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Immunologic serum protein profiles for non-invasive detection of acute cellular rejection after heart transplantation

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Acute cellular rejection (ACR), a T cell-mediated form of organ rejection, remains a common problem after transplantation that compromises long-term allograft survival. Surveillance for ACR has been based on endomyocardial biopsy since the 1970s, which is burdensome due to its invasiveness, risk of complications, significant cost, inter-observer variability, and risk of false negative findings. Circulating biomarkers reflecting inflammation or myocardial injury, such as C-reactive protein or troponins, are not recommended for rejection surveillance due to poor specificity (1). Recent developments in affinity-based technologies have made systematic discovery of more informative biomarkers possible by allowing robust, simultaneous quantification of large sets of low-abundance proteins in blood(2). We aimed to comprehensively explore immunologic protein profiles in heart transplant recipients with ACR.

We applied a proximity extension assay (2) to measure 92 immune-related proteins, selected to provide broad coverage of immune pathways (Inflammation panel, Olink Proteomics, Sweden), in two heart transplant cohorts. Protein abundance is quantified from real-time polymerase chain reaction quantification cycle values, Normalized Protein Expression [NPX] units, expressed on the log2 scale. The discovery cohort consisted of recipients of heart transplants between February 2009 and September 2013 at six Canadian centers, including 22 biopsy-confirmed ACR cases (91% grade 2R, 9% 1R, median 23 days after transplantation [IQR 13-98]) and 38 controls without evidence of rejection in routine surveillance biopsies, all graded by three blinded expert pathologists(3). In this cohort, 10 of the 92 inflammatory proteins were significantly higher in patients with ACR (**Table 1**). Lasso regression analysis retained five proteins as independently informative, which were strongly correlated with the excluded markers (Pearson's pairwise r=0.50-0.86), particularly the chemokines (r=0.86 for CXCL10 and CXCL9). These five proteins were combined into a multimarker protein score based on NPX

sums weighted by beta-coefficients, which allowed significant separation between ACR cases and controls (p<0.0001) and discrimination of ACR (AUC 0.82). A maximally informative score cut-off point was 0.359 (maximum Youden's J statistic), with 82% sensitivity and 76% specificity, and 100% sensitivity was obtained with a cut-off of 0.102.

An increase during ACR was confirmed for four proteins using linear mixed models (**Table 1**) in a validation cohort consisting of serial serum samples (before, during, and after ACR) from ten heart transplant recipients (Skåne University Hospital in Lund, Sweden, 1997-2010) with biopsy-proven ACR (78% grade 3A, 11% 3B, 11% 2, median 356 days after transplantation [IQR 225-731])(4). The multimarker score provided similar diagnostic accuracy as in the discovery cohort (AUC 0.80). The score cutoffs from the discovery cohort for maximum information content and 100% sensitivity provided a sensitivity of 67% and 100%, respectively, and a specificity of 67% and 27%.

The pathway most enriched among the five biomarkers was related to lymphocyte signaling (canonical pathway "Altered T Cell and B Cell Signaling in Rheumatoid Arthritis" from Ingenuity Pathway Analysis Software build 430520M, Qiagen, Germany). Four of the proteins (SLAMF1, LTA, IL-12B, and CSF-1) were included in this pathway as compared to 17 of the 92 proteins in the full assay (p for enrichment = 0.004). *Ex vivo* activation of human CD4+ T cells (n=6) with CD3/CD28 beads (Dynabeads Human T-Activator CD3/CD28, Life Technologies, CA) resulted in increased expression of mRNA transcripts for all five biomarkers (p<0.01).

In summary, circulating proteins associated with T cell activation were increased in heart transplant recipients with ACR. A multimarker model including five such biomarkers provided strong and reproducible discrimination of ACR. The only non-invasive modality currently

recommended for ACR surveillance is gene expression profiling of 20 mRNA transcripts in peripheral blood (AlloMap, CareDx, CA) (1), used at many centers in the United States at a cost similar to endomyocardial biopsy. We note that our score achieves substantially higher AUC than reported for AlloMap in the CARGOII trial (0.80 vs 0.69) and substantially higher specificity at the 100% sensitivity cutoff (27% vs 2%), but recognize that no direct comparison has been performed (5). The encouraging results from this study motivate a large, prospective study to determine if plasma biomarkers reflecting T cell activation can reduce the burden of endomyocardial biopsy in heart transplant recipients.

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Table 1. Immunologic protein biomarkers associated with ACR.

Marker	Discovery cohort				Validation cohort					
	ACR	**NR**	**FDR**	**AUC**	**BR**	**DR**	**AR**	**P-value 1**	**P-value 2**	**AUC**
CCL19	9.01 ±1.37	8.59 ±1.07	0.047	0.59	-	-	-	-	-	-
CD244	6.04 ±0.39	5.75 ±0.43	0.024	0.70	-	-	-	-	-	-
CSF1	8.57 ±0.26	8.43 ±0.25	0.030	0.64	8.50 ±0.19	8.50 ±0.10	8.43 ±0.20	0.97	0.37	-
CXCL9	8.56 ±1.20	7.63 ±1.25	0.008	0.72	-	-	-	-	-	-
CXCL10	10.2	9.20	0.004	0.74	9.33	10.1	9.38	<0.001	<0.001	-

	4±1.24	±1.15			±1.19	3±1.10	±1.44			
CXCL11	8.16±1.34	7.30±1.27	0.017	0.68	-	-	-	-	-	-
IL-6	6.04±1.49	5.42±1.61	0.049	0.63	-	-	-	-	-	-
IL-12B	3.35±1.11	2.57±1.18	0.024	0.71	3.29±0.72	3.76±1.30	3.00±1.05	<0.001	0.01	-
LTA	3.03±0.49	2.67±0.49	0.022	0.69	2.88±0.47	3.07±0.77	2.74±0.69	0.006	<0.001	-
SLAMF1	3.56±0.68	3.06±0.59	0.004	0.72	2.80±0.62	3.30±0.66	2.77±0.50	<0.001	<0.001	-

Multimarker model	0.54 ±0.24	0.25 ±0.19	-	0.82	0.26 ±0.21	0.53 ±0.26	0.38 ±0.20	.33765555-6	0.2829888 89	0.80

Biomarkers with significantly higher expression in acute cellular rejection cases (ACR, n=22) as compared to controls (NR, n=38) in the discovery cohort (false discovery rate [FDR] <0.05 based on p-values from Student's t-tests comparing ACR and NR patients). Mean values ± SD are presented for each group. In the validation cohort, repeat measurements of protein expression were performed in serum samples from 10 heart transplant patients before (BR, n=8), during (DR, N=9) and after rejection (AR, N=9). Measurements were compared using logistic regression analysis and linear mixed models, with p-value 1 referring to AR vs DR and p-value 2 to DR vs AR. AUC, area under the receiver-operating characteristic curve.