Online Supplement

Peripheral blood proteins predict survival and disease progression in

Idiopathic Pulmonary Fibrosis

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Introduction

This online supplement contains Supplementary Methods for the manuscript as well as Supplementary Results. The Supplementary Methods extend those given in the manuscript, providing details on both the Rules Based Medicine and other assays used for derivation and validation cohorts. The Supplementary Results consist of full results tables and plots of martingale residuals for all outcomes and predictors, for both derivation and replication cohorts.

Supplementary Methods

The Discovery Map v1.0 Rules Based Medicine website (http://www.rulesbasedmedicine.com/discoverymap, accessed July 30, 2011).

DiscoveryMAP v1.0 samples are tested on an automated platform in RBM's CLIA-certified laboratory using proprietary reagents and software. The RBM platform is validated to clinical laboratory standards and provides accurate and reproducible biomarker measurements. This enables the discovery of patterns made up of multiple biomarkers, many with small, yet reproducibly detectable changes. Multiplexing allows all of these assays to be performed using less than 750 microliters of serum or plasma, as compared to single-plex platforms, such as ELISA, which would require milliliters.

DiscoveryMAP is a comprehensive quantitative immunoassay service, containing 188 tests. It represents the culmination of 10 years of assay development for cytokines, chemokines, metabolic markers, hormones, growth factors, tissue remodeling proteins, angiogenesis markers, acute phase reactants, cancer markers, kidney toxicity markers, CNS biomarkers and other important serum proteins. DiscoveryMAP is designed for researchers who need a better understanding of their compound's efficacy, safety profile and biological activity as well as of the disease or condition being addressed.

Rules Based Medicine assays

Concentrations of 92 cytokines and chemokines, matrix metalloproteinases (MMPs), and markers of apoptosis and epithelial injury were analyzed using the Human DiscoveryMAP® multiplex bead-based immunoassay (*Rules-Based Medicine,* Austin, TX) (24). The DiscoveryMAP allows testing of samples on an automated platform in RBM's CLIA-certified laboratory using proprietary reagents and software. The RBM platform is validated to clinical laboratory standards and provides accurate and reproducible biomarker measurements. Multiplexing allows all of these assays to be performed in small volumes. All 92 proteins selected on the Human DiscoveryMAP are listed in Table S1 of the online supplement. Associated with each protein in Table S1 is its lowest detectable dose (LDD), defined as the mean plus 3 standard deviations of 20 blank readings. Values below the LDD are considered undetectable. Proteins were excluded from the analysis of prognosis prediction only if concentrations were lower than LDD in at least 95% of samples in the validation or derivation cohort.

Bio-Plex assays

For the validation study, plasma concentrations of S100A12 were analyzed using a CircuLex S100A12 / EN-RAGE ELISA kit according to the manufacturer's instruction. (MBL International, Woburn, MA). Plasma levels of ICAM, VCAM, and IL8 were analyzed using Luminex technology with a Bio-Plex 100 and Bio-Plex manger software 5.0 (Bio-Rad, Hercules, CA). Specifically, ICAM and VCAM were analyzed using MILLIPLEX MAP Human CVD Panel 1 kit (Millipore, Billerica, MA), IL8 was analyzed using a IL8 Ultrasensitive Human Singleplex kit (Invitrogen, Carlsbad, CA).

Statistical Analysis

Time-to-event outcomes analyzed include mortality, transplant free survival and progression free survival (PFS). For mortality analysis, patients were followed from the blood draw until death, or censoring on April 1, 2010; mortality data were complemented by searching The Social Security Death Index (SSDI). For transplant free survival, in addition to mortality transplants were counted as events. For PFS analysis patients were followed from the blood draw until either 1.) disease progression, defined as the first *relative* decline of 10% or more in FVC % predicted within a one year interval; 2.) death without recorded disease progression; or 3.) censoring at the latest pulmonary function test or last contact. Any patient receiving a lung transplant during follow-up was censored at transplant date in both mortality and progression free survival analyses but not in transplant free survival.

