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VEGF-A Is Associated with the Subsequent Development of Moderate or Severe Cardiac Allograft Vasculopathy in Pediatric Heart Transplant Recipients

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Abstract

Background—Cardiac allograft vasculopathy (CAV) is the leading cause of chronic allograft loss following pediatric heart transplantation. We hypothesize that biomarkers of endothelial injury and repair predict CAV development in pediatric heart transplant recipients.

Methods—Blood was collected from pediatric heart transplant recipients at the time of routine annual coronary angiography and the concentrations of 13 angiogenesis-related molecules were determined. The primary endpoint was the presence of moderate or severe CAV by angiography over a 5 y follow up period.

Results—Forty-eight children were enrolled. The cohort was 57% male with a median age of 15.5 y (range 2–22) and median time post-transplant of 5.8 y (range 2–15). Eight recipients developed moderate/severe CAV at a median follow-up of 4.7 y, of whom 3 died, 3 were re-transplanted, 1 had a myocardial infarction and 1 is listed for re-transplantation. Clinical

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Conflict of Interest

The authors declare no conflicts of interest.

K.P.D., M.S., E.D.B. and D.M.B. participated in research design, performance of the research, data analysis, and writing of the manuscript; D.Z. participated in performance of the research, data analysis and editing the manuscript; J.F.K. and M.E. participated in performance of the research and data analysis. EDB and DMB serve as co-senior authors.

DISCLOSURES

None.

characteristics associated with the development of moderate/severe CAV included prednisone use at enrollment ($P=0.03$) and positive recipient CMV IgG at time of transplant ($P<0.01$). In addition, multivariable Cox proportional hazards regression identified plasma VEGF-A concentration greater than 90 pg/mL at the time of blood draw as a significant predictor of time to moderate or severe CAV (hazard ratio of 14.3; 95% CI: 1.3–163). Receiver operating characteristic curve analysis demonstrated that VEGF-A shows moderate performance for association with the subsequent development of CAV (area under the curve of 0.77; CI: 0.61–0.92).

Conclusions—VEGF-A levels in pediatric heart transplant recipients are associated with clinically important CAV progression within the subsequent 5 years.

Keywords

Heart transplantation; Coronary artery disease; Allograft Vasculopathy; VEGF; Biomarkers; transplantation; chronic rejection; diagnosis; angiogenesis

INTRODUCTION

Heart transplantation remains the best treatment option for children with end stage heart failure who have failed maximal medical therapy. While improvements in surgical techniques, better peri-operative care, and prevention of early acute cellular rejection have improved early outcomes, late complications continue to limit allograft survival.^{1, 2} In particular, cardiac allograft vasculopathy (CAV) remains the leading cause of allograft loss and there has been little or no advance in the development of therapeutics to prolong long-term graft survival.^{1, 3} Modest advances in prevention of CAV have occurred through improved prophylaxis against early acute cellular rejection, prevention and/or early treatment of cytomegalovirus (CMV) disease, and possibly chronic treatment with statins.^{4–8} Despite these improvements, persistent injury leads to both microvascular and macrovascular disease, characterized by a proliferative inflammatory process with intimal thickening, limitation of blood flow, local tissue hypoxia, and chronic allograft fibrosis.^{3, 9} CAV affects 20% of pediatric heart transplant recipients by 7 years post-transplant and 50% of patients by 14 years post-transplant.¹ More importantly, CAV is associated with allograft loss in 25–35% of pediatric heart transplant recipients by 1 year post-diagnosis and 40–50% of recipients by 3 years post-diagnosis.¹ Moreover, the degree of CAV defined by angiography carries important prognostic implications because moderate or severe CAV is associated with worse allograft survival than mild disease.^{10–12}

The assessment of allograft function traditionally has taken the form of echocardiography, selective coronary angiography, measurement of invasive cardiac hemodynamics, and histological assessment of endomyocardial biopsies. While these tests have stood the test of time in terms of their use in acute clinical care, they do not allow for the prediction of disease initiation or progression. Furthermore, they have not kept pace with our rapidly expanding knowledge of the human alloimmune system. Currently, there are many new tools and assays that can identify genomic profiles, cellular and antibody-mediated immune activation responses and/or graft endothelial cell responses *in vivo* and thus predict events associated with alloimmune targeting of the graft.^{9, 13–15} Interest in this area has resulted in several NIH- and industry-funded initiatives to advance the development of specific

