Supplementary Methods

Biomarker Development: All blood samples were drawn directly into PAXgene tubes (BD BioSciences, San Jose, CA), processed, and subjected to a previous workflow using the Affymetrix 3' IVT PLUS labeling kit Array hybridization.[1-3] Raw expression (.CEL) data files generated by the GeneTitan from Affymetrix GeneChip HT HG-U133+PM Array plates were processed for QC metrics using the Affymetrix Expression Console software where each sample that passed was used as input for normalization. Normalization was performed using a custom Frozen Robust Multiarray Analysis[4] vector to generate a fixed set of probe level parameters based on a cohort of 560 CEL files previously generated from LTR.[1] In addition to performing the standard steps of background correction, normalization, and summarization, a major advantage of FRMA over the traditional Robust Multiarray Analysis is that only blood samples from LTR were used in normalization vector creation, increasing the confidence in the values of the LTR specific blood transcripts expressed in these samples for our specific LT biomarker development pipeline. Finally, prior to model development, batch effects were corrected using ComBat.[5]

The genomic discovery and validation phases were performed in accordance to Institute of Medicine guidelines.[6] For discovery of gene expression based biomarkers with the sample groups sizes available in this study, the split sample approach (70% training set, 30% testing set) allows for accurate estimation of phenotypic gene expression profiles while retaining a sufficiently large validation cohort to have confidence in the performance metrics.[7] In this approach, all biomarker discovery steps are performed on the training set with the testing set reserved solely for validation of the final "locked" model. An additional consideration when splitting the samples is maintaining the distribution of the phenotypes and pertinent metadata variables (including reported gender, ethnicity, etc.) in both the training and testing cohorts. Therefore, the NU and CTOT-14 samples were merged and then split into the 70% training and 30% testing groups, based on previous discovery work in kidney transplant recipients.[8,9]

Briefly, the dataset of all probes on the GeneChip were filtered to retain the genes with median expression greater than 6 in 50 percent of the samples and in the top 40th percentile of variance across all samples. Five independent classification algorithms (Nearest Shrunken Centroid,[10] Partial Least Squares Discrimination Analysis,[11] Support Vector Machine,[12] Random Forest,[13] Elastic Net[14]) were used to calculate a multivariate score for each probe in the filtered dataset based on metrics (unique for each algorithm) that reflect the relative contribution of a probe towards classification of out of bag samples. For 1000 resamplings of the training datasets, the score represents "the mean of the square accuracy of the models obtained among the set of multivariate methods that selected" the probe.[9] All probes with score greater than 0.2 were retained for use in the next step of the pipeline, with final model generation using random forest. A performance threshold was selected favoring NPV over PPV (above the threshold = AR), and the model and threshold were then locked for validation on the 30% test cohort.

The locked model and threshold were also used on pre–AR and pre–non-AR samples as well as post-AR. As each subject had serial samples collected in the CTOT-14 cohort, a linear mixed effect model with random intercept was used to estimate the pre-biopsy slope for each phenotype to account for within patient correlation. Data first stratified by phenotypes and coefficients were estimated and compared via linear mixed effect model. Another linear mixed effect model was fit to compare the pre- and post-AR slopes. Analysis was performed using R version 3.5.1 (RStudio). Probes from the final locked models were then fed to Ingenuity Core Analysis (Qiagen, Inc., Hilden, Germany) that provides information about enriched pathways and allows comparison to literature data. Enriched pathways were selected based on Fisher's exact test (p-value<0.05 statistically significant).

References

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Table S1: AR vs. Non-AR 59-Probe Model – Ingenuity Pathway Analysis

