCs).

Clinical prediction models of CAV are currently not available and may be useful for noninvasive diagnostic and prognostic purposes. Our hypothesis was that biomarkers of endothelial homeostasis would constitute a solid foundation for the development of such clinical prediction models. Therefore, the objective of the current study was to evaluate whether biomarkers related to endothelial repair (EPC number, EPC function, HPC number) and to endothelial injury ([apoptotic] CECs, [apoptotic] endothelial microparticles) discriminate between CAV-negative and CAV-positive heart transplant recipients.

Methods

Patient population and CAV grading. Fifty-two patients undergoing coronary angiography in the framework of their routine follow-up between 5 and 15 years after heart transplantation were recruited in this cross-sectional study. Heart transplant recipients with prior congenital heart disease and patients who underwent retransplantation were excluded. The study was approved by the Ethics Committee of the University Hospital Gasthuisberg, and written informed consent was obtained from all participating subjects. To establish reference values of selected biomarkers, 25 healthy control subjects age 43.2 ± 2.0 years were selected.

Coronary angiograms were analyzed by 3 transplant cardiologists (A.C., W.D., and J.V.C.). CAV was graded according to the International Society for Heart and Lung Transplantation working formulation of a standardized nomenclature for CAV-2010 (13). Patients with CAV1, CAV2, and CAV3 were pooled and constitute the CAV-negative group. Patients with CAV4, defined as no detectable angiographic lesion, constitute the CAV-negative group.

Endomyocardial biopsies were scored for acute rejection according to Billingham et al. (14), and mean biopsy score of all biopsies in the first year was calculated according to Mehra et al. (15).

Quantification of EPC number and EPC function based on cell culture assays. Peripheral blood was anticoagulated with sodium heparin, and mononuclear cells were isolated from 18 ml of blood by density gradient centrifugation with Ficoll-PAQUE PLUS (Stem Cell Technologies, Grenoble, France), according to the manufacturer’s protocol. Mononuclear cell count was determined using a Nucleocounter (Chemometec, Allerod, Denmark).

Cultivation of early EPCs was performed as described by Vasa et al. (16). Briefly, mononuclear cells were plated onto fibronectin-coated 24-well culture dishes (BD Biosciences, San Jose, California) in endothelial basal medium (Cambrex, East Rutherford, New Jersey) supplemented with endothelial growth medium SingleQuots (Cambrex) and 2% fetal bovine serum (Invitrogen, Carlsbad, California) at a density of 4 × 10^6 cells/well. After 4 days of incubation, EPCs were quantified as the number of1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated LDL (Dil-acLDL) FITC-labeled *Ulex europaeus* agglutinin-I lectin double-positive cells per microscopy field. Experiments were performed in duplicate.

Migration of cultured early EPCs was studied as previously described (16,17). Stromal-derived factor-1 (100 ng/ml; R&D Systems, Inc., Minneapolis, Minnesota) was added in the lower chamber.

HPC culture assay. Cultivation of HPCs was performed using semisolid methylcellulose-based medium (Methocult H4434 Classic; Stem Cell Technologies), according to the manufacturer’s protocol. Briefly, peripheral blood mononuclear cells (1 × 10^5 cells/dish and 2 × 10^5 cells/dish) were seeded in 35-mm culture dishes (Stem Cell Technologies) in 2 ml of Methocult medium (Stem Cell Technologies). After 14 days of incubation, erythroid burst–forming units; erythroid colony–forming units; granulocyte colony–forming units; macrophage colony–forming units; granulocyte, macrophage colony–
forming units; and granulocyte, erythrocyte, macrophage, megakaryocyte colony–forming units were enumerated using an inverted microscope.

Quantification of the number of circulating EPCs, endothelial cells, and endothelial microparticles by flow cytometry. EPC concentration was also measured by fluorescence-activated cell sorting analysis of the circulating number of CD34 vascular endothelial growth factor receptor (VEGFR)-2 double-positive cells, as described previously (18,19).