biomarkers of acute rejection and CAV following cardiac transplantation. In a recent cardiac transplantation Clinical Trial in Organ Transplantation (CTOT)-05, the only biomarker that appeared to have utility in the prediction of early vasculopathy development was one that monitored graft endothelial injury and repair.¹⁶ In prior work, we demonstrated that high levels of Vascular Endothelial Growth Factor (VEGF)-A, VEGF-C, Platelet factor (PF)-4 may serve as biomarkers of late established CAV.^{17,18} In addition, in the CTOT-05 adult cohort, we observed that an early rise in VEGF-C levels was predictive of CAV at 1 year post transplant.¹⁶ On the basis of these observations, we hypothesized that proteins involved in vascular injury and repair could serve as predictive biomarkers of the subsequent development of CAV by angiography in pediatric heart transplant recipients.

MATERIAL AND METHODS

Patient population

Consecutive pediatric heart transplant recipients at least 2 years of age and at least 1.5 years status post heart transplant were consented and enrolled in this study during scheduled annual outpatient visits at Boston Children's Hospital between January and September 2009. We excluded patients less than 1.5 years post-transplant, as CAV is exceedingly rare on the first annual angiogram at our center. Patients were also excluded from primary analysis if their coronary angiogram was inadequate for grading of CAV, or if they had moderate or severe CAV at the time of initial evaluation. Basic demographic information on donor and recipient including maintenance immunosuppression medications, use of a statin, donor/recipient CMV serologic status, donor specific crossmatch status, rejection history, and indication for transplant were collected for all patients. No donor was older than 40 years of age or had a history of coronary artery disease. Blood was collected at the time of enrollment and stored at minus 80°C until analysis. The study protocol was approved by the Committee on Clinical Investigation at Boston Children's Hospital, and written informed consent was obtained from all patients. Assent was obtained for all children over the age of 12 who were developmentally capable.

Clinical management

Immunosuppression at Boston Children's Hospital prior to April 2005 consisted of prednisone, cyclosporine or tacrolimus and either azathioprine or mycophenolate mofetil (MMF) without induction therapy. Cyclosporine trough goals were 180–250 ng/mL immediately post-transplant and were gradually weaned to 75–125 ng/mL by one year post-transplant. Prednisone was weaned off completely in most patients without rejection over the first 6–12 months post-transplant. After April 2005, the immunosuppression protocol included anti-thymocyte globulin (ATG) induction therapy (1.5mg/kg/dose q24 hours × 5 doses) and maintenance therapy with tacrolimus and MMF. Tacrolimus trough goals were 10–12 ng/mL for the first three months post-transplant, 8–10 ng/mL for months 4–12, and 6–8 ng/mL after one year. MMF trough levels were maintained between 2–4 ng/mL at all times post-transplantation. All low risk patients were steroid-free by hospital discharge. In both eras, patients with a positive donor specific crossmatch or other high-risk rejection profiles received long-term maintenance corticosteroids following induction therapy. Peri-operative methylprednisolone was given to patients in both eras.

Per protocol, endomyocardial biopsies are performed at 2, 4, 6, and 9 weeks post transplantation followed by 3, 6, 9, 12, 18, 24, and 30 months. Biopsies are graded for rejection using the prevailing ISHLT criteria.^{19, 20} In addition, all heart transplant recipients are evaluated with an annual coronary angiography and right heart catheterization.

Primary Clinical Endpoint and Classification of CAV

The primary outcome variable for the study was time to development of moderate or severe CAV (ISHLT CAV₂ or ISHLT CAV₃). All coronary angiograms at the time of enrollment and those obtained as part of routine clinical care for up to 5 years following blood sample collection were reviewed by two cardiologist investigators, and any differences in opinion were resolved by conference. Baseline examinations were evaluated by K.P.D. and J.F.K., and follow-up angiograms were evaluated by K.P.D. and one interventional cardiologist including J.F.K. All angiograms were classified according to the published consensus guidelines from the International Society for Heart and Lung Transplantation.¹⁰ The reviewers were blinded to the results of other diagnostic tests performed on the patients or biomarker assay data.

