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CORRESPONDENCE

Research Correspondence

Highly Sensitive Noninvasive Cardiac Transplant Rejection Monitoring Using Targeted Quantification of Donor-Specific Cell-Free Deoxyribonucleic Acid

To the Editor: Approximately 20,000 cardiac transplant recipients currently reside in the United States. 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 deoxyribonucleic acid (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 %DScf-DNA. The targeted approach relies on selected highly-informative genomic regions and potentially provides a rapid (24 to 48 h) cost-efficient (significantly less than whole genome sequencing) method 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 (5 ml) were collected in 3 clinical scenarios:

1. **Scheduled surveillance EMB** from *asymptomatic* heart transplant recipients in the catheterization laboratory immediately before scheduled surveillance EMB.
2. **Unscheduled diagnostic EMB** from *symptomatic* heart transplant recipients before unscheduled diagnostic EMB.
3. **Rejection** from heart transplant recipients with biopsy-proven rejection (> The International Society for Heart and Lung Transplantation grade 2R or antibody mediated rejection 1) before initiation of treatment, during treatment, and at 1 week.

Clinical, laboratory, cardiac catheterization, and echocardiographic data were recorded. Anticoagulated blood was collected to measure cell-free deoxyribonucleic acid (cf-DNA) levels. Plasma separation, cf-DNA extraction, and quantification of total cf-DNA were carried out as previously described (4). Genomic DNA for genotyping was prepared from 1 buffy coat of each recipient, and donor DNA was obtained from the Blood Center of Wisconsin. Determination of the %DScf-DNA in recipient plasma was performed using Digital Analysis of Selected Regions (DANSR, Ariosa Diagnostics, San Jose, California) (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 % 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 *U* 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. The cf-DNA levels were obtained from 26 patients undergoing 38 scheduled surveillance EMBs (Fig. 1A). Thirty-two (84%) samples contained <1% DScf-DNA. No patient with DScf-DNA <1% had pathological rejection. DScf-DNA levels exceeded 1% in 6 samples, and the highest percentage of DScf-DNA was associated with asymptomatic biopsy-proven rejection. The remaining 5 samples had negative biopsies.

Scenario 2. Seven samples were obtained from 6 patients before unscheduled diagnostic EMB to rule out rejection based on clinical criteria (Figs. 1B and 1D). Six samples had DScf-DNA levels >1%, and 1 sample contained DScf-DNA <1%. Four of the 6 were associated with biopsy-proven rejection; the other 2 patients had significant coronary artery vasculopathy on angiography. The single symptomatic patient with a low percentage of DScf-DNA had high levels of total cf-DNA (Fig. 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 intravenous immunosuppressive therapy, all patients demonstrated significantly decreased %DScf-DNA. Interestingly, 3 to 4 days after discontinuing augmented immunosuppression, the %DScf-DNA rebounded in 3 of 4 patients (Fig. 1C). The DScf-DNA was compared with other candidate noninvasive laboratory variables (B-natriuretic peptide, troponin, and C-reactive protein) as well as echocardiographically determined left ventricular ejection fraction in predicting rejection on biopsy; DScf-DNA had the highest sensitivity and specificity (100% and 84%, respectively).

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 1 week post-transplant, which may allow for noninvasive detection of rejection in the vulnerable early post-transplant period (data not shown). A sensitive noninvasive rejection monitoring method could decrease the number of biopsies needed over a lifespan, thereby considerably 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 before clinical