CECs were identified as CD45–CD31bright VEGFR-2+ mononuclear cells (20). Annexin V staining distinguishes between viable and apoptotic CECs (21). Samples were acquired on a high flow rate (120 µl/min) for 3 min using a BD FACSCantoII flow cytometer and BD FACSDIVA software version 1.2.6 (BD Biosciences), with a minimum detection number of 100,000 events within the mononuclear cell gate.

Blood that was used for microparticle quantification by flow cytometry was anticoagulated with sodium citrate. To exclude microparticles derived from nonendothelial cells, mainly platelets, endothelial microparticles were defined as CD144 (VE-cadherin)+ CD42a– microparticles (22). Annexin V binding was used to discriminate between apoptotic and nonapoptotic microparticles. Apoptotic endothelial microparticles were defined as Annexin V+ CD144+ CD42a– microparticles (22).

Statistical analysis. Clinical and biochemical parameters and biomarkers (EPC concentration, EPC function, HPC number, CEC concentration, and number of endothelial microparticles) were compared between CAV-negative and CAV-positive patients using Instat 3 (GraphPad Software, Inc., San Diego, California). Continuous variables were summarized by means, standard error of the mean, and sample size, and were compared by an unpaired t test. Because the distribution of the concentration of CECs is heavily right-skewed, a transformation (natural logarithm) was applied and geometric means were compared. The Fisher exact test was used to compare categoric data between patients with and without CAV. Logistic regression analysis was performed by SAS software, version 9.2 (SAS Institute Inc., Cary, North Carolina). The discriminative ability is quantified using the concordance statistic (C-statistic), which is equal to the area under the receiver-operating characteristic curve. A natural logarithm transformation of CEC data was also applied for logistic regression analysis. A p value <0.05 was considered statistically significant.

Results

Clinical characteristics of heart transplant recipients with and without CAV. The clinical characteristics of heart transplant recipients are shown in Table 1. Patients with CAV were significantly older at the time of inclusion in the study. There was a trend for a greater donor age in patients with CAV, whereas the difference in time after transplantation did not reach statistical significance. Donor age correlated moderately with the age of the recipient at the time of transplantation (r = 0.368; p < 0.01). The age of the transplanted heart (sum of donor age and time after heart transplantation) was significantly greater in CAV-positive patients (Table 1). The proportion of patients with ischemic heart disease did not differ between both groups. Acute rejection episodes (grade ≥3A) were not significantly more frequent in the CAV group than in the non-CAV group (Table 1). The average rejection score of biopsies in the first year also was not significantly different between patients without CAV (0.484 ± 0.057) and patients with CAV (0.546 ± 0.057). Creatinine levels were significantly greater in the CAV group than in the non-CAV group (Table 1). Low-density lipoprotein cholesterol in the CAV group was 16% (p < 0.05) less than in the non-CAV group (Table 1), which reflects a policy to switch to more potent statins once a diagnosis of angiographic CAV is made. The percentage of patients receiving steroids was 3.5-fold (p < 0.01) greater in patients with CAV compared with patients without CAV.

EPC number and function do not significantly differ between CAV-negative and CAV-positive heart transplant recipients. EPCs were quantified as the number of Dil-acLDL and FITC-labeled UEA-1 lectin double-positive cells after 4 days of ex vivo culture or as the number of circulating CD34+ VEGFR-2+ cells. Gating strategy and flow cytometry analysis of EPCs are illustrated in Online Figure 1. The number of FITC-labeled UEA-1 lectin double-positive cells was 49% (p < 0.0001) less in transplant recipients than in healthy controls (Online Fig. 2A) but did not differ between patients without CAV and with CAV (Table 2). The number of circulating CD34+ VEGFR-2+ cells was similar in healthy controls and transplant recipients (Online Fig. 2B) and between CAV-negative and CAV-positive transplant recipients (Table 2).