Data were analyzed using the R environment for statistical data analysis and graphics (25), particularly the survival package (26). Survival curves were estimated using the Kaplan-Meier method. For each outcome, each protein detectable in plasma was dichotomized into high- and low-risk ranges using profile likelihood (27). Briefly, for profile likelihood the risk threshold was used as a parameter in the proportional hazards (PH) model, there being only finitely many possible thresholds based on the observed data. The (profile) partial likelihood was maximized for each threshold and the threshold yielding the highest maximized profile likelihood was chosen for each marker, separately for all outcomes. Model fit was assessed by examining martingale residuals. Additionally we computed quintiles, quartiles, and tertiles, and fit Cox proportional hazards (PH) models with four, three, and two degrees of freedom, respectively, to explore variation in the hazard ratio as a function of each predictors (see supplemental data). Associations of biomarkers with IPF were tested using the log rank test and Cox PH model. The PH model was used to adjust for Age, gender, and baseline pulmonary function assessed by FVC or composite physiologic index (CPI) computed as follows (28): CPI = 91.0 - (0.65 x Dlco % Predicted) - (0.53 x Fvc % Predicted) + (0.34 x FEV_1 % Predicted). For

multiple testing in marker selection the Bonferroni method was used to control the family-wise error rate at 5%.

Derivation of Combined Risk Index

We applied the stepAIC approach for variable selection in the Cox Proportional hazards models that combine peripheral blood biomarkers with known predictors of mortality such as age, gender, and baseline pulmonary function. Starting with a model including Age at blood draw, gender, baseline Fvc and DLco % Predicted, as well as Composite Physologic index (CPI) and all five candidate biomarkers, to avoid overfitting we set the algorithm to use the conservative Schwarz criterion with scope broad enough to encompass models with both main effects and interactions among biomarkers and clinical parameters, with movement of predictors possible in either direction (in or out of the model) at any step. Once a combined set of predictors was derived we computed a risk score, or personal clinical and molecular mortality index (PCMI), on the scale of the log hazard ratio, by multiplying the beta coefficients by 100 and summing(du Bois, Weycker et al. 2011). We then expressed the probability S(t) of t-year survival after the blood draw as $S(t) = S_0(t)^{**}\exp[0.01*PCMI)$, where $S_0(t)$ is the t-year survival probability for a patient with "reference" predictors. Here, to ensure that increasing PCMI values are associated with higher mortality risk, we expressed DLco as the difference from 100% percent predicted and CPI as the difference from 7, which is the CPI computed for a patient with 100% predicted FVC and DLco and also FEV_1. Note that the choice of these reference values for the predictors does not affect variable selection.

Table S1. Alphabetical list of all proteins assayed on Human DiscoveryMAP®

Marker	SwissProt ID(s)	Abbrev.	Units	LDD	Detecable
Adiponectin	Q15848	ADPN	ug/mL	0.2	Yes
Alpha1 Antitrypsin	P01009	A1AT	mg/mL	0.011	Yes
Alpha2 Macroglobulin	P01023	A2M	mg/mL	0.061	Yes
AlphaFetoprotein	P02771	AFP	ng/mL	0.43	Yes
Apolipoprotein A1	P02647	ApoA1	mg/mL	0.0066	Yes
Apolipoprotein CIII	P02656	ApoCIII	ug/mL	2.7	Yes
Apolipoprotein H	P02749	ApoH	ug/mL	8.8	Yes
Beta2 Microglobulin	P61769	B2M	ug/mL	0.013	Yes
BrainDerived Neurotrophic Factor	P23560	BDNF	ng/mL	0.029	Yes
Calcitonin	P01258	CALC	pg/mL	6.0	No
Cancer Antigen 125	Q8WX17	CA125	U/mL	4.2	Yes
Cancer Antigen 19-9	Q9BXJ9	CA199	U/mL	0.25	Yes
Carcinoembryonic Antigen	P06731	CEA	ng/mL	0.84	Yes
CD40	Q6P2H9	CD40	ng/mL	0.021	Yes
CD40 Ligand	P29965	CD40LG	ng/mL	0.02	Yes
Complement 3	P01024	C3	mg/mL	0.0053	Yes
C Reactive Protein	P02741	CRP	ug/mL	0.0015	Yes
Creatine KinaseMB	P12277, P06732	CKMB	ng/mL	0.42	Yes
EGF	P01133	EGF	pg/mL	7.4	Yes
ENA78	P42830	CXCL5	ng/mL	0.076	Yes
Endothelin1	P05305	EDN1	pg/mL	7.2	Yes
Eotaxin	P51671	CCL11	pg/mL	41.0	Yes
Erythropoietin	P01588	EPO	pg/mL	166.0	No
Factor VII	P08709	F7	ng/mL	1.0	Yes
Fatty Acid Binding Protein	P05413	FABP	ng/mL	3.0	No
Ferritin	P02794, P02792	FT	ng/mL	1.4	Yes
FGF basic	P09038	FGF2	pg/mL	98.0	Yes
Fibrinogen	P02671, P02675, P02679	FG	mg/mL	0.0098	Yes
GCSF	P09919	CSF3	pg/mL	5.0	Yes
Glutathione STransferase	P08263	GST	ng/mL	0.4	Yes
GMCSF	P04141	CSF2	pg/mL	57.0	No
Growth Hormone	P01241	GH	ng/mL	0.13	Yes
Haptoglobin	P00738	HP	mg/mL	0.025	Yes
ICAM1	P05362	ICAM1	ng/mL	3.2	Yes
IFNγ	P01579	IFNG	pg/mL	4.6	Yes
IgA		IgA	mg/mL	0.0084	Yes
IgE		IgE	ng/mL	14.0	Yes
IGF1	P01343	IGF1	ng/mL	4.0	Yes
IgM		IgM	mg/mL	0.015	Yes
IL1alpha	P01583	IL1α	pg/mL	0.0025	Yes
IL1beta	P01584	IL1β	ng/mL	1.5	Yes
IL1ra	P18510	IL1RN	pg/mL	15.0	Yes
IL2	P60568	IL2	pg/mL	60.0	No
IL3	P08700	IL3	ng/mL	0.17	No

