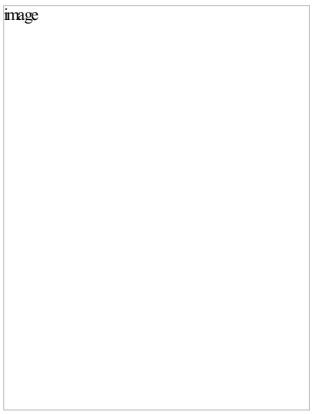

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# Elevated Serum Vascular Endothelial Growth Factor and Development of Cardiac Allograft Vasculopathy in Children

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## Introduction

Cardiac allograft vasculopathy (CAV) is a leading cause of re-transplantation and death in pediatric heart transplant recipients[1]. CAV is a complex process involving alloimmune response, chronic inflammation, and smooth muscle cell proliferation, exemplifying cross talk between cytokines and growth factors[2]. The diagnosis of CAV is challenging: early in the development of CAV, patients are almost universally asymptomatic. Thus, despite the invasive nature, coronary angiography is performed on a routine schedule for CAV surveillance. Nevertheless, coronary angiography is not a highly sensitive method for detecting early CAV[3] since pathologic vascular remodeling is present before the diameter of the coronary artery lumen visibly decreases[4]. The use of intravascular ultrasound (IVUS) has evolved as a valuable adjunct to coronary angiography[5], however cardiac catheterization is still required and its use is limited by expertise and patient size in the pediatric population. Once CAV is diagnosed, modification of immunosuppression to include proliferation signal inhibitors may slow the progression of CAV[6], but no medical treatments are currently proven to reverse CAV. While the diagnosis of moderate or severe CAV portends a significantly higher risk of graft loss[7], outcomes of pediatric patients with angiographically mild CAV are variable: graft survival is similar to those without CAV (stable mild CAV) but in a subset of patients, mild CAV can be rapidly progressive, necessitating listing for re-transplantation[7-9]. Elevated

filling pressures in patients with angiographically mild CAV is associated with a greater risk of graft loss[8], however an indicator to assist with prognostication of pediatric patients with mild CAV prior to hemodynamic derangements is lacking.

The discovery and validation of circulating biomarkers associated with CAV could significantly decrease morbidity and mortality in the pediatric heart transplant population. The utility of a circulating biomarker of CAV in the pediatric heart transplant population is three-fold: 1) optimize timing of invasive catheter-based surveillance, 2) assist with risk stratification, particularly in patients with angiographically mild CAV, and 3) serve as a non-invasive tool for surveillance of disease progression that could inform management decisions[10, 11]. Vascular Endothelial Growth Factor (VEGF, also known as VEGF-A) has established roles in angiogenesis, tumor growth, vascular development, and atherosclerosis[12-14]. Other VEGF-related proteins (VEGF-B, -C, -D, -E) share significant homology with VEGF and bind to VEGF receptors with varying affinities[15]; nevertheless, VEGF is the most well-described pro-angiogenic molecule in the post-transplant population. Specifically, in adult heart transplant recipients, myocardial VEGF is elevated in both acute and chronic allograft rejection[2, 16, 17] and persistent myocardial VEGF expression may predispose to subsequent development of CAV[18]. One of the putative mechanisms by which increased VEGF may promote CAV is through increasing smooth muscle cell migration and enhancing macrophage infiltration[2]. In a murine model of CAV in heterotopically transplanted hearts, treatment with a VEGF inhibitor (soluble VEGF receptor 1) reduced the severity and incidence of CAV[19]. While VEGF-C may concurrently regulate angiogenesis and was found to be elevated in the serum of adult heart transplant recipients with established CAV, it is primarily implicated as a paracrine factor involved in lymphangiogenesis[15] and plasma levels of VEGF-C were recently shown to be similar between pediatric heart transplant recipients with and without CAV[20]. Conversely, plasma VEGF is elevated in pediatric heart transplant recipients with moderate to severe CAV[20] as well as in adult heart transplant recipients with angiographically apparent CAV[21, 22]. The purpose of this study was to evaluate

circulating VEGF as a noninvasive predictive biomarker for the future development of CAV in the pediatric heart transplant population.

