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Multicenter Analysis of Immune Biomarkers and Heart Transplant Outcomes:

Results of the Clinical Trials in Organ Transplantation-05 study

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Abstract

Identification of biomarkers that assess post-transplant risk is needed to improve long-term outcomes following heart transplantation. The Clinical Trials in Organ Transplantation (CTOT)-05

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Supplemental Materials and Methods

Table S1. Eligibility Criteria

Figure S1. Tacrolimus Trough Levels by Visit. Box plots of the tacrolimus trough levels (mg/mL) at each of the five visits at which immunosuppressive trough levels were measured. The value of N below each box indicates the number of subjects who had a trough level reported at that time point. An ad hoc analysis looking at the relationship between ELISPOTs and trough levels showed there was no correlation between the post-transplant ELISPOTs and tacrolimus trough levels among the 271 observations that matched up in terms of time post-transplant (data not shown).

Figure S2. Donor, Recipient CMV Status at Transplant by Composite Endpoint. Numbers and percentages of subjects who met (gray) and did not meet (white) the composite endpoint when classified by donor, recipient CMV infection status.

Figure S3. Donor, Recipient CMV Status at Transplant by CAV Endpoint. Numbers and percentages of subjects who met (gray) and did not meet (white) the CAV endpoint when classified by donor, recipient CMV infection status.

Figure S4. Concordance of pathology scoring. Percent concordance (gray) and discordance (white) of the local pathology reads when compared to the central pathology reads using ISHLT acute cellular rejection scoring criteria.

protocol was an observational, multicenter, cohort study of 200 heart transplant recipients followed for the first post-transplant year. The primary endpoint was a composite of death, graft loss/re-transplantation, biopsy proven acute rejection (BPAR) and cardiac allograft vasculopathy (CAV) as defined by intravascular ultrasound (IVUS). We serially measured anti-HLA- and auto-antibodies, angiogenic proteins, peripheral blood allo-reactivity and peripheral blood gene expression patterns. We correlated assay results and clinical characteristics with the composite endpoint and its components. The composite endpoint was associated with older donor allografts ($p<0.03$) and with recipient anti-HLA antibody ($p<0.04$). Recipient CMV-negativity (regardless of donor status) was associated with BPAR $p<0.001$), and increases in plasma vascular endothelial growth factor-C (OR 20; 95%CI:1.9–218) combined with decreases in endothelin-1: (OR 0.14; 95%CI:0.02–0.97) associated with CAV. The remaining biomarkers showed no relationships with the study endpoints. While suboptimal endpoint definitions and lower than anticipated event rates were identified as potential study limitations, the results of this multicenter study do not yet support routine use of the selected assays as noninvasive approaches to detect BPAR and/or CAV following heart transplantation.

Introduction

Identification and validation of accurate and reproducible, noninvasive biomarkers capable of diagnosing and/or predicting outcomes following heart transplantation has the potential to improve clinical care and patient health. Validated biomarkers for incipient acute rejection (AR) could diminish biopsy-related morbidity and guide decision-making that optimizes immune suppressant dosing, thereby limiting side effects and preventing development of irreversible allograft damage. AR-related morbidity following heart transplantation remains significant (1, 2), supporting the need for predictive biomarkers capable of detecting this endpoint. Median survival of heart allografts remains suboptimal at ~11 years (1, 2), underscoring the pressing need for biomarkers of late outcomes. Cardiac allograft vasculopathy (CAV), a manifestation of chronic injury, is commonly associated with graft deterioration and failure and there are no available therapies capable of reversing CAV once it has been initiated. Thus, identifying and validating markers of early or incipient CAV could be transformative and would support future clinical trials in which preventative interventions could be tested for their ability to improve allograft and patient survival.

With the exception of one multicenter study showing that a peripheral blood gene profile can bypass performing allograft biopsies to detect acute rejection at times >6-months post-transplantation (3), reports of biomarkers in heart transplant recipients have been relatively small, cross-sectional, single center analyses and few have identified predictive biomarkers for CAV at early times post-transplantation (4–20). Several studies have provided evidence that alloreactive T cells detected in peripheral blood (21–26), the quantity of cell-free, donor DNA in recipient's plasma (27, 28), serum angiogenesis-related factors (29–33), serum anti-HLA antibodies and autoantibodies (34–39) and several peripheral blood gene profiles are associated with acute or chronic heart graft injury (40–43). Prospective, multicenter, comparative analyses of candidate biomarkers for AR and CAV have not been reported. How biomarkers relate to known clinical risk factors associated with these endpoints in heart transplant recipients are also not known.

In an effort to address these deficiencies, we designed the Clinical Trials in Organ Transplantation-05 (CTOT-05) trial, a multicenter, observational, study correlating biomarkers with outcomes in first heart transplant recipients. We chose to study a panel of candidate peripheral blood cell biomarkers that were deemed potentially informative based on published single center studies from the heart transplant and/or the kidney transplant literature. We serially collected peripheral blood and biopsy samples over the first year following heart transplantation and assessed independent relationships of the biomarkers with a composite endpoint comprised of graft loss, incidence of rejection and presence of CAV at 12-months, as well as with each of its components.

Methods

Study design and oversight

This prospective multicenter observational trial (clinicaltrials.gov NCT00466804) had a target accrual of 200 adult recipients of primary heart transplantation. The CTOT-05 protocol development team was led by P. Heeger, M. Sayegh and R. Starling. Medical safety oversight was provided by N. Bridges. Statistical analysis was the responsibility of D. Ikle (with the CTOT-05 team). Data were collected by the investigators and coordinators at each site. All authors are responsible for data accuracy and completeness. Each site participated under the auspices of its Institutional Review Board. An independent, NIAID-appointed Data Safety Monitoring Board was responsible for periodic safety review.

Subjects

Adult candidates for heart transplantation were eligible for enrollment. Detailed inclusion and exclusion criteria are shown in Table S1.

Endpoints

The primary endpoint was a composite of death, re-transplantation/re-listing, biopsy proven acute rejection (BPAR), and the incidence of rapidly progressive CAV defined as an incremental change in IVUS-measured coronary artery maximal intimal thickness (MIT) of >0.5 mm from 6–8 weeks post-transplant to 12 months post-transplant in a matched site (44). Secondary endpoints included each component of the composite endpoint.

Central pathology readings of tissue sections were performed blinded by J. Stone (MGH) according to the ISHLT 2005 working formulation (45). We defined BPAR as acute cellular rejection ISHLT >grade 2R. Hemodynamic compromise was not analyzed as an endpoint due to lack of objective evidence to adjudicate. Biopsies were read locally for clinical management. Tissue from the same biopsy (for some centers, different sections) was submitted to and read by the core pathology laboratory.

IVUS was performed at each site using a standardized protocol developed at the Cleveland Clinic (46, 47). Recordings were sent to the central IVUS reading laboratory (M Tuzcu and S Nicholls) where they were assessed by standard quality assurance (QA) criteria (44, 48, 49). If either IVUS reading from an individual subject was not obtained or did not meet QA criteria the subject was deemed ineligible for evaluation of the IVUS endpoint. Subjects who

did not meet the CAV endpoint could still meet the composite endpoint if they died, were re-listed, or developed BPAR.

Interventions and sample collection

Immunosuppression was not standardized; doses and levels of immunosuppressive drugs were defined and maintained within therapeutic ranges as per local practice (Figure S1). Standard of care surveillance endomyocardial biopsies were obtained at weeks 2 and 6 and months 2, 3, 4, 5, 6, 9, and 12 post-transplantation. “For-cause” biopsies were obtained per local center practice.

Blood samples were obtained prior to transplantation, at week 6 and months 3, 6, 9, and 12 post-transplant. Study visits occurred whenever a clinically indicated biopsy was scheduled. Blood samples were collected prior to biopsies or associated treatments.

Surveillance studies for cytomegalovirus (CMV) were performed according to local practice at each participating site. Prophylaxis against CMV and *Pneumocystis jirovecii* was per local standard of care.