Profiling of angiogenesis-related proteins

Plasma levels of 11 angiogenesis-related molecules were assayed using an Angiogenesis Multianalyte Profiling Base Kit, Angio Panel A (LAN000; R&D Systems, Minneapolis, MN) run on a Luminex LX 200 analyzer. The panel includes angiogenin, angiopoietin-1, endostatin, fibroblast growth factor (FGF)-1, FGF-2, platelet-derived growth factor (PDGF)-AA, PDGF-BB, placental growth factor (PIGF), Thrombospondin-2, VEGF-A, and VEGF-D. Plasma was diluted 5-fold as described in the product protocol. Angiogenin was run at a 50-fold dilution. Data was analyzed using xPonent Software (Version 3.1; Milipore, Billerica, MA). In addition, VEGF-A, VEGF-C and PF-4 plasma concentrations were analyzed using a sandwich enzyme-linked immunoassay (ELISA) according the protocol provided by the manufacturer (DVE00, DEVEC00 and DY795 respectively, R&D systems, Minneapolis, MN). PF-4 concentrations were measured in plasma diluted 400-fold in 30% Fetal Calf Serum (FCS). Bovine serum albumin (BSA) 1% was used as the reagent diluent. A two-fold dilution series was created for the PF-4 standard using 30% FCS and a high standard concentration of 1000 pg/ml. Tetramethylbenzidine (TMB) substrate solution (N301; Thermo Scientific, San Jose, CA) was used and addition of 50µl of 1 M sulfuric acid was used to stop the reaction. Optical density of samples were read at 450 nm using a Molecular devices microplate reader and analyzed using Softmax software (Sunnyvale, CA).

Statistical analysis

Initial calculations indicated that approximately 40 patients would provide 80% statistical power for analysis of biomarker data. In order to account for dropouts and meet our statistical goal, we targeted enrollment at 48 patients. Baseline patient characteristics at the time of transplant, at the time of enrollment, as well as donor characteristics were analyzed using a time-to-event approach with univariate Cox regression for continuous variables and Kaplan-Meier product-limit method for categorical variables. Time-dependent covariates in Cox regression were applied to evaluate the assumption of proportional hazards (with the likelihood ratio and Wald tests) to evaluate any possible violation. The primary outcome

measure for the study was time to development of moderate or severe CAV. Variables with $P < 0.05$ by univariate analysis were included as covariates in the multivariable Cox proportional-hazards regression using backward selection to identify independent predictors of moderate to severe CAV with the likelihood ratio test to assess significance of the tested variables.²¹ The analysis adhered to Vittinghoff guidelines²² to avoid overfitting of the model. Median concentrations for angiogenesis factors were compared by the Mann-Whitney U-test. The log-rank test was used to compare Kaplan-Meier time-to-event curves,²² and Greenwood's formula was used to determine 95% confidence intervals. Correlation between variables was determined using the Pearson correlation coefficient. Receiver operating characteristic curve (ROC) curve analysis was applied to determine diagnostic accuracy based on area under the curve (AUC) for VEGF-A. Statistical analysis was performed by using IBM SPSS Statistics Version 23.0 (IBM Corporation, Armonk, NY), and all reported P -values are 2-tailed.

RESULTS

Patient Characteristics

Overall, 48 patients were enrolled in the study. Two patients had a non-diagnostic coronary angiogram and two patients had severe CAV (ISHLT CAV₃) diagnosed at the time of enrollment, leaving a cohort of 44 patients for analysis. The study cohort was predominately male (57%) and Caucasian (80%) (Table 1). The median age at transplant was 6.8 years old (Interquartile range, IQR:11.9) and the main indications for heart transplantation were cardiomyopathy (61%) and congenital heart disease (27%). Ten patients (29%) were sensitized prior to transplant and 4 (10%) had a positive T- or B-cell CDC donor specific crossmatch at the time of transplant. Seven patients (16%) were transplanted in the later era in which anti-thymocyte globulin induction therapy was used as part of the clinical immunosuppression protocol.

At the time the blood sample was drawn, patients were a median of 5.8 years (IQR: 5.8) removed from their heart transplant (Table 1). A total of 64% patients (N=28) experienced at least one episode of acute cellular rejection and 11% (N=4) experienced antibody-mediated rejection. At the time of enrollment, no patient had moderate or severe acute cellular rejection on biopsy. The majority of patients (N=34, 77%) had no evidence of CAV by selective coronary angiography and the remainder (N=10) had only mild CAV (CAV₁) at the time of sample collection. Fifty-two percent (N=23) patients were on statin therapy at the time of enrollment. Of the patients who were not on a statin, 62% (N=13) were over the age of 8 years of age, when statin medications are prescribed to recipients by protocol in our program. Tacrolimus and mycophenolate mofetil were the most commonly used maintenance immunosuppression medications. Forty three percent (N=19) patients were on prednisone at the time of study sample.