## Methods

**Patient population.** Heart transplant recipients cared for at Children's Hospital Colorado were enrolled in an Institutional Review Board-approved study of circulating biomarkers in pediatric heart disease. All patients included in this study were less than 18 years of age at the time of heart transplantation, had available blood samples drawn at least one year post-transplant, and (if in the CAV group) had an initial diagnosis of CAV between 2008-2015. In subjects with CAV, samples were obtained at two time points: within 5 years prior to CAV diagnosis (Pre-CAV) and at the time of initial CAV diagnosis (CAV). In subjects without CAV (No CAV), only one time point was used. Patients were excluded if they were less than one year post-transplant, or had acute cellular or antibody-mediated rejection at either blood draw time point or within one year of CAV diagnosis. Similarly, patients without CAV were included in the No CAV group if banked serum was available from a time point more than one year post-transplant and they had no history of rejection at any point post-transplant.

**Sample collection.** Blood samples are obtained from consented participants at the time of clinically-indicated cardiac catheterization. Serum is isolated and stored at -80°C.

**Diagnosis of CAV.** All patients included in this study had right and left coronary artery selective angiography and IVUS evaluation of the left anterior descending (LAD) or circumflex coronary artery at the time of blood sample collection. IVUS is performed as part of all catheter-based coronary assessments in patients 20 kilograms or greater; IVUS is occasionally deferred if a patient has already been diagnosed with CAV by angiography for safety considerations. Coronary angiography is performed beginning at one year post-transplant (regardless of recipient age or size), and every two years until CAV is diagnosed. After the diagnosis of CAV by either IVUS or angiography, the frequency of coronary angiography surveillance is shortened to annually or more frequently if clinically indicated. Angiograms were performed in standard fashion with biplane angiography with identical camera angles for all patients. The presence of CAV by angiography was determined by the clinician performing the cardiac catheterization,

according to the published consensus guidelines from the International Society for Heart and Lung Transplantation (ISHLT)[23]. If angiography was normal, CAV was alternatively diagnosed by the presence of ≥0.5mm of intimal proliferation seen in any portion of the LAD or circumflex artery on IVUS imaging as determined by the catheterizing physician.

**Quantification of VEGF levels.** VEGF concentrations were measured in banked serum samples, stored at -80°C. Serum VEGF concentrations were assayed in duplicate using the Human VEGF Quantikine ELISA kit (R&D Systems, Minneapolis, MN), which is specific for VEGF (no cross-reactivity for VEGF-B, -C, or –D). 5 (out of 74 serum samples) VEGF levels were excluded for a greater than 20% variation between duplicates.

**Data Analysis.** Statistical analyses were performed with Prism version 6.0h (GraphPad Software, Inc., San Diego, CA) and R. Outliers were defined as greater or less than 2 times the standard deviation from the mean and excluded from analyses (1 of 28 values at the Pre-CAV time point, 2 of 26 values at the CAV time point, and 1 of 13 in the No CAV group). Normality of the data was confirmed using the D'Agostino & Pearson omnibus normality test. Non-parametric data was analyzed using Wilcoxon matched-pairs signed rank test or Kruskal-Wallis or Friedman tests with Dunn's multiple comparisons, as indicated. When multiple comparisons were performed, adjusted p-values are reported. Recipient characteristics of the No CAV and CAV groups were compared using Fisher's exact test, t-test, ANOVA, and Chi-squared analysis. Comparison of VEGF values in the No CAV group and CAV group (Pre-CAV and CAV time points) was performed via ANOVA with a post-hoc Tukey's multiple comparisons test. Receiver-operating characteristic (ROC) curve was constructed using VEGF values from the No CAV group (true negative) versus the Pre-CAV time point (true positive); sensitivity and specificity were reported for a VEGF cut-off of >226.3 pg/ml. Paired analyses were performed using a paired t-test. Sub-analysis of variables was performed by t-test, ANOVA, and correlation between variables and VEGF level was assessed by linear regression.

## Results

**CAV subject characteristics.** Recipient characteristics are listed in Tables 1 and 2. No significant differences exist between the groups with and without CAV with regard to the characteristics listed, although there is a trend toward a higher proportion of males in the group with CAV. Notably, there are no subjects that underwent repeat heart transplantation in the No CAV group. Of the 29 subjects with CAV, 25 subjects had paired Pre-CAV and CAV blood samples available for intra-individual comparison of VEGF levels over time (Table 2). The median time between paired Pre-CAV and CAV time points is 2 years, reflecting institutional practice regarding frequency of cardiac catheterization. At the time of CAV diagnosis, only one patient had ISHLT CAV2 disease by angiography and none had CAV3 disease.