EPC migration induced by stromal-derived factor-1α (100 ng/ml) was 31% (p < 0.05) less in patients than in healthy controls (Online Fig. 2C). No difference in EPC migration was observed between CAV-negative and CAV-positive patients (Table 2), indicating similar EPC function. Granulocyte colony–forming units are significantly less in the peripheral blood of patients with CAV than in patients without CAV. Online Figure 3 compares HPC number between healthy controls and transplant recipients. Erythroid burst–forming units; granulocyte, macrophage colony–forming units; macrophage colony–forming units; and granulocyte colony–forming units were significantly less in transplant recipients than in healthy controls, whereas no significant differences were observed for erythroid colony–forming units and granulocyte, erythrocyte, macrophage, megakaryocyte colony–forming units (Online Fig. 3). The number of granulocyte colony–forming units was reduced by 59% (p < 0.05) in patients with CAV compared with
patients without CAV, but no significant differences were observed in other types of colonies (Table 2).

**Circulating apoptotic endothelial cells are significantly greater in patients with CAV than in patients without CAV.** The geometric mean of the concentration of circulating endothelial (CD45− CD31bright VEGFR-2+) cells, apoptotic endothelial (CD45− CD31bright VEGFR-2+ Annexin V positive) cells, and viable endothelial (CD45− CD31bright VEGFR-2+ Annexin V negative) cells was increased by 1.7-fold (p = 0.062), 1.5-fold (p = NS), and 1.8-fold (p = 0.053), respectively, in transplant recipients compared with healthy controls (Online Fig. 4). The geometric mean of total, apoptotic, and viable endothelial cells was 2.0-fold (p = 0.063), 2.6-fold (p < 0.01), and 1.9-fold (p = NS) greater, respectively, in patients with CAV compared with patients without CAV (Table 3). Gating strategy and flow cytometry analysis of apoptotic CECs are illustrated in Online Figure 5.

**Circulating apoptotic endothelial microparticles are significantly greater in patients with CAV than in patients without CAV.** The concentration of circulating endothelial (CD42a− CD144+) microparticles, apoptotic endothelial (CD42a− CD144+ Annexin V positive) microparticles, and viable endothelial (CD42a− CD144+ Annexin V negative) microparticles was 2.6-fold (p < 0.0001), 2.0-fold (p < 0.001), and 2.6-fold (p < 0.0001) greater, respectively, in transplant recipients compared with healthy controls (Online Fig. 6). The concentration of total, apoptotic, and viable endothelial microparticles was 1.8-fold (p < 0.05), 2.0-fold (p < 0.01), and 1.7-fold (p < 0.05) greater,
respectively, in patients with CAV compared with patients without CAV (Table 3). Gating strategy and flow cytometry analysis of apoptotic endothelial microparticles are illustrated in Online Figure 7.

**Discrimination between CAV-positive and CAV-negative transplant recipients based on logistic regression and receiver-operating characteristic analysis.** Table 4 summarizes C-statistic values of univariable models. The odds ratio per standard deviation increase of apoptotic CECs (natural logarithm transformed) and apoptotic endothelial microparticles was 2.32 (95% CI: 1.14 to 4.71; p = 0.0196) and 3.24 (95% CI: 1.17 to 8.96; p = 0.0234), respectively. The receiver-operating characteristic curve for the logistic regression function containing apoptotic CECs and apoptotic endothelial microparticles as independent predictors is shown in Figure 1. The C-statistic was 0.812 (95% CI: 0.692 to 0.932).

In a logistic regression model with recipient age and creatinine as covariates, apoptotic CECs and apoptotic endothelial microparticles added significant information (chi-square = 18.4; df = 2; p = 0.0001) and were both independent predictors (Table 5). The C-statistic corresponding to this model was 0.855 (95% CI: 0.756 to 0.953). When recipient age in these models was substituted by time after heart transplantation or age of the transplanted heart, results were essentially unaltered (data not shown).

