ELSEVIER

The Journal of Heart and Lung Transplantation
http://www.jhltonline.org

ORIGINAL CLINICAL SCIENCE

Late graft dysfunction after pediatric heart transplantation is associated with fibrosis and microvasculopathy by automated, digital whole-slide analysis

Brian Feingold, MD, MS,[a,b] Jennifer Picarsic, MD,[c] Andrew Lesniak, BS,[c,d] Benjamin A. Popp, BS,[d] Michelle A. Wood-Trageser, PhD,[c,d] and Anthony J. Demetris, MD, PhD[c,d]

From the [a]Department of Pediatrics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA; [b]Clinical and Translational Science, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA; [c]Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA; and the [d]Division of Transplant Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA.

KEYWORDS:
pediatric;
heart;
transplant;
fibrosis;
allograft;
vasculopathy

BACKGROUND: Histopathologic features of late graft dysfunction (LGD) in endomyocardial biopsies (EMBs) after pediatric heart transplantation (HT) have been incompletely described and rarely quantified. We employed automated, morphometric analysis of whole-slide EMB images to objectively quantify fibrosis and microvasculopathy after pediatric HT.

METHODS: Nine recipients with clinical LGD were matched with controls on age, listing diagnosis, crossmatch and time since HT. Fibrosis was quantified as percent tissue area with fibrosis and capillary density as capillaries per unit area, number of capillary "neighbors" within 30 μm of each myocyte and myocyte-to-nearest-capillary diffusion distance. Clinical data, including all EMB reports, were also reviewed.

RESULTS: The groups were well matched for age at HT (median 4.0 vs 3.1 years), listing diagnosis (50% congenital heart disease for each), positive crossmatch (11% each) and days post-HT (2,628 vs 2,894, $p = 0.69$). Despite a similar number of previous EMBs (median 23 each, $p = 0.43$), areas occupied by fibrosis were greater in LGD cases (44.5% vs 23.2%, $p = 0.012$). Capillary number/area data were not statistically different between LGD cases and controls (378/mm^2 vs 559/mm^2, $p = 0.57$), but LGD cases more commonly had zero capillary neighbors (35% vs 20%, $p = 0.02$) and greater myocyte-to-nearest-capillary distances (27.1 μm vs 18.7 μm, $p = 0.005$). Cumulative rejection history correlated with fibrosis ($r = 0.49$, $p = 0.039$) and myocyte-to-nearest-capillary distance ($r = 0.5$, $p = 0.036$).

CONCLUSIONS: LGD after pediatric HT is associated with previous rejection and characterized histologically by fibrosis and microvasculopathy, which are not readily appreciated by traditional semi-quantitative EMB analysis. Software-assisted EMB analysis may enable greater pathophysiologic understanding of LGD and identification of targets for future study and intervention.

J Heart Lung Transplant ■■■;■:■■■–■■■

Late allograft dysfunction (LGD) after pediatric heart transplantation (HT) is an ominous finding associated with a high risk of death and/or allograft loss.[1–3] In the absence of acute rejection, LGD is usually attributed to coronary

Reprint requests: Brian Feingold, MD, MS, Children's Hospital of Pittsburgh of UPMC, 4401 Penn Avenue, Faculty Pavilion, Fifth Floor, Pittsburgh, PA 15224. Telephone: 412-692-5541; Fax: 412-692-6991.
E-mail address: brian.feingold@chp.edu

1053-2498/$ - see front matter
http://dx.doi.org/10.1016/j.healun.2017.09.012

allograft vasculopathy (CAV).[4] Histopathologic hallmarks of CAV include intimal proliferation with medial thinning of coronary vessels, resulting in luminal stenosis.[5,6] This process is often diffuse, silently progressive and poorly detected by angiography in its earliest stages. Microvascular blood flow may be diminished more than flow in larger epicardial vessels,[7,8] and microcirculation rarefaction of cardiac allografts may be associated with worse outcomes independent of epicardial vasculopathy.[9,10] Symptomatic heart failure and systolic ventricular dysfunction on echocardiography are the most frequently recognized clinical features of CAV-associated LGD in children, although isolated elevation of ventricular filling pressures (i.e., diastolic dysfunction) has also been described.[11,12] The prognostic significance of isolated diastolic dysfunction for allograft loss in children was recently confirmed in a large, multi-institutional analysis.[13]

Myocardial fibrosis is a well-described feature of cardiac histopathology in adult HT recipients,[14] where it has been linked to repeated endomyocardial biopsies (EMBs), advanced CAV and poor allograft survival.[15] In the native heart, myocardial fibrosis is implicated in arrhythmogenesis, diastolic dysfunction, impaired myocardial oxygenation and pathologic ventricular remodeling.[16–18] With the exception of a single descriptive pathology study of 14 explanted pediatric allografts,[19] the literature is devoid of analyses of fibrosis and microvasculopathy after pediatric HT, with no studies of fibrosis or microvascular quantification using clinical EMB samples.

