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Highly Sensitive Non-Invasive Cardiac Transplant Rejection Monitoring Using Targeted Quantification of Donor Specific Cell Free DNA

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Brief title: Donor specific cf DNA to monitor cardiac transplant

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To the Editor: Approximately 20,000 cardiac transplant recipients currently reside in the US. Rejection remains a major cause of graft failure and requires lifelong surveillance. The current gold standard for monitoring rejection is catheter based endomyocardial biopsy (EMB) which is associated with risk and expense (1). Donor specific cell free DNA (DScf-DNA) has been proposed as a marker for cellular injury caused by rejection (2). Shotgun whole genome sequencing (WGS) has been used to detect DScf-DNA (3). The complexity and cost of the analysis required by WGS limits its application as a surveillance tool. We have employed targeted quantitative genotyping to determine the percent DScf-DNA. The targeted approach relies on selected highly informative genomic regions and provides a rapid (24-48 hours) cost-efficient (<$1000) method potentially suitable for clinical surveillance. We applied this method to detect DScf-DNA in pediatric cardiac transplant recipients in a prospective blinded study.

Cardiac transplant recipients followed at the Herma Heart Center at the Children's Hospital of Wisconsin were invited to participate. Blood samples (5cc) were collected under three clinical scenarios. 1) **scheduled surveillance EMB -** from asymptomatic heart transplant recipients in the catheterization laboratory immediately prior to scheduled surveillance EMB. 2) **unscheduled diagnostic EMB** - from symptomatic heart transplant recipients prior to unscheduled diagnostic EMB. 3) **rejection** - from heart transplant recipients with biopsy proven rejection (ISHLT grade 2R or AMR1) before initiation of treatment, during treatment, and at one week. Clinical, laboratory, cardiac catheterization and echocardiographic data were recorded. Anti-coagulated blood was collected to measure cf-DNA levels. Plasma separation, cf-DNA extraction and quantification of total cf-DNA (Tcf-DNA) was carried out as previously described (4). Genomic DNA for genotyping was prepared from one buffy coat of each recipient, and donor DNA was obtained from the Blood Center of Wisconsin. Determination of

the percent DScf-DNA in recipient plasma was performed using Digital Analysis of Selected Regions (DANSR^{TM}, Ariosa Diagnostics, San Jose, CA) (5). Genotyping of donor and recipient genomic DNA was carried out by the same assay. Loci are informative when recipient genotypes are homozygous and donor genotypes are either heterozygous or homozygous for the other allele. The minor allele frequency for informative loci was modeled as a binomial distribution. The percent DScf-DNA was defined as the peak from this modeling. Summary statistics included median and range. Unpaired samples (i.e. rejection-group vs. surveillance-group) were compared using a Mann-Whitney test. Rejection samples were compared with a Friedman analysis of variance. A Pearson correlation summarized correlations. A P-value < 0.05 was considered significant.

Fifty-three samples from 32 patients were analyzed. **Scenario-1)** cf-DNA levels from 26 patients undergoing 38 scheduled surveillance EMBs (Figure 1A). Thirty-two (84%) samples contained <1% DScf-DNA. No patient with DScf-DNA <1% had pathological rejection. DScf-DNA levels exceeded1% in 6 samples with the highest percentage DScf-DNA associated with asymptomatic biopsy proven rejection. The remaining five samples had negative biopsies. **Scenario-2)** Seven samples were obtained from six patients prior to unscheduled diagnostic EMB to rule out rejection based on clinical criteria (Figure 1B and D). Six had DScf-DNA levels >1% and one sample contained DScf-DNA <1%. Four of the six were associated with biopsy proven rejection; the other two patients had significant coronary artery vasculopathy on angiography. The single symptomatic patient with low percentage DScf-DNA had high levels of Tcf-DNA (Figure 1D), implying that the dominant pathology was global rather than confined to the donor organ. This patient was diagnosed with culture positive sepsis, the accompanying EMB was negative for rejection and coronary angiography was normal. **Scenario-3)** Four

patients with biopsy proven rejection were analyzed. All pre-treatment samples collected at diagnosis contained DScf-DNA>1% (sensitivity 100%). Following IV immunosuppressive therapy, all patients demonstrated significantly decreased %DScf-DNA. Interestingly, 3-4 days after discontinuing augmented immunosuppression, the percent DScf-DNA rebounded in three of four patients (Figure 1C). DScf-DNA was compared to other candidate non-invasive laboratory variables (BNP, Troponin, CRP) as well as echocardiographically determined LVEF in predicting rejection on biopsy; DS-cfDNA had the highest sensitivity and specificity (100%/84%).

DScf-DNA may be sufficiently sensitive to detect rejection and injury to the donor organ earlier than currently available methods. Levels of Dscf-DNA fall consistently by one week post-transplant which may allow for non-invasive detection of rejection in the vulnerable early post-transplant period (data not shown). A sensitive non-invasive rejection monitoring method could decrease the number of biopsies needed over a lifespan considerably thereby decreasing complications, discomfort, and cost. We were able to detect all rejection episodes including both cellular and antibody mediated rejection at the earliest onset and even prior to clinical indicators of disease. However, these results are based on a limited sample size. A larger validation study is needed.

In summary, targeted quantitative genotyping was employed to determine circulating levels of DScf-DNA in pediatric heart transplant recipients. The percentage of DScf-DNA was elevated in all patients diagnosed with rejection. Further, all patients with DScf-DNA levels less than 1% were shown by biopsy and clinical parameters to be negative for rejection (negative predictive value 100%). Targeted quantitative genotyping of circulating DScf-DNA constitutes a

sensitive, rapid, and cost-effective non-invasive tool potentially suitable for rejection surveillance as an alternative to EMB.

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Figure legend

Figure 1: cf-DNA in patients undergoing surveillance EMB and during rejection. Panels **A** & **B**) Percent DScf-DNA in scheduled surveillance EMB (A) and unscheduled diagnostic EMB samples (B). Panel **C)** Boxplot of surveillance EMB samples (black circles) and rejection samples (red circles) collected at 3 time points. Panel **D)** Total cf-DNA in unscheduled diagnostic EMB samples. Data in panels A, B and D are sorted on the x-axis according to increasing percent DScf-DNA. Samples in panels B and D align vertically. The dashed line in panels A, B and C highlights the 1% DScf-DNA level. The vertical solid line in panel A orient the picture so all samples containing less than 1% DScf-DNA are on the left-hand side and all samples greater than 1% are on the right.

Figure1 (Hidestrand *et al*)