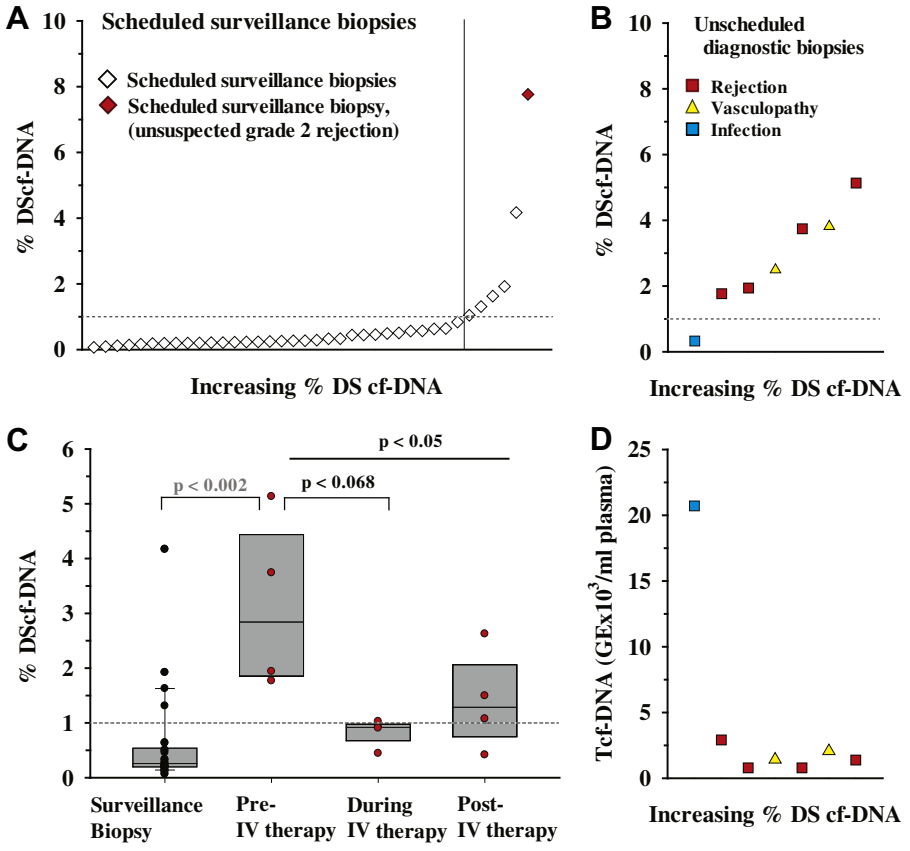

Figure 1 **cf-DNA in Patients Undergoing Surveillance EMB and During Rejection**

Percent donor-specific cell-free deoxyribonucleic acid (DScf-DNA) in scheduled surveillance endomyocardial biopsy (EMB) **(A)** and unscheduled diagnostic EMB samples **(B)**. **(C)** Boxplot of surveillance EMB samples **(black circles)** and rejection samples **(red circles)** collected at 3 time points. **(D)**Total cell-free deoxyribonucleic acid (cf-DNA) in unscheduled diagnostic EMB samples. Data in **A, B, and D** are sorted on the x-axis according to increasing %DScf-DNA. Samples in **B and D** align vertically. The **dashed line in A, B, and C** highlights the 1% DScf-DNA level. The **vertical solid line in A** orients the picture so that all samples containing <1% DScf-DNA are on the **left-hand side** and all samples >1% are on the **right**. IV = intravenous.

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 <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 noninvasive tool potentially suitable for rejection surveillance as an alternative to EMB.

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Letters to the Editor

Triggering of Cardiac Arrhythmias

The Problem of Multicollinearity Among Air Pollution and Meteorological Factors

Link et al. (1) recently suggested the influence of ambient air pollutants on the occurrence of atrial fibrillation, with a 26% increase in the risk of arrhythmic incidence for each 6-$\mu g/m^3$ increase in particulate matter <2.5 μg ($PM_{2.5}$) aerodynamic diameter. Arguably, the association of acute cardiac events with environmental influences may not be as simple because of strong correlations between the atmospheric constituents, which may also act as triggers of cardiac arrhythmias. For example, meteorological factors correlate with each other, but also substantially influence the concentration of air pollutants (2–8). A growing body of evidence has suggested a strong relationship of suspended particulate matter to wind speed, air pressure and temperature, and relative humidity (2–5). The problem of multicollinearity may be particularly important for the relationship between meteorological factors and ambient $PM_{2.5}$ (3), the particles that showed a significant association with the occurrence of atrial fibrillation in the study by Link et al. (1).

Link et al. (1) made adjustments for air temperature and dew point for 24-h periods, which could partly be the reason for nonsignificant associations between atrial fibrillation and air pollution in this analysis. Two previous studies from Poland investigated the association of the incidence of atrial fibrillation also using daily meteorological data, but without adjustments. The first study has suggested that meteorological factors such as temperature, relative humidity, and atmospheric pressure are implicated in the occurrence of atrial fibrillation in 87% of patients (9). The second study failed to confirm the influence of individual meteorological factors, but found an increased incidence of atrial fibrillation associated with the passage of a cold front and an occlusion of the cold front type (10).

The most important advantage of the study by Link et al. (1) is the 2-h time window of exposure measurements. However, using comparable time windows (i.e., 2- and 3-h periods), we reported that all 4 meteorological factors mentioned above may be involved in the triggering of both ventricular (11,12) and supraventricular arrhythmias (13,14), independently of other external triggering factors including physical activity and emotional stress. High absolute levels of atmospheric pressure and increasing air moisture were strong predictors of supraventricular tachycardia in all patient subgroups, as well as both wind speed and blowing of southeasterly and southwesterly wind directions in male patients (13). Increasing air moisture was also a strong common predictor of single supraventricular premature beats, whereas atmospheric temperature correlated with ectopy in participants younger than 65 years of age (14). In addition, we reported independent associations of atmospheric temperature and pressure, increasing relative air moisture, and wind speed and direction with the occurrence of ventricular tachyarrhythmia (11,12).

Possible mechanisms of the influence of wind speed and direction on cardiac arrhythmias, such as wind-induced turbulence and rapid atmospheric pressure oscillations (11–13,15), are highly speculative. Adverse effects of higher temperatures or increasing relative air moisture may burden and disturb the human homeostasis, particularly thermoregulatory, hemorheological, cardiovascular, and nervous systems (11,12,16). Higher absolute levels or decreasing atmospheric pressure have been associated with enhanced sympathetic activity (12,17,18). In considering their close inter-relations (2–5), one might expect that such meteorological conditions cause a more marked endogenous response and triggering burden than air pollution itself.

The main limitation of the research on environmental triggers is the lack of an adequate methodology. Link et al. (1) used a case-crossover design, comparing the risk of an incident during exposure and during control periods using conditional logistic regression (19,20). Although each patient serves as his or her own control, this design cannot control for meteorological variables and multiple correlations among environmental factors. To account for the possibility of multiple environmental triggers of cardiac arrhythmias (11–14), we applied a framework of multiple regression analysis with multiple successive measurements for each patient. We suggested that these 2 designs may be considered complementary (11). However, a more precise estimation of triggering and interaction effects of environmental factors may only be addressed through individual patient-based meta-analyses of large-scale data on air pollution and meteorological factors.

The acute effects of short-term exposure to higher pollution probably increase the myocardial vulnerability in some patients, but some concerns about the causal relationship with atrial fibrillation and other cardiac arrhythmias remain. There is a scenario in which increased air pollution is not the only or the direct culprit. Instead of a single trigger, a cluster of adverse atmospheric conditions may cause an arrhythmic incident. Further studies on interactions among all atmospheric triggers could further our understanding of the mechanisms of cardiovascular risk associated with certain atmospheric factors.

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