Association among Plasma levels of Angiogenesis-related Proteins with early CAV

We initially performed a univariate cross sectional analysis of associations among angiogenesis factors and CAV₁ at the time of blood draw. As illustrated in Table 2, multianalyte and ELISA screens failed to demonstrate any significant association among

most biomarkers and mild CAV (CAV₁) at the time of blood sampling. Nevertheless, there were trends for increased levels of biomarkers in the patients with CAV (Figure 1), with angiogenin showing a strong trend in this cohort ($P=0.06$) (Table 2 and Figure 1). Thus, it is possible that mild CAV₁ is not detectable using plasma endothelial-based biomarker assays, and/or that low biomarker levels are suggestive that early mild CAV is not clinically meaningful.

Levels of Angiogenesis Factors as Predictors of Time to Development of Moderate or Severe CAV

Patients were followed for a median of 4.7 years (range 4.3 to 4.9 years in survivors) after the study sample was drawn. In follow up, 8 patients (18%) developed moderate or severe CAV. Amongst the patients who developed moderate/severe CAV, the median time to first diagnosis was 1.8 years post sample draw. Three of the eight patients with moderate/severe CAV died (at 1.2, 2.3 and 3.6 years after the study sample), three required re-transplantation, one had a myocardial infarction, and one was listed for re-transplantation. There were no deaths, re-transplants, or myocardial infarctions amongst the 36 patients who did not develop CAV as evaluated by angiography.

As illustrated in Figure 2A, we initially found that VEGF-A levels were increased ($P=0.019$) in patients who subsequently developed moderate/severe CAV. We further evaluated the effect of VEGF-A by stratifying patients according to the overall median VEGF-A level (~90 pg/mL). When our cohort was stratified by plasma VEGF-A levels, we found that patients with levels above 90 pg/mL (N=22) have a shorter time interval to the development of moderate or severe CAV (ISHLT CAV₂/CAV₃) compared to patients (N=22) with a VEGF-A level less than or equal to 90 pg/mL (Figure 2B; Log-rank=5.09, $P=0.02$). The assumption of proportional hazards was checked using the likelihood ratio and Wald tests. ROC analysis demonstrated fair performance of plasma VEGF-A ELISA in identifying patients who developed moderate/severe CAV in follow-up (Figure 2C; AUC=0.77; 95% CI: 0.61–0.92). This analysis suggests that a VEGF-A level of 100 pg/ml would provide optimal diagnostic separation between progressors and non-progressors. In addition, multivariable Cox proportional hazards regression identified plasma VEGF-A concentrations greater than 90 pg/mL, use of prednisone at study enrollment, and a positive recipient CMV IgG at transplant as predictors of time to moderate or severe CAV (Table 3). In particular, plasma VEGF-A concentrations above 90 pg/mL were associated with a hazard ratio of 14.3 (95% CI: 1.3–163).

We also compared results obtained from the plasma VEGF-A ELISA and VEGF-A multianalyte profiling testing. While the results are highly correlated (Pearson's correlation coefficient of 0.96), the multianalyte VEGF-A results consistently had lower values.

DISCUSSION

In this pilot prospective pediatric heart transplant cohort, we found that an elevated plasma concentration of VEGF-A is associated with the subsequent development of moderate or severe CAV within 5 years. Specifically, recipients with plasma VEGF-A concentrations above 90 pg/mL, as measured by ELISA, had a higher risk for the development of moderate

or severe CAV. These findings support the hypothesis that VEGF-A is pathophysiological in the development of CAV and supports its use as a biomarker of disease initiation. Furthermore, our findings suggest that serial VEGF-A testing could be trialed in the future as a means to risk stratify pediatric heart transplant recipients and allow for the modification of invasive CAV screening intervals.