When steroid dose was introduced in the model, apoptotic CECs and apoptotic endothelial microparticles remained independent predictors (data not shown). The C-statistic corresponding to this model was 0.926 (95% CI: 0.851 to 1.00). There was significant added value of introduction of apoptotic CECs together with endothelial apoptotic microparticles in the model with age, creatinine, and steroid dose (chi-square = 16.3; df = 2; p = 0.0003).

**Discussion**

This is the first study to demonstrate that apoptotic CECs and apoptotic endothelial microparticles are independent predictors of CAV and that the combination of these 2 biomarkers has a high discriminative ability between patients without CAV and with CAV.

**Clinical parameters and CAV.** A salient finding of the current study is that steroid use was more prevalent in CAV-positive patients than in CAV-negative patients. This is in line with a report of the Stanford group in 2004 (23) in a population of patients receiving an immunosuppressive background therapy predominantly comprising cyclosporine

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**Table 2**

<table>
<thead>
<tr>
<th>Comparison of EPC Number, EPC Function, and HPC Number Between CAV-Negative and CAV-Positive Patients</th>
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<tbody>
<tr>
<td><strong>Patients Without CAV</strong> (n = 22)</td>
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<tr>
<td>---------------------------------</td>
</tr>
<tr>
<td>Dil-acLDL/lectin^+^ cells (n/mm^2)</td>
</tr>
<tr>
<td>CD34^+^ VEGFR-2^+^ cells (n/μl blood)</td>
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<tr>
<td>Migrated cells (n/mm^2)</td>
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<tr>
<td>CFU-E (number per 2 × 10^5 MNC)</td>
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<tr>
<td>BFU-E (number per 2 × 10^5 MNC)</td>
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<tr>
<td>CFU-GEMM (number per 2 × 10^5 MNC)</td>
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<tr>
<td>CFU-GM (number per 2 × 10^5 MNC)</td>
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<tr>
<td>CFU-M (number per 2 × 10^5 MNC)</td>
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<tr>
<td>CFU-G (number per 2 × 10^5 MNC)</td>
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*Values are mean ± SEM.*

**Table 3**

<table>
<thead>
<tr>
<th>Comparison of CEC and Circulating Endothelial Microparticles Between CAV-Negative and CAV-Positive Patients</th>
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<tbody>
<tr>
<td><strong>Patients Without CAV</strong> (n = 22)</td>
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<tr>
<td>---------------------------------</td>
</tr>
<tr>
<td>Total CECs (ln [n/μl blood])</td>
</tr>
<tr>
<td>Apoptotic CECs (ln [n/μl blood])</td>
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<tr>
<td>Viable CECs (ln [n/μl blood])</td>
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<tr>
<td>Total CEMPs (n/μl plasma)</td>
</tr>
<tr>
<td>Apoptotic CEMPs (n/μl plasma)</td>
</tr>
<tr>
<td>Viable CEMPs (n/μl plasma)</td>
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</table>

*Values are mean ± SEM.*

CAV = cardiac allograft vasculopathy; CEC = circulating endothelial cell; CEMP = circulating endothelial microparticle; ln = natural logarithm; SEM = standard error of the mean.
and azathioprine. Our observation cannot be explained by a continuation of steroids after severe acute rejection episodes in the first year, because the proportion of patients with severe acute rejection episodes in the first year was similar in patients with CAV compared with patients without CAV. Second, difference of steroid use also cannot be the result of tailoring of therapy according to renal function because creatinine levels were not different between steroid users and nonsteroid users (data not shown). On the other hand, the time after heart transplantation was significantly (p < 0.05) longer in steroid users (11.6 ± 0.9 years) compared with nonsteroid users (9.1 ± 0.5 years). This reflects an evolution to steroid-free immunosuppressive regimens in patients who more recently underwent transplantation. However, steroid use was also predictive of the presence of CAV in models in which the time after transplantation was introduced as a covariate. Therefore, we cannot exclude the possibility that continued steroid use plays a causative role in the development of CAV.