Laboratory studies

Anti-HLA antibody analysis—Anti-HLA antibodies were measured at the core laboratory at the Brigham and Women’s Hospital (I. Guleria) using Luminex LABScreen® Single Antigen HLA Class I and Class II Antibody Detection. Assignment of a DSA required a median fluorescent intensity >1000 (a predetermined albeit relatively low threshold chosen to minimize the false negative rate) and an appropriate donor-specific epitope pattern following review of recipient and donor HLA types and the anti-HLA specificities.

ELISPOT Panel of Reactive T cell (PRT) assays—PBMCs obtained at baseline were stimulated against a panel of 6 allogeneic B cell lines in IFNγ ELISPOT assays, in triplicate as described previously in detail (50, 51). Mean values for responses to each stimulator were summed to derive the PRT value.

Plasma angiogenesis-related proteins—Plasma collected at the time of transplant and 1-year post-transplant was stored at −80°C. Concentrations of 17 angiogenesis-related proteins were initially measured in batches using a multiplex magnetic bead based assay (Millipore, Billerica, MA) on a LX 200 analyzer (Luminex, Austin, TX), according to the manufacturer’s instructions. Initially, 20 subjects who met the CAV endpoint and 40 controls were randomly selected from the 106 subjects with paired IVUS evaluations. Based on these results, ELISAs for VEGF-A, VEGF-C, Leptin, and Endothelin-1 (ET-1) (R&D Systems; Minneapolis, MN) were performed on all available baseline and 1-year post- transplant samples collected from the 106 subjects with evaluable paired IVUS results.

Peripheral blood and tissue gene expression profiling—Biopsy samples were immediately placed in 150μl RNA*later* (Life Technologies, Grand Island, NY) and stored at −70°C. After thawing, samples were homogenized using Tissue*lyser* (Qiagen, Hilden, Germany), and total RNA was extracted using Purelink micro to midi Total RNA

Purification System (Invitrogen, Carlsbad, CA). Peripheral blood was collected in PAXgene RNA collection tubes (BD Diagnostics, Valencia, CA), stored at room temperature for 6–24 hours, then frozen at −70°C. Total RNA was by PAXgene blood miRNA kit (PreAnalytiX, QIAGEN, Hilden, Germany). 1μg of total RNA/100 μl was converted into complementary DNA (cDNA) using Taqman Reverse Transcription kit (Applied Biosystems/Life Technologies, Foster City, CA, USA).

Real-time PCR (RT-qPCR) analysis was performed by a two-step process—a 10-cycle preamplification step (AmpliTaq® DNA Polymerase Kit; Applied Biosystems/Life Technologies, Foster City, CA, USA) followed by measurement of mRNA copies with an ABI PRISM 7900HT Sequence Detection System (for details and primers see supplemental methods).

Statistical Methods

Original sample size calculations were based on the reported incidence of anti-HLA antibodies and their relationship with CAV. A two-sided Chi-square test at the alpha level of 0.05 was expected to achieve 80% power to detect odd ratios in the range of 2.7 to 3.5 (comparing presence of CAV to absence of CAV) with a sample size of 150.

Data are summarized using descriptive statistics for categorical (counts/percentages) and continuous (mean and standard deviations) variables. Univariate analyses were performed using chi-square, Fisher's Exact, or Cochran-Mantel-Haenszel tests for categorical variables and t-tests for continuous variables. Log_{10} transformations applied as necessary to satisfy normal distribution assumptions. Univariate and multivariable logistic regression methods were used to model the relationships between markers and endpoints of interest. See the Supplemental Methods section for greater detail. All statistical analyses were performed in SAS Version 9.3 (SAS, Cary, NC).

Results

Description of cohort

We enrolled 263 heart transplant candidates at 12 sites in the US between 2007 and 2010 (Table 1). The final study cohort was composed of the first 200 subjects who underwent heart transplantation. This cohort was predominantly Caucasian (74.5%) and male (81%) with a mean age of 54 years. 43% were CMV IgG-negative (regardless of donor serology) and 36% were supported by left ventricular assist devices (LVAD) at the time of transplantation. Organ donors had a mean age of 31 years, 67.5% were Caucasian, and 39.5% were CMV-antibody negative.

Post-transplant immunosuppression was determined by local practice (Table 1): 25.3% of subjects received induction therapy with rabbit anti-thymocyte globulin (ATG) and 18% received anti-IL-2 receptor (anti-IL-2R) induction. Maintenance immunosuppression varied among centers but generally included tacrolimus, mycophenolate mofetil (MMF) or its equivalent and a variable course of corticosteroids. Immunosuppression at one site differed from the others: no high dose steroids were administered at transplant, no ATG and/or anti-IL-2R was used for induction, all recipients were treated with MMF for 3–6 weeks only and

post-transplant corticosteroids were administered for 8–12 weeks only; tacrolimus was initiated at the time of transplantation and was used as the only maintenance therapy beyond 8–12 weeks.

The 12-month outcomes of the 200 transplanted subjects included 14 deaths, 1 re-listing for transplantation, and 50 subjects with at least one episode of BPAR >2R (Figure 1).

106 of the 200 transplanted subjects had evaluable paired IVUS studies (Figure 1, Table 2), a similar percentage as reported in published studies (52–54). Clinical characteristics of the CAV subset were similar to those not evaluable for CAV with the exception of a lower frequency of LVAD support, less ATG induction, and a higher prevalence of CMV-IgG positivity (not shown). Twenty-three of the 106 subjects (22%) met the CAV endpoint. 79 of 136 evaluable subjects reached the composite endpoint (Figure 1, Table 2).

Clinical characteristics and outcomes

Clinical characteristics associated with the primary composite endpoint (Table 2) included older donor age (33.3±11.3 vs. 28.8±11.32 years, p=0.03), higher recipient weight and body mass index (p <0.05 for each), recipient waiting list status (p=0.006), recipient CMV-negative status (regardless of donor status, p<0.001, Figure S2), and absence of induction therapy with either anti-IL-2R or ATG (use of either was associated with a lower rate of reaching the composite endpoint, p<0.001, Table 2). Pre-transplant LVAD support trended toward significance (p=0.06). A higher frequency of recipient CMV-negative but donor CMV-positive (D^+R^-) subjects met the composite endpoint compared to CMV^+ recipients regardless of donor status (74% vs. 46%, p=0.005, univariate analyses, Figure S2). Post-transplant CMV infection was diagnosed in 12 subjects. We did not observe significant correlations between CMV infection and BPAR or CAV (only 7/12 had IVUS data evaluable for CAV, not shown).

Regarding the individual components of the composite, recipient weight correlated directly with developing >1 episode of BPAR, while induction therapy was associated with a lower incidence of BPAR (Table 2). CAV occurred more commonly in males (p=0.039) and in CMV^- recipients (p=0.03). CAV trended higher in CMV^- recipients who had CMV^+ donors as compared to CMV^+ recipients regardless of donor status (33% vs. 15%, p=0.073, Figure S3). We also observed a trend toward a lower incidence of CAV in subjects given ATG (p=0.07, Table 2).

Serum antibodies and outcomes

We detected serum anti-HLA antibodies in 24% (46/195) of subjects either pre- or post-transplantation. 12% (n=24) were reactive to class I HLA alone, 6% (n=11) to class II HLA alone and 6% (n=11) to both class I and II. There were 132 subjects evaluable for the primary composite endpoint and who had serum samples available for analysis (Figure 2A). The prevalence of anti-HLA antibodies was greater among those who met the endpoint (22/75, 29%) than among those who did not (8/57, 14%, p=0.04). Anti-class I antibodies were present in 20/75 (27%) subjects who met the endpoint vs. 4/57 (7%) who did not (p<0.01). There were no differences in anti-class II antibodies between subjects who met the endpoint (10/75, 13%) and those who did not (6/57, 11%, p=ns).

Of 193 evaluable subjects with donor typing available, serum from 21 subjects contained DSA. In 14 instances DSA was present in the pre-transplant sample. In 6 subjects DSA developed *de novo* post-transplant. In 1 subject DSA was detected post-transplant but the pre-transplant sample was not adequate (we could not determine the timing of DSA development). Of 130 subjects evaluable for the primary composite endpoint with available donor/recipient HLA typing data, DSA was present in 15: 10/15 (67%) subjects with DSA met the endpoint vs. 64/115 (56%) subjects without DSA (p=ns). Within the 104 subjects evaluable for the CAV endpoint, 2/11 with DSA met the endpoint compared with 21/93 without DSA (p=ns).