We previously reported that serum levels of angiogenesis factors including VEGF-A, VEGF-C and PF4 were statistically associated with CAV at an average of 12 years post transplantation in adult cardiac transplant recipients.¹⁷ In the current pediatric cohort, in a cross-sectional analysis, we found that plasma levels of angiogenesis factors were not associated with existing mild CAV (CAV₁) at a median of 5.8 years post-transplantation. Since we had previously found that these same factors were associated with CAV at later times post transplantation¹⁷, it is possible that they do not correlate with mild degrees and/or clinically insignificant CAV at early times post transplantation. It is also possible that early CAV is driven by different factors and/or is a mechanistically different disease than chronic CAV at later times post transplantation. Indeed, consistent with this possibility, and our findings in this cohort, we failed to find an association among plasma levels of angiogenesis factors and early CAV in the CTOT-05 cohort of adult recipients.¹⁶

Knowing that CAV develops over time, in this study, we examined whether any of the 13 angiogenesis biomarkers tested were associated with the development of CAV over a subsequent 5-year period. We initially assessed whether the median levels of each biomarker within the entire cohort (as a cut-off value) could stratify patients for multivariable Cox proportional hazards regression analysis. Using the median, we found that VEGF-A concentrations greater than 90 pg/mL at the time of blood draw are associated with the development of moderate or severe CAV within 5 years. ROC analysis suggests that a cutoff of 100 pg/mL is optimal in diagnostic test performance in this cohort. However, given the small sample size of patients in this study, we are wary of selecting a single cutoff value for VEGF-A to identify patients at risk of progression to moderate/severe CAV. Instead, we believe that serial measurements of VEGF-A could enhance predictive analysis. Therefore, in future observational studies, it is possible that serial sampling of other angiogenesis plasma biomarkers may enhance their clinical performance and allow for better risk stratification of patients.

VEGF-A expression is induced in association with cell-mediated immune responses, acute and chronic inflammation.²³ It has also been identified in allografts in association with leukocytic infiltrates,^{18, 24–26} and we propose that it is central to the initiation of chronic rejection.⁹ VEGF-A may be expressed within the intragraft microenvironment as a result of local tissue ischemia^{9, 27}, it may be delivered by infiltrating leukocytes^{9, 28} or it may be induced locally by contact between activated T-cells or platelets and graft endothelial cells and/or APCs.²⁸ Once expressed, it serves as an angiogenesis factor²³ and as a potent chemoattractant for monocytes and CD4+ T cells.²⁵ Consistent with its proposed pro-inflammatory function within allografts, intragraft VEGF-A overexpression has repeatedly been reported to be associated with cellular rejection and antibody-mediated rejection in both heart and kidney transplant recipients.^{9, 24, 26, 29, 30} Furthermore, genetic polymorphisms leading to increased VEGF-A production have been found to be associated

with increased risk of acute rejection in pediatric heart³¹ and kidney³² transplant recipients. In heart transplant recipients with antibody-mediated rejection, increased localization of VEGF-A to allograft vascular endothelial cells is associated with a higher risk of development of CAV twelve months after the rejection episode.³³ Studies in rodent models have confirmed the pathophysiologic function of VEGF-A in rejection and CAV development.^{25, 34, 35} Thus, early identification of its expression could open a window of opportunity for therapeutic intervention to attenuate the progression of CAV.

Registry studies have demonstrated small improvements in the incidence of CAV amongst pediatric heart transplant recipients, possibly related to the use of newer immunosuppressive agents and induction therapy.¹ However, CAV and non-specific graft failure remain the leading causes of allograft loss beyond 5 years post-transplantation.¹ Thus, the ability to risk stratify patients and predict the development of moderate or severe CAV is of great prognostic importance. Investigations from the Pediatric Heart Transplant Study have demonstrated that moderate or severe CAV (ISHLT CAV₂/CAV₃) carries the worst prognosis, while the outcome of patients with mild CAV₁ is similar to those without angiographically apparent CAV.^{11, 12} Indeed, consistent with this observation, we find no difference in plasma angiogenesis factor profiles among recipients with/without mild CAV. However, amongst the 8 patients who developed moderate or severe CAV, there were 3 deaths, 3 re-transplants, 1 myocardial infarction and 1 patient was listed for re-transplant, and all these patients had higher levels of VEGF-A. None of these events were present in the patients with lower levels of VEGF-A who did not develop moderate or severe CAV. We acknowledge that there is potential for confounding by indication when making this type of comparison, particularly for those patients who were listed for re-transplantation. However, as expected, the difference in outcomes is striking between those who developed moderate/severe CAV and those who did not.