In this pilot analysis, we compared clinical right ventricular (RV) EMB specimens from pediatric HT recipients who met a pre-defined, clinical LGD phenotype to matched controls using automated morphometric analysis of fibrosis and microvasculature density on whole-slide imaging (WSI). We hypothesized that automated analysis of WSI can quantify myocardial fibrosis and microvasculopathy on EMB, and that recipients with LGD have more myocardial fibrosis and decreased microvascular density compared with matched controls lacking LGD.

Methods

After institutional review board approval, we identified all pediatric HT recipients at our center for whom we could establish a clinical phenotype of LGD ($n = 9$). We defined LGD as meeting at least 2 of the following 4 criteria in the absence of T-cell–mediated rejection (TCMR): (1) right atrial pressure ≥10 mm Hg; (2) pulmonary capillary wedge pressure ≥12 mm Hg; (3) cardiac index ≤2.2 liters/min/m^2; and (4) ≥3-month diuretic requirement in the absence of moderate to severe kidney disease (glomerular filtration rate [GFR] <45 ml/min/1.73 m^2). Each case was matched to a control recipient without LGD for: age at HT; listing diagnosis; and donor-specific, complement-dependent cytotoxicity (CDC) crossmatch result. For each LGD case, the EMB at (or nearest following) the onset of the LGD clinical phenotype (i.e., index case EMB) was selected for automated WSI analysis. For controls, the EMB closest in time after HT to the matched case's index EMB was selected for comparison (i.e., index control EMB). In both groups, we also selected the EMB nearest to 6 months post-HT (baseline) to compare features early after transplantation. Pertinent clinical data were collected from the medical record, including EMB rejection grades and treatment.

Selected EMBs were formalin-fixed, paraffin-embedded, sectioned at 4 μm, then routinely stained with hematoxylin and eosin, Masson's trichrome (fibrosis), C4d and CD31 (capillary staining). All slides were assessed blindly for staining quality and converted to high-resolution WSI (40× scan: 0.13 μm/pixel; Mirax Midi, Zeiss, Oberkochen, Germany). Automated WSI analysis using NEARCYTE (http://nearcyte.org/) was performed, employing a standard deconvolution-based methodology to identify tissue edges; trichrome-stained areas; and segmented cardiomyocyte nuclei, distinguished from other cell-type nuclei by size. A 2-pass method was used to identify interstitial capillaries on CD31-immunostained slides: (1) objects with an elongation value of ≥3 were excluded to eliminate longitudinally cut microvessels; and (2) remaining objects were screened by following the pixel direction of connections on the shape to verify the structure approached a circle (i.e., a "splat"). Results with overlays showing identified capillaries were blindly assessed to confirm accuracy.

Percent fibrosis area was calculated by dividing the collagen-occupied area by the total tissue area using standard deconvolution techniques without human thresholding, eliminating any potential selection bias. The number of CD31 splats was calculated and divided by total tissue area to determine traditional capillary density. Two additional capillary spacing measurements were calculated, including: (1) the number of nearest capillary neighbors within a 30-μm radius from the geographic center of each myocyte nucleus; and (2) the shortest distance between the geographic center of each myocyte to its closest capillary (myocyte-to-nearest-capillary diffusion distance). Our reasoning for these analyses was that immunologically-mediated microvascular disruption leads to localized areas of ischemia, which in turn triggers microvascular growth and remodeling to ameliorate hypoperfused areas.[20] Uneven restitution of the normal microvascular network causes changes in the shape/distribution of the capillaries and at least temporarily increases the distance from capillaries to the nearest cardiac myocyte, which can be automatically and precisely measured in high-resolution WSIs.

Although we collected the number of EMBs that resulted in treatment for rejection, this does not account for lower grades of TCMR that did not prompt treatment, nor does it account for antibody-mediated rejection (AMR) because some EMBs date to the mid-1990s when C4d staining and AMR assessments were not routinely performed. Thus, we performed a blinded review of the detailed histologic descriptions from all clinical pathology reports to better classify the presence and severity of TCMR and AMR using current International Society for Heart and Lung Transplantation (ISHLT) criteria.[21,22] To quantify cumulative TCMR "exposure," we assigned a rank score to each EMB using the ISHLT's 1990 criteria[22] as follows: Grade 0 = 0 point; Grade 1A = 1 point; Grade 1B = 2 points; Grade 2 = 3 points; Grade 3A = 4 points; and Grade 3B/4 = 5 points.

Data are presented as count (%) or median (interquartile range), unless otherwise noted. Group comparisons were performed using Fisher's exact test or the rank-sum test, as appropriate. Results of the capillary nearest-neighbor analysis were assessed by frequency histograms, comparing proportions of 0, 1 or 2, and ≥3 capillary nearest neighbors within a 30-μm radius between cases and controls. Pearson's coefficient or Spearman's rank test was used, as appropriate, to assess whether histologic features correlated with patient or allograft characteristics. All statistical tests used a 2-sided $\alpha < 0.05$ and were performed using STATA SE version 14.1 software (StataCorp LP, College Station, TX).