Impaired renal function was an independent predictor of the presence of CAV. The natural history of renal function after heart transplantation is characterized by a decline in renal function (24). Age, pre-transplant glomerular filtration rate, pre-transplant diabetes, and pre-transplant hypertension are important risk factors for a decrement of renal function (24). Nephrotoxicity of calcineurin inhibitors is largely responsible for the progressive development of renal dysfunction (25,26). Our results are in line with those of Schober et al. (27). However, impaired renal function was not a predictor in a study with angiographic follow-up limited to 4 years (28).

The observation that time after transplantation does not predict the CAV status may seem illogical. However, the recruitment of patients in the current study was restricted to heart transplant recipients between 5 and 15 years after transplantation undergoing coronary angiography during follow-up. Because of this selection procedure, time after transplantation tends to be similar in the CAV-positive and CAV-negative patients.

**Biomarkers of endothelial repair and CAV.** We did not observe a difference between CAV-positive and CAV-negative patients in the number of EPCs quantified as the number of CD34+/VEGFR-2+ cells by flow cytometry analysis or as the number of Dil-acLDL and FITC-labeled UEA-1 lectin double-positive cells after 4 days of ex vivo culture. Furthermore, EPC function was similar in patients with CAV and without CAV. In contrast, Simper and colleagues (29) observed that the number of EPC colony-forming units (late outgrowth EPCs) appearing over a 6-week culture period was significantly less in 8 patients with CAV compared with 7 patients without CAV. However, our results are in line with those of Thomas et al. (30) and Schober et al. (27), who quantified EPC count as the number of CD34+/VEGFR-2+ cells. Schober et al. (27) demonstrated that the number of CD34+ CD140b+ smooth muscle progenitor cells is independently associated with the presence of CAV.

### Table 4

<table>
<thead>
<tr>
<th>Discrimination Between CAV-Positive and CAV-Negative Transplant Recipients Based on Receiver-Operating Characteristic Analysis</th>
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<tbody>
<tr>
<td><strong>C-Statistic (95% CI)</strong></td>
</tr>
<tr>
<td><strong>Age</strong></td>
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<tr>
<td><strong>Creatinine</strong></td>
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<tr>
<td><strong>LDL cholesterol</strong></td>
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<tr>
<td><strong>Steroid dose</strong></td>
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<tr>
<td><strong>CFU-G</strong></td>
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<tr>
<td><strong>Apoptotic CECs</strong></td>
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<tr>
<td><strong>Apoptotic endothelial microparticles</strong></td>
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</table>

The C-statistic corresponds to the area under the receiver-operating characteristic curve.

CAV = cardiac allograft vasculopathy; CEC = circulating endothelial cell; CFU-G = granulocyte colony-forming unit; CI = confidence interval; LDL = low-density lipoprotein.

### Table 5

**Logistic Regression Model for Prediction of CAV**

<table>
<thead>
<tr>
<th>OR (95% CI)</th>
<th><strong>p Value</strong></th>
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<tbody>
<tr>
<td>Age (yrs)</td>
<td>1.38 (0.66–2.88)</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>3.50 (1.27–9.66)</td>
</tr>
<tr>
<td>Apoptotic CECs (ln number/µl)</td>
<td>4.34 (1.40–13.5)</td>
</tr>
<tr>
<td>Apoptotic endothelial microparticles (number/100 µl)</td>
<td>5.30 (1.40–20.0)</td>
</tr>
</tbody>
</table>

Odds ratios (OR) are expressed per standard deviation increase.