Continued focus on medical therapies that can decrease the incidence and/or the progression of CAV³⁶⁻³⁸ are vital to improving long-term outcomes in children and adults after heart transplantation. It is for these reasons that our finding that elevated concentration of plasma VEGF-A predicts which pediatric heart transplant recipients are most likely to develop moderate or severe CAV is a most significant observation. We acknowledge that this finding may also be related to other biological variables such as ongoing cellular or antibody-mediated rejection, and it will be important to study why it remains elevated in high-risk patients. It is possible, for instance, that VEGF-A levels are reflective of production by different cell types at early vs. late times post transplantation. For example, early increases in VEGF-A levels may be reflective of ongoing alloimmunity and later increases in levels may be reflective of intra-graft response(s) to ischemia/fibrosis. Nevertheless, it would be timely to further validate the utility of these biomarkers in future clinical trials, for example to determine whether a modified invasive screening approach for CAV using plasma VEGF-A levels (and/or other angiogenesis factors, including VEGF-C levels¹⁶) can result in equivalent clinical outcomes.

In conclusion, CAV remains a major challenge for the heart transplant clinician. We found that an elevated plasma VEGF-A concentration above 90 pg/mL (by ELISA) in pediatric heart transplant recipients at least 1.5yrs post transplantation is associated with the

subsequent development of moderate or severe CAV. Recipient CMV IgG positivity at transplantation and maintenance prednisone therapy are also independent risk factors for the development of moderate or severe CAV. Since maintenance prednisone is typically administered to clinically evident high risk recipients, these data provide a basis for the use of serial measurements of plasma angiogenesis factors, including VEGF-A, as biomarkers to stratify patients into those requiring less invasive CAV screening and/or those in whom specific medical interventions to prevent CAV should be trialed in the future.

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ABBREVIATIONS

CAV	cardiac allograft vasculopathy
EC	endothelial cells
ISHLT	International Society for Heart and Lung Transplantation
IVUS	intravascular ultrasound
mTOR	mechanistic target of rapamycin
mTORi	mechanistic target of rapamycin inhibitor/medication
ROC	receiver-operating characteristic
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

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Figure 1. Cross sectional analysis among proteins involved in vascular injury and repair and mild CAV at the time of blood sampling

A total of 13 proteins were assayed in plasma by multianalyte profiling and/or by ELISA.

Data for all proteins are illustrated in Table 2, and representative box-and-whisker plots of selected proteins are displayed below. By univariate analysis, angiogenic proteins were not significantly elevated ($P=NS$) in association with mild CAV (CAV₁) at the time of blood sampling.

Figure 2. Plasma VEGF-A concentrations are associated with the development of moderate/severe CAV

A. Box-and-whisker plot of VEGF-A levels at the time of enrollment and the subsequent development of no/mild CAV (N=36) or moderate/severe CAV (N=8; $P=0.019$, Mann-Whitney U -test). **B.** Kaplan Meier plot of freedom from moderate or severe CAV (ISHLT CAV₂ or CAV₃) stratified by the median VEGF-A concentration of 90 pg/mL. There were 22 patients in each strata (total 44) and patients with VEGF-A concentrations above 90 pg/mL had a shorter time to development of moderate or severe CAV ($P=0.02$). In Panels A

and B, the median follow up time was 4.7 years. **C.** Receiver-operating characteristic (ROC) curve analysis for VEGF-A levels and the subsequent development of moderate or severe CAV.

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Table 1

Patient, Donor, and Graft Characteristics

	Overall Cohort N=44
<i>Patient Characteristics</i>	N (%)
Male	25 (57%)
Ethnicity: Caucasian	35 (80%)
Median Age at Time of Transplant, y [IQR]	6.8 [11.9]
Indication for Transplant	
Cardiomyopathy	27 (61%)
Congenital Heart Disease	12 (27%)
Re-transplant	4 (9%)
Myocarditis	1 (2%)
<i>Transplant Characteristics</i>	
Recipient CMV IgG+ at Transplant (N=42)	18 (43%)
Donor CMV IgG+ at Transplant (N=33)	22 (67%)
CMV Risk Status (N=33)	
Low (D-/R-)	4 (12%)
Intermediate (D+/R+ or D-/R+)	19 (58%)
High (D+/R-)	10 (30%)
Donor Specific CDC Crossmatch Positive	4 (10%)
Highly Sensitized Pre-transplantation (PRA > 10%)	11 (29%)
Induction therapy with anti-thymocyte globulin	7 (16%)
<i>Patient Characteristics at Time of Study Sample</i>	
Median Time Since Transplant, y [IQR]	5.8 [5.8]
History of ACR 3A/2R or greater	28 (64%)
History of AMR	4 (11%)
Immunosuppression at Time of Study	
Tacrolimus	34 (77%)
Cyclosporine	6 (14%)
Sirolimus	8 (18%)
Mycophenolate mofetil	30 (68%)
Azathioprine	6 (14%)
Prednisone	19 (43%)
On Statin at Enrollment	23 (53%)
ISHLT CAV Grade at Enrollment	
CAV ₀ (Not Significant)	34 (77%)
CAV ₁ (Mild)	10 (23%)
ISHLT ACR Grade at Enrollment	
Grade 0R	37 (88%)