In an effort to extend/validate previous studies suggesting that autoantibodies reactive to VM or CM correlate with worse heart transplant outcomes (34–39), we quantified serum anti-VM and anti-CM auto-antibodies pre-transplant and at 12-months post-transplant and correlated the results with the 1-year outcomes. Neither positive serum anti-CM antibodies (Figure 2B) nor positive serum anti-VM antibodies (data not shown) were associated meeting the composite endpoint. We did not observe associations between anti-CM autoantibody (Figure 2B) or anti-VM (not shown) and the incidence of BPAR or CAV.

Cellular alloimmunity and outcomes

Based on previous work showing frequencies of alloreactive IFNγ-producing PBMCs correlated with worse outcomes in kidney transplant recipients (55, 56), we determined the frequency of primed/memory cellular alloimmunity pre-transplant using an IFNγ-ELISPOT assay. Low rates of donor cell collection prevented us from delineating frequencies of donor-reactive cellular immunity in the recipients. As an alternative, we quantified allo-reactivity by stimulating PBMC with a panel of allogeneic stimulator cells as described in panel of reactive T cells (PRT) assay (51, 55, 56). Interpretable assays were available from 130 subjects. We did not observe significant associations between the strength of the pre-transplant, or the post-transplant, PRT and either the composite endpoint, BPAR >2R, or CAV (Figure 3). This was true regardless of whether the PRT was analyzed as a continuous variable or as a dichotomous variable based on a pre-defined threshold derived from kidney transplant recipients (51).

Peripheral blood and tissue gene expression profiles and outcomes

Based on previous publications indicating that various effector T cell gene expression patterns detected in peripheral blood cells correlate with BPAR (57, 58) we serially quantified peripheral blood expression of 6 candidate genes, FasL, Foxp3, GZMB, HPRT, CXCL10, and PRF1 in our study cohort, and correlated the results with the rejection outcomes. We observed no significant correlations between any of the mRNAs and BPAR ≥2R at the time of rejection (Figure 4A). Nor did we observe any correlations between any of the peripheral blood cell mRNA levels and CAV (data not shown).

When we examined expression patterns of the same genes within biopsy tissue with and without BPAR, we did observe significantly higher levels of granzyme B, Foxp3 and CXCL10/IP-10 mRNA in association with BPAR ≥2R (Figure 4B). The expression levels of

individual genes in the peripheral blood did not correlate with their expression levels in cardiac tissue (all correlation values were r<0.2).

We extended the analysis to include a larger number of potential biomarker genes using Nanostring®, quantifying >500 immune-related RNAs from serially collected peripheral blood samples using a subset of 9 subjects without BPAR or CAV and 9 subjects with at least one episode of BPAR. Again, the analyses did not show any significant relationships (data not shown). We did not specifically evaluate the association of gene used in the AlloMap® assay with BPAR, but 4/11 genes in AlloMap® (PDCD1, ITGAM, ITGA4, IL1R2) were present in the CTOT-05 nanostring panel. We performed univariate and multivariate logistic regression using these 4 markers to test for associations with central BPAR and locally treated rejection; none were significant univariately or in combination with others.

Plasma levels of angiogenesis-related proteins and CAV

We performed a Luminex screen for 17 angiogenesis/vascular injury-related proteins (Table 3) using plasma from a subset of subjects within the IVUS cohort (n=46 with paired samples). While none of the markers individually correlated with the development of CAV, multivariate analysis showed increases in VEGF-C (OR: 2.7; 95% CI 1.15–6.23) and FGF1 (OR: 14.1; 95% CI 1.06–188) combined with decreases in ET-1 (OR: 0.2; 95% CI 0.06–0.71) from pre-transplant to 12-months post-transplant were associated with CAV (Table 3). We subsequently performed ELISAs for ET-1, Leptin, VEGF-C and VEGF-A on all plasma samples obtained at study entry and 12-mo post-transplant from the subjects with paired IVUS evaluations (Figure 5, Table 4). The baseline plasma protein concentrations did not predict CAV, but increases in VEGF-C (p=0.013) and decreases in serum ET-1 (p=0.017) correlated with the development of CAV. Multivariable regression analyses (Table 4) that included clinical characteristics identified in Table 2 showed that the changes in VEGF-C (OR 20; 95% CI 1.9–218) and ET-1 (OR 0.14; 95% CI 0.02–0.97) were associated with CAV independent of ATG induction, CMV status or male sex. Changes in ET-1 or VEGF-C did not correlate with the composite endpoint.

Discussion

CTOT-05 was designed as a multicenter, observational study to assess relationships between markers and endpoints in a group of first heart transplant recipients treated with heterogeneous immunosuppression and CMV prophylaxis that reflects current standard of care in the US. In contrast to findings from previous, predominantly small, single-center biomarker reports of heart transplant recipients (4–20), the results of CTOT-05 did not show significant associations between the majority of the tested biomarkers and the composite endpoint or BPAR. Our findings suggest that the majority of the tested biomarkers are unlikely to be clinically useful surrogates for outcomes.

One weakness of the study design derives from heterogeneity in practice among study centers, including suboptimal standardization of clinically relevant endpoints. With regard to BPAR, significant variability of endomyocardial biopsy interpretation among expert pathologists reading the same biopsy slides has been previously documented (59, 60). In

CTOT-05, treatment decisions were based on local rather than core lab biopsy interpretation, and thresholds for initiating treatment likely differed among sites. Core lab biopsy diagnoses derived from analyses of sections from the same tissue block as those used for the local reading, but for some centers, unique sections were sent to the core lab, adding to variability. These differences likely contributed to the observed discordance between central reads of BPAR>2R and local decisions to treat for rejection (Figure S4), which could have confounded detection of associations between biomarkers and the composite endpoint and/or the BPAR component. In support of this concept a previously published report (59) indicated that a diagnosis of BPAR ≥2R made in isolation (without considering clinical context) was deemed insufficient for making clinical decisions or for use as a research criterion. We speculate that previously reported biomarker analyses from single center heart transplant studies provided more consistent correlations with BPAR because the grading of BPAR and the decision to treat are more uniform at a single site.

Regarding the IVUS component of the endpoint, while changes in IVUS measurements over 1–2 years post-transplant have been shown to be associated with graft survival (44, 61–65), limitations of using this technique for biomarker analyses also exist. The percentage of subjects with interpretable IVUS results in our study (53%) was similar to that previously reported from multicenter studies (53). Nonetheless, with 106 interpretable IVUS pairings and a lower than anticipated rate of developing CAV (23/106, ~20%), absence of detected associations between CAV and the tested biomarkers could have been a result of a type 2 error. In addition, the IVUS-defined endpoint in CTOT-05 was a measured increase in maximal intimal thickness of >0.5 mm in a matched coronary segment over 1 year. In the ensuing years since the initiation of CTOT-05 the heart transplant research community has adopted volumetric analyses as more sensitive and reliable measures of CAV than measurements of intimal thickness (66). Combined with other documented limitations of IVUS (67) and known coefficients of variance of most biomarkers of ~30% (50, 68), our findings suggest that future multicenter studies of biomarkers in heart transplantation may need to include several hundred evaluable subjects studied with volumetric-based IVUS analyses to have sufficient power to identify meaningful relationships with CAV.

One CTOT-05 study site employed a nontraditional immunosuppression protocol (no induction and tacrolimus only) that could have influenced detectable relationships between biomarkers and outcomes in the entire cohort. However, when we re-analyzed the data excluding subjects from this site we observed similar relationships compared to those observed in the entire cohort (not shown).