Ingenuity Canonical Pathways	-log(p-value)	-log(B-H p-value)	Molecules
LXR/RXR Activation	5.45	3.19	CD36,CLU,IL1R2,LYZ,MMP9
Glucocorticoid Receptor Signaling	4.57	2.8	ADRB2,CD3G,FCGR1A,IL1R2,MMP9,NFAT5,SLPI
Role of NFAT in Regulation of the Immune Response	4.6	2.8	CD3G,FCGR1A,GNA12,HLA-DQB1,NFAT5
B Cell Development	4.34	2.69	HLA-DQB1,IGHM,PTPRC
CD28 Signaling in T Helper Cells	4.06	2.65	CD3G,HLA-DQB1,NFAT5,PTPRC
iCOS-iCOSL Signaling in T Helper Cells	4.21	2.65	CD3G,HLA-DQB1,NFAT5,PTPRC
Systemic Lupus Erythematosus Signaling	4.11	2.65	CD3G,FCGR1A,IGHM,NFAT5,PTPRC
Atherosclerosis Signaling	3.98	2.63	CD36,CLU,LYZ,MMP9
Dendritic Cell Maturation	3.36	2.06	CD1D,FCGR1A,HLA-DQB1,HLA-DRB4
OX40 Signaling Pathway	3.16	1.9	CD3G,HLA-DQB1,HLA-DRB4
Protein Kinase A Signaling	3	1.79	FLNA,LEF1,NFAT5,PTPN4,PTPRC
T Cell Receptor Signaling	2.95	1.77	CD3G,NFAT5,PTPRC
Th1 Pathway	2.78	1.64	CD3G,HLA-DQB1,HLA-DRB4
Inhibition of Angiogenesis by TSP1	2.71	1.6	CD36,MMP9
Antigen Presentation Pathway	2.59	1.56	HLA-DQB1,HLA-DRB4
Neuroinflammation Signaling Pathway	2.58	1.56	HLA-DQB1,MMP9,NFAT5,SNCA
Th2 Pathway	2.64	1.56	CD3G,HLA-DQB1,HLA-DRB4
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	2.51	1.51	FCGR1A,IL1R2,LEF1,NFAT5
PKCθ Signaling in T Lymphocytes	2.48	1.5	CD3G,HLA-DQB1,NFAT5
Cdc42 Signaling	2.32	1.47	CD3G,HLA-DQB1,HLA-DRB4
Hematopoiesis from Pluripotent Stem Cells	2.39	1.47	CD3G,IGHM
Primary Immunodeficiency Signaling	2.38	1.47	IGHM,PTPRC
Systemic Lupus Erythematosus In T Cell Signaling Pathway	2.41	1.47	CD3G,GNA12,HLA-DQB1,HLA-DRB4
T Cell Exhaustion Signaling Pathway	2.33	1.47	HLA-DQB1,HLA-DRB4,NFAT5
Th1 and Th2 Activation Pathway	2.36	1.47	CD3G,HLA-DQB1,HLA-DRB4
B Cell Receptor Signaling	2.26	1.42	IGHM,NFAT5,PTPRC
ILK Signaling	2.23	1.42	FLNA,LEF1,MMP9
Oxidized GTP and dGTP Detoxification	2.24	1.42	DDX6
IL-8 Signaling	2.17	1.38	DEFA1 (includes others),GNA12,MMP9
Calcium-induced T Lymphocyte Apoptosis	2.14	1.36	CD3G,HLA-DQB1
Osteoarthritis Pathway	2.06	1.31	IL1R2,LEF1,MMP9
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	2.07	1.31	IL1R2,LEF1,NFAT5
Allograft Rejection Signaling	1.92	1.2	HLA-DQB1,HLA-DRB4
IL-4 Signaling	1.93	1.2	HLA-DQB1,NFAT5
Phosphatidylcholine Biosynthesis I	1.87	1.17	CHPT1
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	1.88	1.17	CD3G,NFAT5
Cardiac Hypertrophy Signaling (Enhanced)	1.82	1.14	ADRB2,GNA12,IL1R2,NFAT5
Communication between Innate and Adaptive Immune Cells	1.83	1.14	HLA-DRB4,IGHM
PD-1, PD-L1 cancer immunotherapy pathway	1.75	1.09	HLA-DQB1,HLA-DRB4
Type I Diabetes Mellitus Signaling	1.71	1.06	CD3G,HLA-DQB1
MSP-RON Signaling In Macrophages Pathway	1.7	1.05	HLA-DQB1,HLA-DRB4

Ingenuity Toxicity Lists	-log (p-value)	Molecules
LXR/RXR Activation	5.41	LYZ,CD36,MMP9,CLU,IL1R2
Increases Cardiac Dysfunction	3.53	ADRB2,CD36,MMP9
Liver Necrosis/Cell Death	3.44	MMP9,DICER1,FLNA,ADM,PTPRC
Increases Cardiac Dilation	3.43	ADRB2,MMP9
Increases Heart Failure	3.09	ADRB2,MMP9
Increases Renal Damage	3.06	FCGR1A,KLRB1,ADM
Persistent Renal Ischemia-Reperfusion Injury (Mouse)	2.82	LYZ,CLU
Acute Renal Failure Panel (Rat)	2.19	CLU,ADM
Renal Safety Biomarker Panel (PSTC)	1.94	CLU
Cardiac Fibrosis	1.88	ADRB2,MMP9,DICER1
Mechanism of Gene Regulation by Peroxisome Proliferators via PPARα	1.84	CD36,IL1R2
Cardiac Necrosis/Cell Death	1.67	ADRB2,DICER1,ADM
Hepatic Fibrosis	1.54	ADRB2,MMP9,HLA-DQB1
Renal Necrosis/Cell Death	1.52	SNCA,CLU,GNA12,NFAT5
Increases Glomerular Injury	1.5	FCGR1A,ADM
Genes associated with Chronic Allograft Nephropathy (Human)	1.4	MMP9
Cardiac Hypertrophy	1.39	ADRB2,MMP9,DICER1

Figure S1.