	Overall Cohort N=44
Grade 1R	5 (12%)
Grade 2R/3R	0

CMV, cytomegalovirus; ISHLT, International Society for Heart and Lung Transplantation; CAV, cardiac allograft vasculopathy; ACR, acute cellular rejection; AMR, antibody mediated rejection; CDC, cytotoxicity dependent crossmatch; D, donor; R, recipient or revised;

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Table 2

Cross Sectional Analysis of Associations among Angiogenesis Factors and CAV at the time of blood draw

Variable	Mild CAV (CAV ₁) (N=10)	No CAV (N=34)	P-Value
Angiogenin	380269 [365123, 635705]	354509 [304751, 390217]	0.06
Angiopoietin-1	11093 [7667, 23355]	9834 [7704, 14880]	>0.2
Endostatin	175840 [147947, 206899]	209275 [156679, 257832]	>0.2
Erythropoietin	5.7 [4.2, 7.3]	5.4 [4.2, 7.3]	>0.2
FGF-1	13.4 [11.5, 13.4]	11.5 [8.1, 14.9]	>0.2
FGF-2	33.6 [16.5, 46.6]	46.6 [21.3, 64.4]	>0.2
PDGF AA	970 [568, 1256]	705 [463, 1137]	>0.2
PDGF BB	1468 [1019, 1853]	1505 [928, 2176]	>0.2
PF-4	54429 [48486, 178831]	74098 [46221, 101702]	>0.2
PIGF	5.7 [3.1, 5.8]	3.9 [3.1, 4.8]	>0.2
Thrombospondin-2	43399 [30114, 63413]	31221 [25112, 47114]	>0.2
VEGF-A Luminex	41 [28, 52]	27 [13, 50]	>0.2
VEGF-A ELISA	107 [82, 176]	88 [50, 127]	>0.2
VEGF-C	1423 [1007, 1891]	1574 [1138, 2177]	>0.2
VEGF-D	138 [91, 207]	137 [102, 162]	>0.2

Data in each column represent the median [25th percentile, 75th percentile].

CAV, cardiac allograft vasculopathy; VEGF-A, vascular endothelial growth factor-A; PDGF AA, platelet derived growth factor AA; FGF, fibroblast growth factor; PIGF, placental growth factor; VEGF-D, vascular endothelial growth factor-D; VEGF-C, vascular endothelial growth factor-C; PF-4, platelet factor 4; PDGF BB, platelet derived growth factor BB;

Table 3

Univariate and Multivariable Predictors of Time to Development of Moderate or Severe CAV (N=44)

Variable	Univariate	Multivariable Cox Proportional Hazards Regression		
	<i>P</i> value	Hazard Ratio	95% CI	<i>P</i> value
Gender	0.64			
History of antibody mediated rejection	<0.01 [*]	6.8	0.5 – 88	0.26
Recipient CMV IgG+ at transplant	<0.01 [*]	18.3	1.6 – 204	<0.01 [†]
ISHLT CAV grade	0.06			
Prednisone Maintenance	0.04 [*]	8.4	1.1 – 62	0.03 [†]
Plasma VEGF-A > 90 pg/mL	0.02 [*]	14.3	1.3 – 163	0.01 [†]
Plasma VEGF-C	0.09			
Plasma angiogenin	0.10			

All variables at study enrollment unless specified.

^{*} Statistically significant univariate predictor. CI = confidence interval.

[†] Statistically significant independent multivariable predictor. Variables with an asterisk from univariate analysis were included as covariates in the multivariable regression analysis.