One perceived strength of CTOT-05 is that it was intentionally designed to identify biomarkers of heart transplant outcomes in the context of current clinical practice, which involves heterogeneous immunosuppression and CMV prophylaxis/therapy protocols. We acknowledge the possibility that the tested biomarkers may behave differently if immunosuppression/CMV therapy was identical across sites. Despite these acknowledged limitations we did observe that plasma levels of peripheral blood proteins associated with vascular injury and remodeling are promising biomarkers for development of CAV. Increases in the plasma VEGF-C together with decreases in ET1 (plasma ELISAs) over the first post-transplant year were strongly associated with developing CAV [AUC=0.794 from single

model; 0.750 from bootstrapped model]. VEGF-C is a well-established growth factor for lymphatic endothelial cells, and overexpression has been previously reported to contribute to immune-mediated chronic allograft injury (69, 70). The associated decreases in ET1 were not predicted from the known angiogenic mechanisms of action of this protein (71) and mechanistic links with VEGF-C remain speculative. Several studies have identified additional vascular growth factors including VEGF-A in blood (33, 72) and tissue (20, 69, 73, 74) as biomarkers of established CAV at later times post-transplantation. Most of these studies, including a 2013 publication (33) showed relationships between the markers and established angiographic CAV >5 years after transplantation. Our findings extend this and other previous work (10) by showing that select angiogenesis markers in the peripheral blood have the potential to inform risk of incipient CAV during the initial post-transplant year. Longer term follow-up will be required to directly assess relationships of angiogenesis markers obtained within the first year to the incidence of major adverse cardiac events detected at >2 years and to graft survival. Toward this end, an analysis of relationships between biomarkers obtained within the initial year post-transplant and 4-year outcomes of the CTOT-05 cohort is ongoing (CTOT-18, www.ctot.org).

Previous studies by our group among others (50, 51, 75–77) indicate that many of the tested biomarkers can provide diagnostic/prognostic information in kidney transplant recipients, raising the additional possibility that absence of correlations in the CTOT-05 cohort reflects organ specific differences. On the other hand, our observation that 3 genes found to be informative in acute kidney injury (Foxp3, GZMB and CXCL10 (78, 79) were significantly upregulated in heart graft tissue with histological evidence of acute cellular rejection (and the absence of a correlation between expression levels in the blood and the tissue, not shown) suggests that local events within the graft may result in the similar alloactivation of a subset(s) of T cells but that they cannot be detected in peripheral blood. The absence of detectable correlations between peripheral blood gene expression profiles and histological BPAR and/or IVUS-defined CAV in CTOT-05 contrasts with previous studies that reported AlloMap® is a useful biomarker in heart transplantation (3). We speculate that one key reason for what would appear as disparate conclusions is that the AlloMap® was shown to limit the requirement for endomyocardial biopsies (3) as opposed to being a diagnostic marker for rejection and/or CAV. We did not include the AlloMap® or all of its gene targets in the CTOT-05 analysis and are thus unable comment on AlloMap®'s diagnostic utility. We observed an association between serum anti-HLA antibodies and rejection/CAV, which together with results from other studies (80–82) supports the conclusion that anti-HLA antibodies are pathogenic in human heart transplant recipients. While the CTOT-05 analyses did not demonstrate a statistically significant association between DSA and outcomes, few evaluable subjects (n=15) developed DSA over this 12-month study, further supporting the need for evaluating larger cohorts followed for longer time periods.

The CTOT-05 results validate and extend previously identified clinical characteristics associated with an elevated risk of graft loss/death, BPAR or CAV (1). These factors include older donor age, recipient BMI, and worse pre-transplant clinical status as indicated by more urgent UNOS status and LVAD support (Table 1). Recipient CMV-negative serum status was also strongly associated with reaching these endpoints, consistent with previous studies (83–85). While the findings from our study suggest that subjects given ATG induction may be

protected from developing CAV, CTOT-05 was not designed to prospectively test this. We therefore caution the heart transplant community from reaching any conclusions about the efficacy of ATG induction based on our results.

In summary, the CTOT-05 findings indicate that reliable biomarkers for heart transplant outcomes remain elusive. They also highlight the limitations of BPAR and traditionally analyzed IVUS measurements as endpoints in multicenter, observational, biomarker studies. Suggestive evidence that plasma levels of VEGF-C and ET-1 along with serum alloantibodies identify heart transplant recipients at elevated risk for allograft injury require further validation. The CTOT-05 results provide useful lessons for improving future design of biomarker validation trials and biomarker-guided interventional trials in heart transplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Anti-IL-2R	anti-IL-2 receptor monoclonal antibody
AR	acute rejection
ATG	anti-thymocyte globulin
BPAR	biopsy proven acute rejection
CAV	coronary artery vasculopathy
CM	cardiac myosin

CMV	cytomegalovirus
CTOT	Clinical Trials in Organ Transplantation
DSA	donor specific antibody
ET-1	endothelin-1
FGF	fibroblast growth factor
HLA	Human Leukocyte Antigen
ISHLT	International Society of Heart and Lung Transplantation
IVUS	intravascular ultrasound
IFNγ	interferon gamma
LVAD	left ventricular assist device
MIT	maximal intimal thickness
MMF	mycophenolate mofetil
OR	odds ratio
QA	quality assurance
PBMC	peripheral blood mononuclear cells
PRT	Panel of Reactive T cells
VEGF	vascular endothelial growth factor
VM	vimentin

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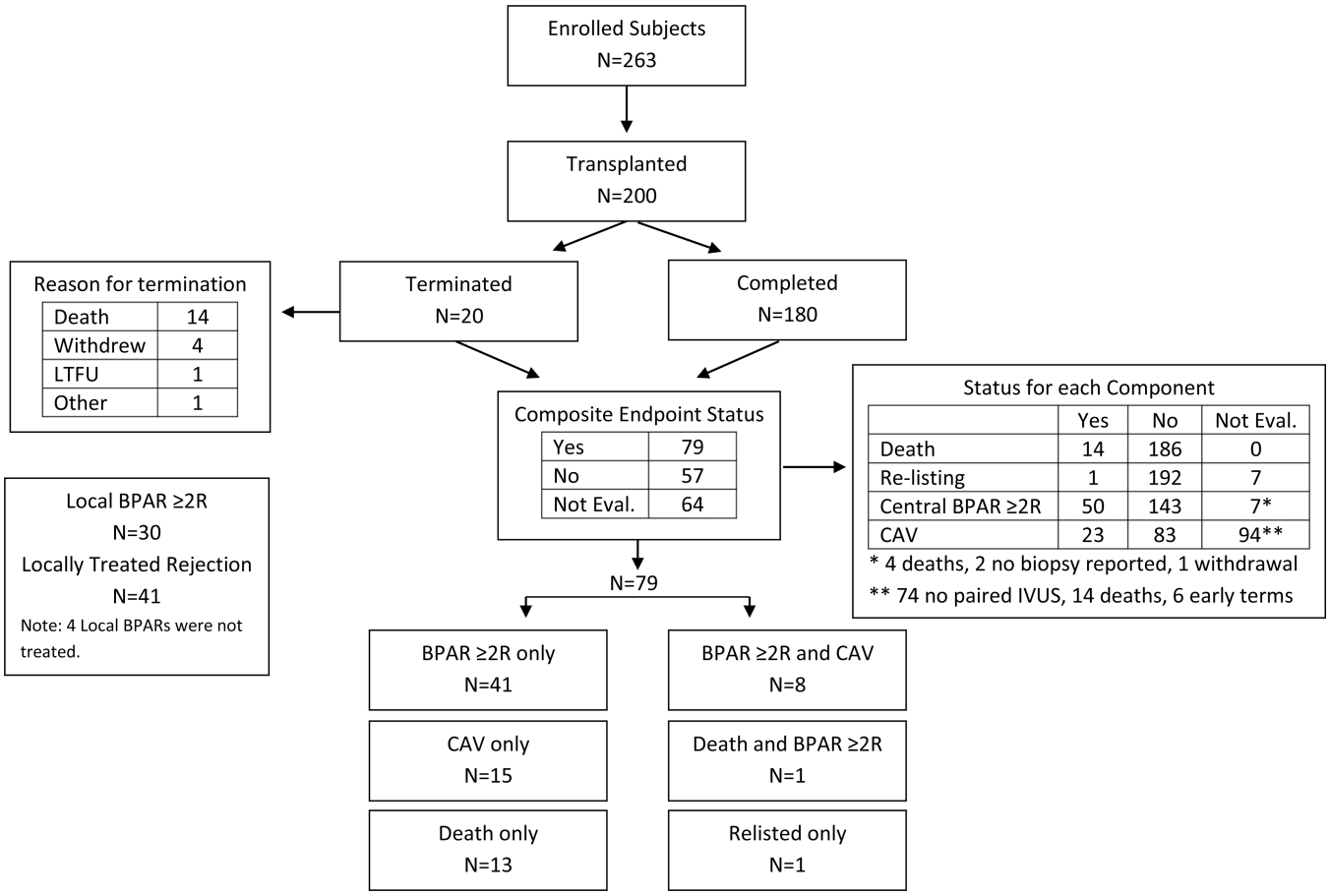

Figure 1. Overview of study outcomes

Consort diagram illustrating the outcomes of subjects throughout the course of the study including numbers and results of biopsies performed and numbers of subjects who reached the 12-month endpoint. 0f 200 transplanted subjects (and of 180 who completed the study), 64 were not evaluable for the primary composite endpoint because IVUS data were missing (not evaluable for the CAV component) and the subject did not have an episode of BPAR >2R. Subjects without IVUS data but who had an episode of BPAR component were considered evaluable because they met the BPAR component of the composite endpoint.