**Serum VEGF is elevated in pediatric heart transplant recipients with CAV.** Serum VEGF is significantly higher in pediatric heart transplant recipients with CAV (mean 241.7 ± 119.7 pg/ml) compared to those without CAV (mean 144.0 ± 89.05 pg/ml) (Figure 1A No CAV vs. CAV, p=0.048). Notably, in patients who have a future subsequent diagnosis of CAV, VEGF concentrations are elevated prior to their catheter-based CAV diagnosis (mean 316.2 ± 118.3 pg/ml) (Figure 1A No CAV vs. Pre-CAV, p=0.0002). Paired analysis of Pre-CAV and CAV VEGF levels from the same patient demonstrates a trend toward higher serum VEGF at the Pre-CAV time point (Figure 1B, p=0.059). ROC analysis of Pre-CAV VEGF levels demonstrates an area under the curve of 87.7% (Figure 1C, p=0.0002), with a serum VEGF level of 226.3 pg/ml predicting future CAV development with 77.8% sensitivity and 91.7% specificity. Linear regression analysis of paired samples demonstrates a strong positive relationship between VEGF levels at the Pre-CAV and CAV time points, however only 27% of the variation of CAV values is explained by the Pre-CAV value (Figure 1D, Pearson r=0.52, $r^2$=0.27, p=0.01).

**Serum VEGF concentrations are similarly elevated in both IVUS-only and angiographically-apparent CAV**. VEGF levels are equally elevated among those with IVUS-only CAV and angiographic CAV (Figure 2A IVUS vs. Angio, p=0.77). In particular, subjects with IVUS-only CAV have significantly elevated serum VEGF concentrations when compared to subjects without CAV (Figure 2A

No CAV vs. IVUS, p=0.0004).

**Recipient characteristics associated with elevated serum VEGF.** The number of values included in each analysis varies between Tables 1-3 secondary to a combination of factors: exclusion of values >2 standard deviations above the mean and those with >20% variation in duplicates (Table 3), unavailable data (donor specific antibody determination, CMV status), and presentation of only paired samples (Table 2). At the Pre-CAV time point, VEGF levels are similar between recipients with different degrees of sensitization (PRA ≤ 10% versus PRA >10%, Table 3, p=0.81). However, there are differential VEGF levels based on sensitization at the time of CAV diagnosis: in subjects who are not highly sensitized (PRA ≤ 10%), VEGF levels at the time of CAV diagnosis are lower than at the Pre-CAV time point (p=0.016) and are lower than in those with a PRA >10% (Figure 2B, p=0.023). VEGF serum concentration is not significantly influenced by the other recipient variables listed in Table 3, including sex, years post-transplant, age at transplant, presence of donor specific antibodies, CMV status, and indication for transplant. There is no correlation between VEGF levels and ischemic time, age at blood collection, fractional shortening, left ventricular end diastolic pressure (LVEDP) and B-type natriuretic peptide (BNP) (Table 4). There is a mild inverse correlation between BNP and serum VEGF at the time of CAV diagnosis (Table 4, $r^2$=0.229, p=0.021).

**Discussion**

Our retrospective cohort study demonstrates that elevated serum VEGF is predictive of subsequent CAV development in pediatric heart transplant recipients. In our population, a serum VEGF concentration of 226 pg/ml or greater predicts future CAV development with 77.8% sensitivity and 91.7% specificity. The increase in serum VEGF prior to the onset of detectable CAV by angiography or IVUS is fundamental to its utility as a predictive biomarker and suggests further investigations of VEGF in the pathogenesis of CAV are warranted in the pediatric heart transplant population.

We evaluated VEGF levels in pediatric heart transplant recipients with a spectrum of CAV disease severity, with patients having IVUS-only disease representing early CAV[25, 26] and those with angiographic evidence of CAV having relatively more advanced CAV. Although not directly comparable, our results demonstrating elevated serum VEGF concentrations in patients with IVUS-only disease contrasts with prior work demonstrating similar plasma VEGF concentrations between patients with no CAV and mild CAV[20]. Importantly, serum VEGF concentrations are known to be higher than that in plasma secondary to the release of platelet-derived VEGF during the process of clotting[27, 28]. Therefore, similar cut-offs cannot be used for serum and plasma samples and serial VEGF measurements in the same patient over time (performed with the same methodology) will likely yield the highest predictive value. Our study suggests that increased serum VEGF may be useful in distinguishing patients who will develop CAV, regardless of severity.