CAV = cardiac allograft vasculopathy; CEC = circulating endothelial cell; CI = confidence interval; ln = natural logarithm.
The EPC number based on ex vivo culture assay was lower and EPC function was impaired in transplant recipients compared with healthy controls. Different classes of immunosuppressive drugs may affect EPC biology (31,32). Therefore, the lack of discriminative ability of EPC number and EPC function to detect the presence of CAV may be related to a generalized impairment of these parameters in heart transplant recipients receiving different classes of immunosuppressive drugs. In contrast, in a murine model of transplant vasculopathy without immunosuppression, increased EPC number and enhanced EPC function attenuated progression of the disease (33,34).

Because the term EPC is used in a very broad sense in the literature, EPCs encompass different categories of cells with different phenotypic and functional properties that affect neovascularization and re-endothelialization. A salient observation in the current study is that EPC number defined as CD34+/VEGFR-2+ cells did not differ between healthy controls and transplant recipients in contrast to EPC number determined by ex vivo cell culture. This basically reflects that the same term is used for entirely different categories of cells. Many so-called “EPCs” are in fact hematopoietic lineage cells. HPCs may differentiate into proangiogenic cells (12) and may promote neovascularization and endothelial repair in a paracrine way. HPC culture assays showed a significantly lower number of granulocyte colony–forming units in the peripheral blood of CAV-positive compared with CAV-negative patients. However, in multivariate logistic regression, the number of granulocyte colony–forming units was not an independent predictor of the presence of CAV (data not shown). Taken together, biomarkers of endothelial repair that were evaluated in the current study are not suited for clinical prediction models of CAV. However, we cannot exclude that other assays of endothelial repair discriminate between CAV-negative and CAV-positive patients in multivariable models.

**Biomarkers of endothelial injury and CAV.** The concentration of apoptotic endothelial microparticles and apoptotic CECs was significantly different between CAV-positive and CAV-negative patients. As indicated by the C-statistic value of 0.812, the logistic regression model combining these 2 biomarkers provides high discriminative ability between CAV-positive and CAV-negative patients.

In several logistic regression models, the introduction of apoptotic CECs and apoptotic endothelial microparticles consistently resulted in added value, indicating that these biomarkers are robust independent predictors. Whereas the final model in the current study is restricted to age, creatinine, apoptotic CECs, and apoptotic endothelial microparticles, the latter 2 parameters remain significant predictors in models with creatinine and steroid dose. Of note, the discriminative ability of these 2 biomarkers was also preserved in models in which recipient age was replaced by time after transplantation or age of the transplanted heart.

**Study limitations.** Because the number of subjects in the current study is limited to 22 CAV-negative patients and 30 CAV-positive patients, models with more than 3 predictors should be interpreted with caution (35). Inclusion of too many predictors leads to overfitting of the data and C-indices are overestimated (35). However, the additional models strongly suggest that the discriminative ability of apoptotic endothelial microparticles and apoptotic CECs is not affected by potential confounders such as age, renal function, and steroid dose.

Annexin V positivity, corresponding to phosphatidylserine externalization, should be interpreted with caution. It is already positive in an early and potentially reversible stage of apoptosis (36,37) and is not entirely specific for apoptosis (38,39). However, these issues do not undermine the discriminative ability of Annexin V positive categories that were evaluated in the current study.

**Future studies.** The high discriminative ability of apoptotic CECs and apoptotic endothelial microparticles provides a solid foundation for the further development of clinical prediction models of CAV in the framework of prospective studies that evaluate CAV by coronary intravascular ultrasound. Prospective prediction models may lead to a more rational and tailored use of coronary angiography and intravascular ultrasound that are not without risk. In addition, risk prediction models may allow quicker intervention and may help in the design of new randomized clinical trials to optimize therapy in heart transplant recipients.

**Conclusions**

Apoptotic CECs and apoptotic endothelial microparticles predict the presence of CAV independent of the age of the recipient, age of the transplanted heart, creatinine level, steroid use, and number of granulocyte colony–forming units. The results of the current study are compatible with the hypothesis that endothelial activation and injury are involved in the development of CAV.

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**REFERENCES**