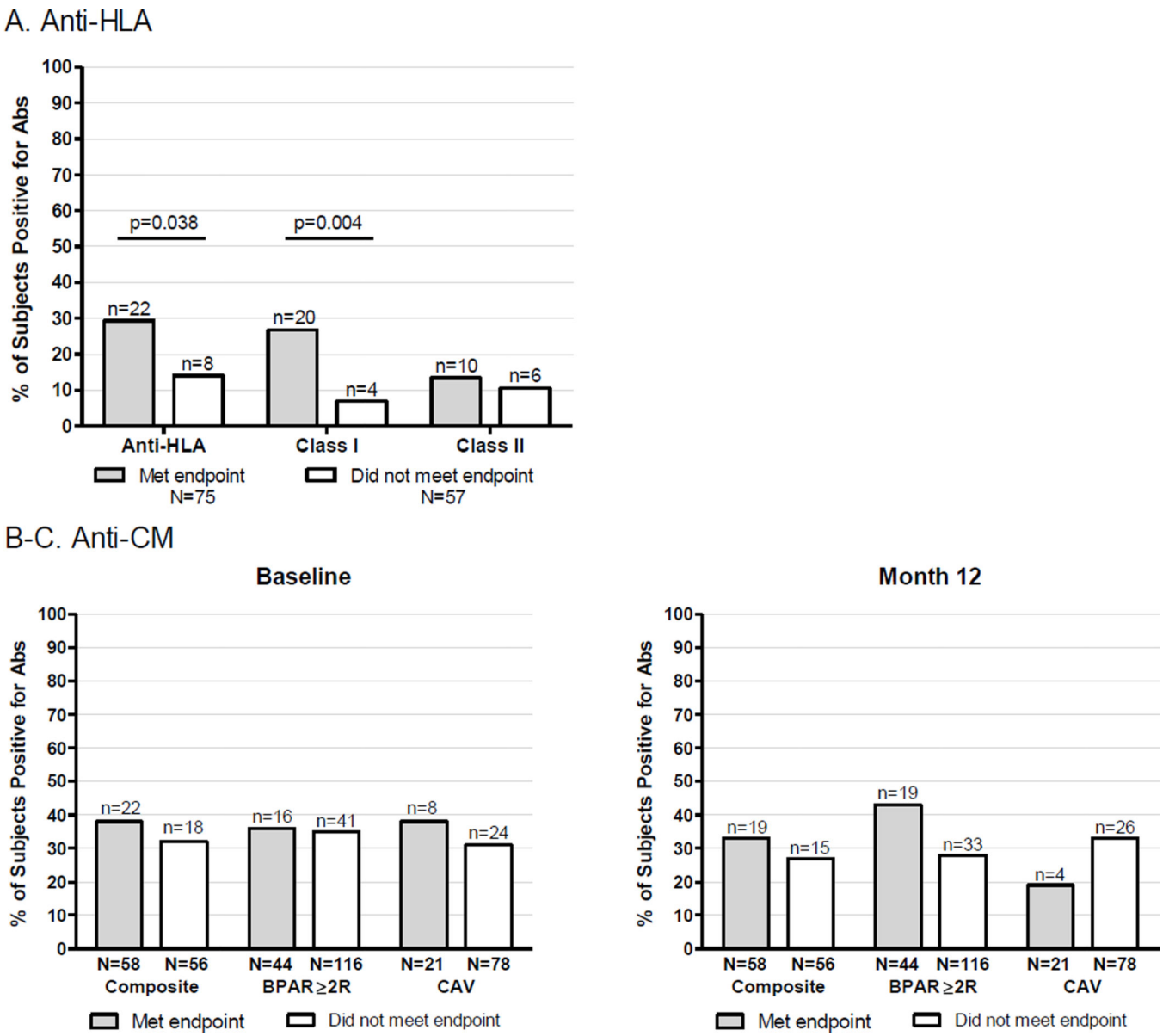

Figure 2. Relationships between serum anti-HLA or anti-CM antibodies and study outcomes

A. Percentages of study subjects with any serum anti-HLA antibodies (left), antibodies reactive to class I HLA (middle) and antibodies reactive to class II HLA (right), stratified by meeting (gray) or not meeting (white) the composite endpoint. B-C. Percentages of study subjects with serum anti-CM antibodies at baseline (B) or at 12-months (C) who met (gray) or did not meet (white) the composite endpoint (left), BPAR endpoint (middle), CAV endpoint (right). Anti-VM antibodies were also tested baseline and 12-months, and no relationships were observed with any of the endpoints at either time point (not shown).