Comparatively little is known regarding serum VEGF concentration in the setting of pediatric cardiovascular disease, thus we sought to evaluate relevant recipient factors that may influence VEGF levels. Highly sensitized recipients (PRA >10%) have been identified as being at increased risk for early development of CAV (within 5 years post-transplant)[30]. While VEGF levels are predictive of future CAV development irrespective of PRA level, we demonstrated that there are differential levels of circulating VEGF over time based on recipient sensitization. Specifically, patients with CAV who are not highly sensitized, on average, had decreasing VEGF levels over time. Persistent elevation of VEGF in highly sensitized recipients who developed CAV is consistent with the hypothesis that VEGF is an intermediary between cell-mediated immune inflammation and the associated angiogenesis reaction[16]. While the clinical significance of persistently elevated circulating VEGF cannot be elucidated in this study, this finding highlights the importance of considering sensitization of patients and the utility of trending VEGF levels over time. There was only one subject in the No CAV group with a PRA >10%, so it is not possible to know if sensitization could influence the predictive value of VEGF.

Older age at transplant has been consistently described as a CAV risk factor in pediatric recipients[7, 30, 31], yet serum VEGF concentrations are similar across all age groups. CMV disease and infection predispose adult heart transplant recipients to development of CAV[32] and there are conflicting reports regarding the association between pre-transplant CMV positivity and risk of CAV in pediatric recipients[33, 34]. Our study did not identify any significant differences in VEGF concentration based on recipient and donor CMV status at transplant. The incidence of CAV in pediatric recipients increases with time from transplant[7, 30, 31], nevertheless VEGF levels are unchanged based on number of years post-transplant and similar VEGF criteria may be applicable regardless of time post-transplant. Specific evaluation of VEGF levels in pediatric patients is necessary, given possible differences in normative values of biomarkers based on age[35, 36]. Nevertheless, we did not identify any significant association between age at blood collection and serum VEGF concentration within our pediatric cohort, and VEGF levels in our pediatric population (with or without CAV) are similar to those reported in adult heart transplant recipients measured by the same method[22]. While an association between recipient sex and CAV development has not been reported in the literature, there have been notable differences in cardiovascular biomarkers (including natriuretic peptide levels and soluble suppressor of tumorigenicity 2, sST2) between men and women[37]. In our study, there is a trend toward a higher proportion of males in the CAV group, but VEGF levels are similar between males and females, with the majority of patients being post-pubertal at the time of sample collection. Lastly, serum VEGF concentration did not correlate with factors that may influence or represent graft function, including ischemic time, fractional shortening, and BNP.

**Limitations**

There are several limitations to our current study. First, although our findings appear to support clinical observations, our study is a retrospective cohort study and cannot establish causality. Due to the small number of patients with moderate or severe CAV by angiography, no conclusions could be made with regard to VEGF concentrations and CAV grade by angiography. Additionally, we are unable to comment on the effects of proliferation signal inhibitors, statin therapy, or maintenance prednisone

therapy (determined to be an independent risk factor for the development of moderate or severe CAV[20]) on VEGF levels, as none of our study subjects were on any of these medications at the time of blood sampling. Despite a non-significant statistical difference, a definitive association between donor specific antibodies, indication for transplant, or elevated left ventricular end diastolic pressure and elevated VEGF cannot be ruled out given the small number of subjects within each of these groups. Lastly, VEGF is elevated in numerous other pathologic conditions, and the relationship between VEGF and post-transplant lymphoproliferative disorder or other potential comorbidities remains unknown. The described association between elevated serum VEGF concentration and development of CAV is part of a growing body of literature demonstrating that VEGF is an important contributor in the pathogenesis of CAV[2, 19] and could have potential as a clinically relevant biomarker in pediatric heart transplant recipients.

**Conclusions**

In conclusion, elevation in serum VEGF concentration predicts subsequent CAV development in pediatric heart transplant recipients. Given that the estimated incidence of CAV more than doubles from 10 years to 15 years after transplant[31], serial measurement of VEGF levels in conjunction with other non-invasive investigations[40] may assist in individual risk-stratification and influence the frequency with which surveillance catheterization are performed to evaluate for CAV. Future multicenter, prospective trials evaluating circulating VEGF in the pediatric heart transplant population will be required prior to widespread incorporation as a clinical CAV biomarker. Additionally, there may be a role in evaluating VEGF polymorphisms (resulting in persistent elevations of circulating VEGF) which could be linked to CAV development, akin to disease progression of major solid tumors[41] and in association with acute rejection in pediatric heart[42] and kidney[43] transplant recipients. Furthermore, complementary mechanistic studies are needed to further define the role of VEGF in the pathogenesis of CAV.