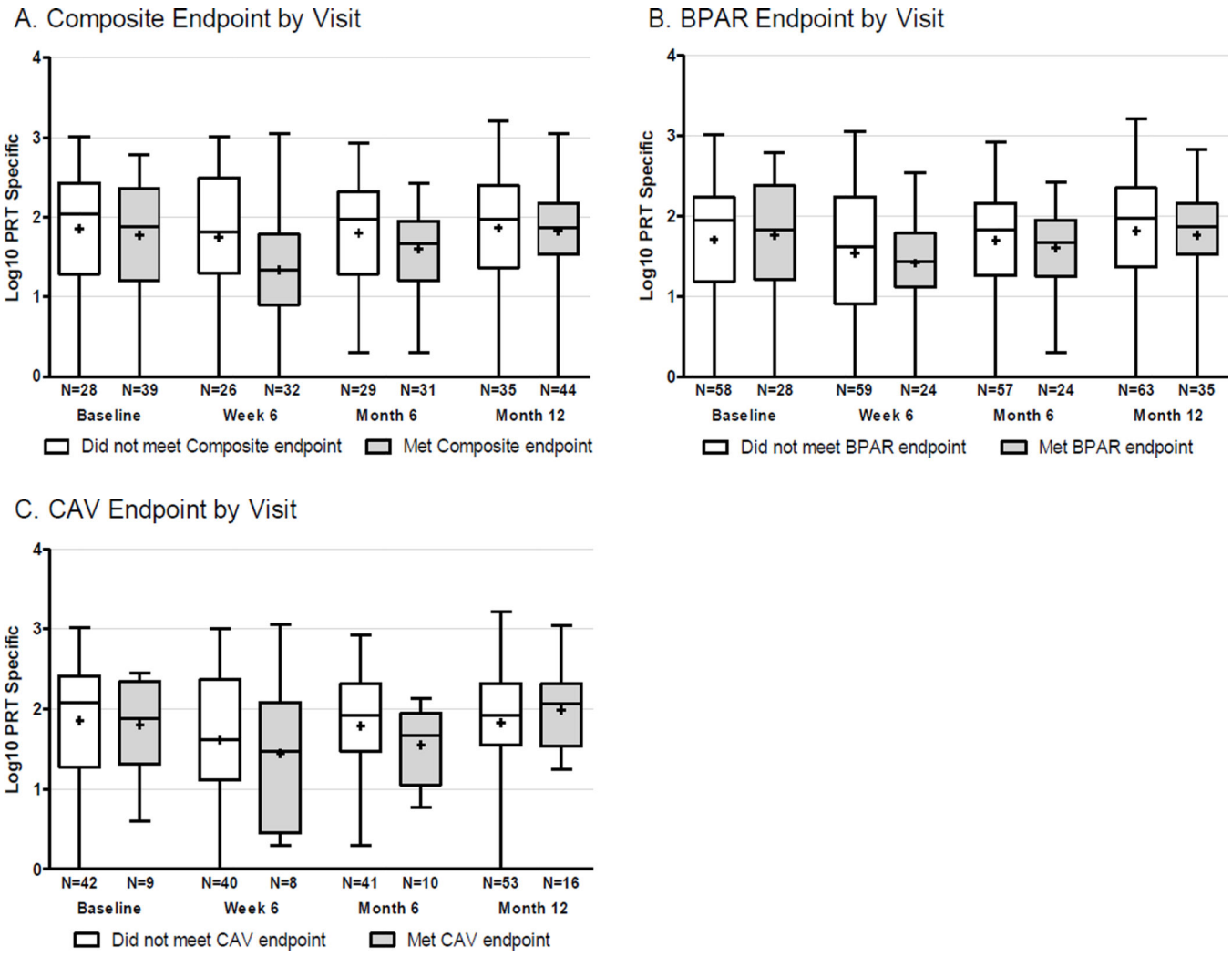

Figure 3. Panel of reactive T cell (PRT) and study outcomes

Frequencies of alloreactive IFNγ-producing PBMCs at baseline, 6-weeks, 6-months and 12-months post-transplant who met (grey) or did not meet (white) the composite endpoint (A), the BPAR endpoint (B) or CAV endpoint (C). The values (n) below each bar represent the number of subjects with available ELISPOT results who were evaluable for each endpoint. There were no statistically significant differences among groups.

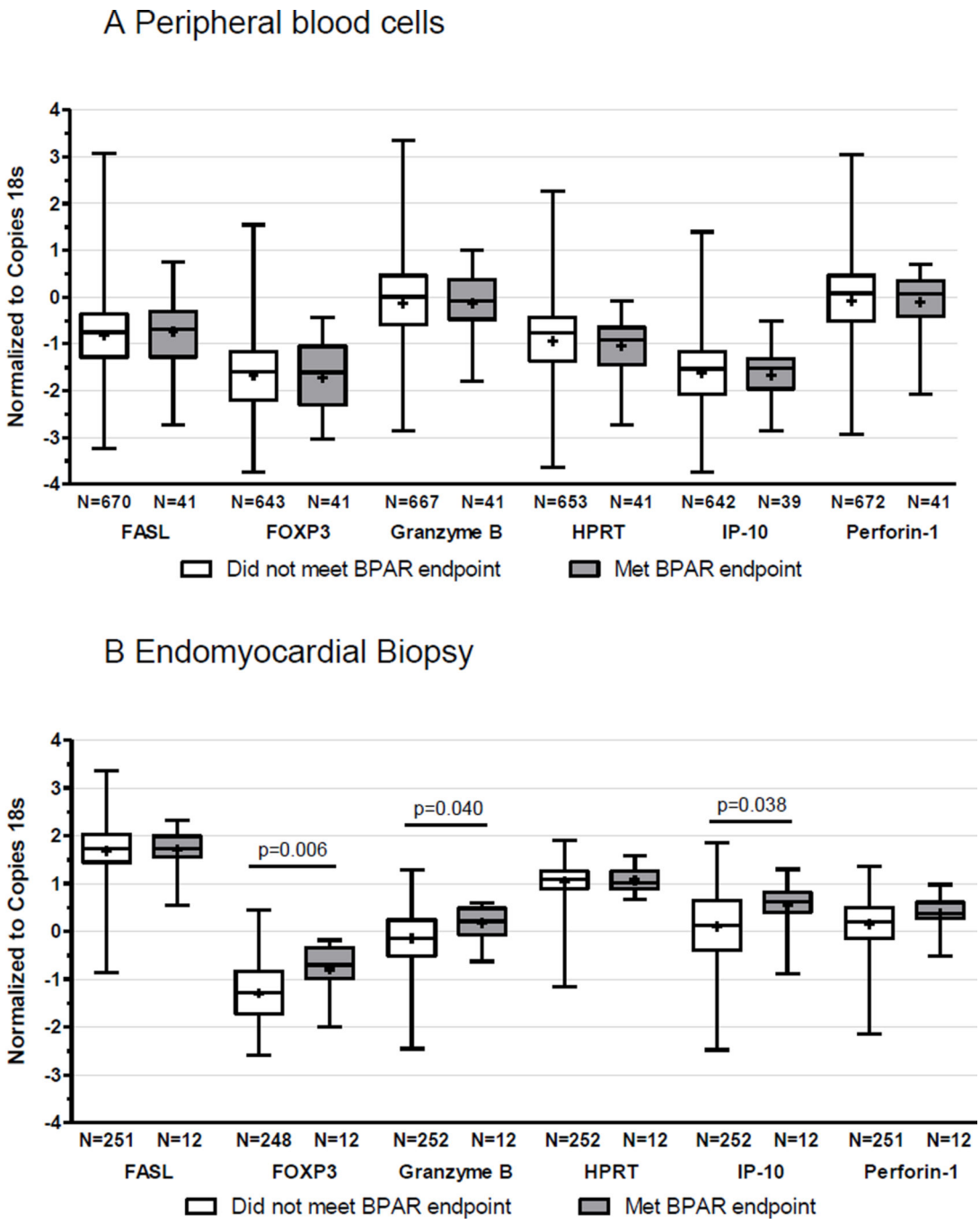

Figure 4. Gene expression profiling and study outcomes

A. Absolute copy numbers for each of the listed genes normalized to the copy number of 18S–rRNA in peripheral blood cell samples obtained up to the first episode of BPAR in each individual, stratified by meeting (gray) or not meeting (white) the BPAR endpoint. The values (n) below each bar represent the number of evaluable subjects with available PCR results. B. Absolute copy numbers for each of the listed genes normalized to the copy number of 18S–rRNA in endomyocardial tissue samples obtained at the time of protocol or for-cause biopsy <2 weeks prior to obtaining a biopsy sample that were read by the core

pathology laboratory as meeting the BPAR endpoint (gray) or not (white). The values (n) below each bar represent the number of evaluable subjects with available PCR results.