**Figure Legends**

**Figure 1.** Serum VEGF is increased in pediatric heart transplant recipients with CAV, both prior to and at the time of initial CAV diagnosis. A. Serum VEGF concentrations are elevated at both the Pre-CAV and CAV time points in comparison to the No CAV group. ANOVA p=0.0002, with Tukey's multiple comparisons test adjusted p values as noted. B. Paired analysis of VEGF levels in the same patient over time demonstrates a trend toward lower VEGF levels at the CAV time point compared to the Pre-CAV time point. C. ROC curve. Area under the curve (AUC) = 0.877. A Pre-CAV VEGF ≥226.3 pg/ml predicts development of CAV with a sensitivity of 77.8% and specificity of 91.7%. D. Serum VEGF concentrations at Pre-CAV and CAV time points demonstrates a strong positive relationship, but a relatively small percentage of the variation in CAV values is explained by the Pre-CAV value.

**Figure 2.** A. VEGF is equally elevated in patients with CAV regardless of degree of severity. In the IVUS group, patients had normal angiography but IVUS evidence of CAV, representing early CAV. ANOVA p=0.0003 with Tukey's multiple comparisons test adjusted p values as noted. B. At the time of CAV diagnosis, VEGF levels in highly sensitized patients (PRA >10%) are significantly higher than in patients with PRA ≤10%.

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**Table 1. Comparison of Subject Characteristics (with and without CAV).** CMV = cytomegalovirus. D = donor. R = recipient. PRA = panel reactive antibody. CHD = congenital heart disease. Results are reported as median (interquartile range) or number of subjects (percent of group total).

| | **No CAV** | **CAV** | **p-value** |
|---|---|---|---|
| ***Characteristic*** | n = 16 | n = 29 | |
| *Male sex* | 5 (31%) | 18 (62%) | 0.065 |
| *Median age at transplant (years)* | 1.0 (0.3-13.5) | 0.5 (0.2-13.2) | 0.853 |
| *CMV status* | | | 0.250 |
| *High risk (D+/R-)* | 8 (50%) | 8 (28%) | |
| *Intermediate risk (D+/R+, D-/R+)* | 4 (25%) | 13 (45%) | |
| *Low risk (D-/R-)* | 3 (19%) | 4 (14%) | |
| *Not available* | 1 (6%) | 4 (14%) | |
| *PRA >10%* | 1 (6%) | 6 (21%) | 0.393 |
| *Cyclosporine monotherapy* | 8 (50%) | 14 (48%) | 1.000 |
| *Indication for transplant* | | | 0.132 |
| *CHD* | 9 (56%) | 17 (59%) | |
| *Re-transplant* | 0 | 5 (17%) | |
| *Cardiomyopathy* | 7 (44%) | 7 (24%) | |
| *Race* | | | 0.988 |
| *White* | 13 (81%) | 23 (79%) | |
| *Non-white (other)* | 3 (19%) | 6 (21%) | |

**Table 2. Paired CAV Subject Characteristics, prior to and at the time of CAV diagnosis.** LVEDP = left ventricular end diastolic pressure. Results are reported as medians (interquartile range) or number of subjects (percent of group total).

| ***Characteristics*** | **Pre-CAV** | **CAV** |
|---|---|---|
| *B-type Natriuretic Peptide (ng/L)* | 93 (59-110) | 105 (66-180) |
| *CAV diagnosed by angiography* | 0 | 12 (48%) |
| *CAV grade* | | |
| *CAV0* | 25 (100%) | 15 (60%) |
| *CAV1* | 0 | 9 (36%) |
| *CAV2* | 0 | 1 (4%) |
| *CAV3* | 0 | 0 |
| *Donor Specific Antibodies* | 4 (16%) | 6 (24%) |
| *LVEDP* | | |
| *≤10 mmHg* | 21 (84%) | 20 (80%) |
| *>10 mmHg* | 2 (8%) | 5 (20%) |
| *Not available* | 2 (8%) | 0 |
| *Median age at sample collection (y)* | 14.7 (12.1-18.8) | 17.0 (13.3-20.4) |
| *Median time between Pre-CAV & CAV (y)* | 2.0 (1.1-2.2) | |
| *Median time post-transplant (y)* | 9.2 (3.3-13.2) | 11.1 (5.6-16.0) |

**Table 3. Association Between Recipient Variables and Serum VEGF Concentrations.** CHD = congenital heart disease. CMV = cytomegalovirus. IVUS = intravascular ultrasound. SD = standard deviation.