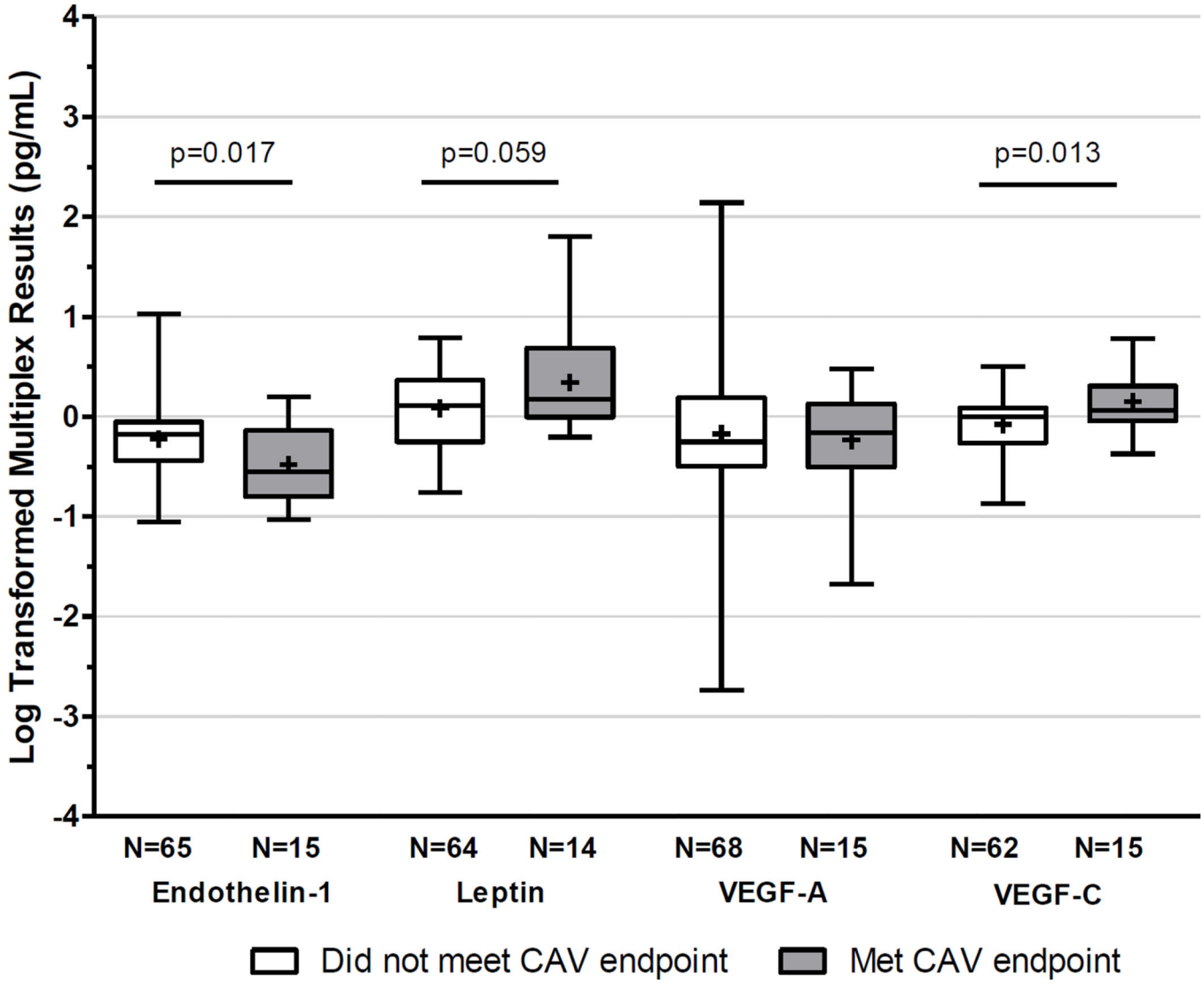

Figure 5. Plasma angiogenesis-related proteins and CAV

Change in plasma concentration by ELISA for each of the listed proteins between the pre-transplant and 1 year post-transplant visit stratified by meeting (gray) or not meeting (white) the CAV endpoint. The values (n) below each bar represent the number of IVUS evaluated subjects with available paired ELISA results.

Table 1

Baseline characteristics for all 200 transplanted subjects

Characteristics	Total Transplanted (N=200)
Donor Characteristics	
Age	
Mean ± SD	31.1 ± 11.84
Male Gender	139 (69.5)
Race	
White	135 (67.5)
Black or African American	34 (17.0)
Other	6 (3.0)
Unknown or Not Reported	25 (12.5)
Ethnicity	
Hispanic or Latino	33 (16.5)
Not Hispanic or Latino	141 (70.5)
Unknown or Not Reported	26 (13.0)
Cause of Death	
Anoxia	31 (15.5)
Cerebrovascular/Stroke	27 (13.5)
Head Trauma	102 (51.0)
Other	40 (20.0)
CMV IgG Status [1]	
Positive	118 (59.0)
Negative	79 (39.5)
Recipient Characteristics	
Age (year)	
Mean ± SD	53.6 ± 12.40
Male Gender	162 (81.0)
Race	
White	149 (74.5)
Black or African American	32 (16.0)
Other	9 (4.5)
Unknown or Not Reported	10 (5.0)
Ethnicity	
Hispanic or Latino	14 (7.0)
Not Hispanic or Latino	173 (86.5)
Unknown or Not Reported	13 (6.5)
Weight (kg) (N=188)	
Mean ± SD	81.6 ± 15.63

Characteristics	Total Transplanted (N=200)
BMI (kg/m²) (N=173)	
Mean ± SD	26.7 ± 4.51
Pre-operative Cardiac Diagnosis	
Idiopathic Dilated Cardiomyopathy	76 (38.0)
Ischemic Cardiomyopathy	70 (35.0)
Other	54 (27.0)
LVAD Support at the time of transplant	72 (36.0)
UNOS status at the time of transplant	
1A	110 (55.0)
1B	78 (39.0)
2	12 (6.0)
CMV IgG Status [1]	
Positive	112 (56.0)
Negative	86 (43.0)
Donor,Recipient CMV IgG Status	
D+,R−	53 (26.5)
D+,R+	64 (32.0)
D−,R+	47 (23.5)
D−,R−	31 (15.5)
Use of Induction Therapy (N=194)	
Anti-IL-2R	35 (18.0)
ATG	49 (25.3)
Anti-IL-2R or ATG	83 (42.8)
Use of Maintenance Therapy within 1st Month (N=176)	
Tacrolimus/Cyclosporine	173 (98.3)
MMF	173 (98.3)
Steroids	172 (97.7)

[1]Three subjects have no donor CMV status summarized here: 1 reported as ‘Indeterminate’ and 2 reported as ‘Not Done’. Two subjects have no recipient CMV status summarized here: 1 reported as ‘Not Done’ and 1 is missing.

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Table 2

Baseline Characteristics of Study Subjects Stratified by Primary Composite, Central BPAR, and CAV Endpoints

Characteristic	Primary Composite Endpoint = Yes (N=79)	Primary Composite Endpoint = No (N=57)	p-val	Central BPAR ≥2R = Yes (N=50)	Central BPAR ≥2R = No (N=143)	p-val	CAV Endpoint = Yes (N=23)	CAV Endpoint = No (N=83)	p-val
Donor Characteristics									
Age									
Mean ± SD	33.3 ± 11.53	28.8 ± 11.32	0.027	32.5 ± 11.56	30.5 ± 12.01	0.313	34.1 ± 10.96	29.6 ± 11.62	0.099
Male Gender	53 (67.1)	39 (68.4)	0.870	31 (62.0)	102 (71.3)	0.220	16 (69.6)	54 (65.1)	0.686
Race									
White	55 (69.6)	39 (68.4)	0.264	33 (66.0)	99 (69.2)	0.257	18 (78.3)	54 (65.1)	0.482
Black or African American	15 (19.0)	7 (12.3)		12 (24.0)	19 (13.3)		3 (13.0)	15 (18.1)	
Other	1 (1.3)	3 (5.3)		1 (2.0)	5 (3.5)		0	3 (3.6)	
Unknown or Not Reported	8 (10.1)	8 (14.0)	NA	4 (8.0)	20 (14.0)	NA	2 (8.7)	11 (13.3)	NA
Ethnicity									
Hispanic or Latino	14 (17.7)	12 (21.1)	0.641	8 (16.0)	25 (17.5)	0.860	6 (26.1)	16 (19.3)	0.557
Not Hispanic or Latino	56 (70.9)	39 (68.4)		35 (70.0)	101 (70.6)		15 (65.2)	60 (72.3)	
Unknown or Not Reported	9 (11.4)	6 (10.5)	NA	7 (14.0)	17 (11.9)	NA	2 (8.7)	7 (8.4)	NA
Cause of Death									
Anoxia	16 (20.3)	7 (12.3)	0.281	10 (20.0)	20 (14.0)	0.563	4 (17.4)	15 (18.1)	0.599
Cerebrovascular/Stroke	10 (12.7)	4 (7.0)		5 (10.0)	21 (14.7)		3 (13.0)	7 (8.4)	
Head Trauma	35 (44.3)	34 (59.6)		23 (46.0)	74 (51.7)		9 (39.1)	44 (53.0)	
Other	18 (22.8)	12 (21.1)		12 (24.0)	28 (19.6)		7 (30.4)	17 (20.5)	
CMV IgG Status *[1]*									
Positive	50 (63.3)	34 (59.6)	0.532	30 (60.0)	84 (58.7)	0.799	16 (69.6)	50 (60.2)	0.414
Negative	27 (34.2)	23 (40.4)		19 (38.0)	58 (40.6)		7 (30.4)	33 (39.8)	
Recipient Characteristics									
Age (year)									
Mean ± SD	53.9 ± 12.30	53.5 ± 11.68	0.838	53.5 ± 12.86	53.9 ± 11.92	0.838	52.0 ± 12.04	53.4 ± 12.31	0.622
Male Gender	68 (86.1)	43 (75.4)	0.114	43 (86.0)	113 (79.0)	0.281	22 (95.7)	63 (75.9)	0.039

Characteristic	Primary Composite Endpoint = Yes (N=79)	Primary Composite Endpoint = No (N=57)	p-val	Central BPAR ≥2R = Yes (N=50)	Central BPAR ≥2R = No (N=143)	p-val	CAV Endpoint = Yes (N=23)	CAV Endpoint = No (N=83)	p-val
Race									
White	58 (73.4)	44 (77.2)	0.234	37 (74.0)	108 (75.5)	0.624	19 (82.6)	61 (73.5)	0.371
Black or African American	13 (16.5)	5 (8.8)		10 (20.0)	20 (14.0)		1 (4.3)	13 (15.7)	
Other	3 (3.8)	5 (8.8)		3 (6.0)	6 (4.2)		2 (8.7)	6 (7.2)	
Unknown or Not Reported	5 (6.3)	3 (5.3)	NA	0	9 (6.3)	NA	1 (4.3)	3 (3.6)	NA
Ethnicity									
Hispanic or Latino	2 (2.5)	5 (8.8)	0.238	2 (4.0)	11 (7.7)	0.736	1 (4.3)	5 (6.0)	>0.999
Not Hispanic or Latino	68 (86.1)	49 (86.0)		41 (82.0)	127 (88.8)		21 (91.3)	73 (88.0)	
Unknown or Not Reported	9 (11.4)	3 (5.3)	NA	7 (14.0)	5 (3.5)	NA	1 (4.3)	5 (6.0)	NA
Weight (kg)	N=72	N=55	0.010	N=45	N=136	0.029	N=21	N=79	0.088
Mean ± SD	85.0 ± 16.80	77.4 ± 15.94		86.2 ± 17.47	80.3 ± 14.94		85.9 ± 17.19	78.9 ± 16.59	
BMI (kg/m²)	N=63	N=50	0.048	N=38	N=128	0.165	N=20	N=69	0.285
Mean ± SD	27.5 ± 4.35	25.8 ± 4.69		27.6 ± 4.56	26.4 ± 4.58		27.4 ± 4.62	26.1 ± 4.79	
Pre-operative Cardiac Diagnosis									
Idiopathic Dilated Cardiomyopathy	30 (38.0)	23 (40.4)	0.785	22 (44.0)	54 (37.8)	0.420	7 (30.4)	36 (43.4)	0.380
Ischemic Cardiomyopathy	28 (35.4)	17 (29.8)		19 (38.0)	50 (35.0)		10 (43.5)	24 (28.9)	
Other	21 (26.6)	17 (29.8)		9 (18.0)	39 (27.3)		6 (26.1)	23 (27.7)	
LVAD Support at the time of transplant	30 (38.0)	13 (22.8)	0.061	19 (38.0)	50 (35.0)	0.700	7 (30.4)	22 (26.5)	0.708
UNOS status at the time of transplant									
1A	48 (60.8)	28 (49.1)	0.006	32 (64.0)	72 (50.3)	0.151	10 (43.5)	47 (56.6)	0.056
1B	30 (38.0)	20 (35.1)		17 (34.0)	60 (42.0)		13 (56.5)	27 (32.5)	
2	1 (1.3)	9 (15.8)		1 (2.0)	11 (7.7)		0	9 (10.8)	
CMV IgG Status [1]									
Positive	37 (46.8)	43 (75.4)	<0.001	24 (48.0)	84 (58.7)	0.172	10 (43.5)	57 (68.7)	0.027
Negative	42 (53.2)	14 (24.6)		26 (52.0)	58 (40.6)		13 (56.5)	26 (31.3)	
Donor,Recipient CMV IgG Status									
D+,R−	26 (32.9)	9 (15.8)		15 (30.0)	38 (26.6)		8 (34.8)	16 (19.3)	

Characteristic	Primary Composite Endpoint = Yes (N=79)	Primary Composite Endpoint = No (N=57)	p-val	Central BPAR ≥2R = Yes (N=50)	Central BPAR ≥2R = No (N=143)	p-val	CAV Endpoint = Yes (N=23)	CAV Endpoint = No (N=83)	p-val
D+,R+	24 (30.4)	25 (43.9)	0.015	15 (30.0)	46 (32.2)	0.566	8 (34.8)	34 (41.0)	0.112
D−,R+	13 (16.5)	18 (31.6)		9 (18.0)	37 (25.9)		2 (8.7)	23 (27.7)	
D−,R−	14 (17.7)	5 (8.8)		10 (20.0)	20 (14.0)		5 (21.7)	10 (12.0)	
Use of Induction Therapy	N=73	N=57		N=49	N=139		N=22	N=83	
Anti-IL-2R	10 (13.7)	18 (31.6)	0.014	6 (12.2)	28 (20.1)	0.217	5 (22.7)	19 (22.9)	0.987
ATG	11 (15.1)	15 (26.3)	0.112	5 (10.2)	42 (30.2)	0.005	1 (4.5)	18 (21.7)	0.070
Anti-IL-2R or ATG	20 (27.4)	33 (57.9)	<0.001	11 (22.4)	70 (50.4)	<0.001	6 (27.3)	37 (44.6)	0.142

[1]Counts of ‘Indeterminate’ and ‘Not Done’ results are not presented here.

Table 3

Change in plasma concentrations of angiogenesis-related proteins (Luminex) from pre-transplant to 1 year post-transplant is associated with the development of CAV by IVUS

		Univariate			Multivariate		
Parameter	**n**	**OR**	**95% CI**	**p-value**	**OR**	**95% CI**	**p-value**
Change in Angiopoietin-2	46	1.50	0.22–10.49	0.680			
Change in BMP-9	46	1.11	0.21–5.90	0.906			
Change in EGF	46	2.01	0.51–7.98	0.319			
Change in Endoglin	46	0.86	0.36–2.08	0.738			
Change in Endothelin-1	46	0.50	0.21–1.20	0.119	0.20	0.06–0.71	0.012
Change in FGF-1	46	3.45	0.47–25.17	0.221	14.11	1.06–188.22	0.045
Change in FGF-2	46	3.74	0.08–168.41	0.497			
Change in Follistatin	46	0.80	0.25–2.56	0.704			
Change in G-CSF	46	0.78	0.30–2.01	0.606			
Change in HB-EGF	46	5.25	0.20–138.31	0.320			
Change in HGF	46	0.45	0.18–1.17	0.103			
Change in IL-8	46	1.10	0.53–2.26	0.803			
Change in Leptin	46	1.89	0.37–9.66	0.442			
Change in PLGF	46	1.06	0.55–2.05	0.860			
Change in VEGF-A	46	1.20	0.61–2.37	0.599			
Change in VEGF-C	46	1.69	0.86–3.33	0.128	2.68	1.15–6.23	0.022
Change in VEGF-D	46	1.33	0.42–4.22	0.632			

N= 46 subjects. Subjects were randomly selected from the CAV cohort who met or did not meet the endpoint (2:1 ratio).

Table 4

Change in plasma concentrations of angiogenesis-related proteins (ELISA) from pre-transplant to 1 year post-transplant is associated with the development of CAV by IVUS

		Univariate			Multivariate (n=74)		
Parameter	**n**	**OR**	**95% CI**	**p-value**	**OR**	**95% CI**	**p-value**
Male	106	6.98	0.88–55.13	0.065			
CMV+ Recipient	106	0.35	0.14–0.90	0.030	0.35	0.09–1.28	0.112
ATG Induction	105	0.17	0.02–1.37	0.096			
Change in Endothelin-1	80	0.12	0.02–0.69	0.017	0.14	0.02–0.97	0.047
Change in Leptin	78	3.71	0.95–14.49	0.059			
Change in VEGF-A	83	0.90	0.43–1.86	0.770			
Change in VEGF-C	77	15.98	1.79–142.49	0.013	20.11	1.85–218.04	0.014

N=106 subjects with IVUS evaluations