| | | n | Mean VEGF (pg/ml) | SD | Adjusted p-value |
|---|---|---|---|---|---|
| **No CAV** | | | | | |
| Sex | Male | 2 | 150.4 | 62.01 | 0.93 |
| | Female | 10 | 142.7 | 96.20 | |
| **Pre-CAV Time Point** | | | | | |
| Age at transplant | ≤1 | 16 | 292.5 | 118.0 | 0.23 |
| | 2-10 | 2 | 399.9 | 22.15 | |
| | 11-17 | 6 | 298.3 | 125.0 | |
| CAV diagnosis method | Angiography | 13 | 300.9 | 110.0 | 0.53 |
| | IVUS only | 14 | 330.3 | 128.0 | |
| CMV status | High | 8 | 368.2 | 85.18 | 0.11 |
| | Intermediate | 12 | 295.9 | 127.9 | |
| | Low | 4 | 224.0 | 82.66 | |
| Donor Specific Antibodies | No | 15 | 353.9 | 114.4 | 0.083 |
| | Yes | 5 | 219.9 | 116.4 | |
| Indication for transplant | Cardiomyopathy | 5 | 354.7 | 109.5 | 0.59 |
| | CHD | 17 | 297.9 | 116.4 | |
| | Re-transplant | 5 | 339.7 | 144.4 | |
| Panel Reactive Antibody | ≤10% | 23 | 313.8 | 126.4 | 0.81 |
| | >10% | 4 | 329.6 | 62.45 | |
| Sex | Male | 16 | 333.2 | 107.1 | 0.35 |
| | Female | 11 | 291.4 | 134.4 | |
| Years post-transplant | ≤1 | 5 | 356.3 | 143.7 | 0.76 |
| | 2-7 | 3 | 256.5 | 75.10 | |
| | 8-13 | 8 | 300.0 | 131.4 | |
| | 14-19 | 8 | 317.5 | 84.65 | |
| | ≥20 | 3 | 348.6 | 194.0 | |
| **CAV Time Point** | | | | | |
| Donor Specific Antibodies | No | 15 | 220.0 | 121.4 | 0.16 |
| | Yes | 7 | 306.5 | 79.11 | |
| Panel Reactive Antibody | ≤10% | 18 | 210.3 | 110.6 | 0.023 |
| | >10% | 6 | 335.8 | 100.8 | |
| Sex | Male | 15 | 266.7 | 127.3 | 0.17 |
| | Female | 9 | 200.0 | 98.38 | |

**Table 4. Correlation Analysis (Linear Regression) Between Recipient Characteristics and Serum VEGF Concentrations.** BNP = B-type natriuretic peptide. LVEDP = left ventricular end diastolic pressure.

| | Correlation | $r^2$ | p-value |
|---|---|---|---|
| No CAV VEGF Level | Age at blood collection (No CAV) | 0.06 | 0.239 |
| | LVEDP (No CAV) | 0.167 | 0.187 |
| Pre-CAV VEGF Level | Age at blood collection (Pre-CAV) | 0.107 | 0.096 |
| | BNP (Pre-CAV) | 0.141 | 0.103 |
| | Fractional shortening (Pre-CAV) | 0.015 | 0.551 |
| | Ischemic time | 0.00066 | 0.899 |
| | LVEDP (Pre-CAV) | 0.106 | 0.098 |
| CAV VEGF Level | BNP (Pre-CAV) | 0.229 | 0.021 |
| | LVEDP (CAV) | 0.011 | 0.621 |

Figure 1

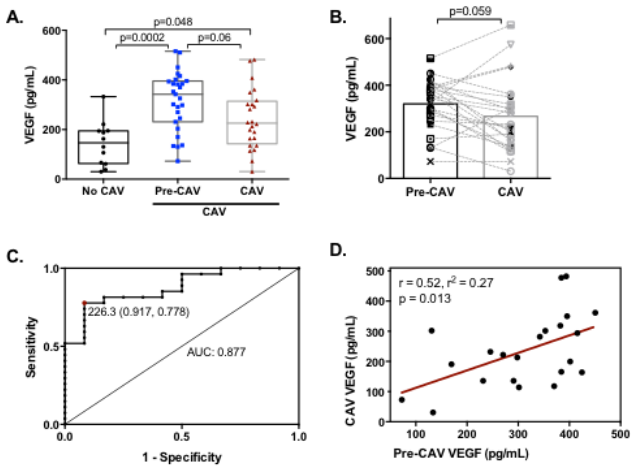

Figure 2