Marker	SwissProt ID(s)	Abbrev.	Units	LDD	Detecable
IL4	P05112	IL4	pg/mL	104.0	No
IL5	P05113	IL5	pg/mL	33.0	No
IL6	P05231	IL6	pg/mL	12.0	No
IL7	P13232	IL7	ng/mL	53.0	Yes
IL8	P10145	IL8	pg/mL	3.5	Yes
IL10	P22301	IL10	pg/mL	15.0	No
IL12p40	P29460	IL12p40	pg/mL	1.2	No
IL12p70	P29459	IL12p70	ng/mL	94.0	No
IL13	P35225	IL13	ng/mL	57.0	Yes
IL15	P40933	IL15	pg/mL	1.3	No
IL16	Q14005	IL16	pg/mL	66.0	Yes
IL17	Q16552	IL17	pg/mL	2.7	Yes
IL18		IL18	pg/mL	54.0	Yes
IL23	Q9NPF7	IL23	pg/mL	0.67	No
Insulin	P01308	INS	uIU/mL	0.86	Yes
Leptin	P41159	LEP	ng/mL	0.1	Yes
Lipoprotein a		LPA	ug/mL	3.7	Yes
Lymphotactin	P47992	LPTN	ng/mL	0.38	No
MCP1	P13500	CCL2	pg/mL	52.0	Yes
MDC	O00626	CCL22	pg/mL	14.0	Yes
MIP1alpha	P10147	CCL3	pg/mL	13.0	Yes
MIP1beta	P13236	CCL4	pg/mL	38.0	Yes
MMP2	P08253	MMP2	ng/mL	150.0	No
MMP3	P08254	MMP3	ng/mL	0.2	Yes
MMP9	P14780	MMP9	ng/mL	37.0	Yes
Myeloperoxidase	P05164	MPO	ng/mL	68.0	Yes
Myoglobin	P02144	MB	ng/mL	1.1	Yes
PAI1	P05121	PAI1	ng/mL	0.9	Yes
PAPPA	Q13219	PAPPA	mIU/mL	0.037	No
Prostate Specific Antigen Free	P07288	KLK3	ng/mL	0.023	Yes
Prostatic Acid Phosphatase	P15309	ACPP	ng/mL	0.034	Yes
RANTES	P13501	CCL5	ng/mL	0.048	Yes
S100A12	P80511	S100A12	ng/mL	0.25	Yes
Serum Amyloid P	P02743	SAP	ug/mL	0.058	Yes
SGOT	P17174	GOT1	ug/mL	3.7	Yes
SHBG	P04278	SHBG	nmol/L	1.3	Yes
Stem Cell Factor	P21583	SCF	pg/mL	56.0	Yes
Thrombopoietin	P40225	THPO	ng/mL	3.2	No
Thyroid Stimulating Hormone	P01215, P01222	TSH	uIU/mL	0.028	Yes
Thyroxine Binding Globulin	P05543	TBG	ug/mL	0.34	Yes
TIMP1	P01033	TIMP1	ng/mL	8.4	Yes
Tissue Factor	P13726	TF	ng/mL	0.84	No
TNFalpha	P01375	TNFA	pg/mL	4.0	Yes
TNFbeta	P01374	TNFB	pg/mL	46.0	No
TNF RII	Q92956	TNFRSF14	ng/mL	0.13	Yes
VCAM1	P19320	VCAM1	ng/mL	2.6	Yes
VEGF	P15692	VEGF	pg/mL	7.5	Yes

Marker	SwissProt ID(s)	Abbrev.	Units	LDD	Detecable
von Willebrand Factor	P04275	VWF	ug/mL	0.4	Yes

Supplementary Results: Derivation and Validation Cohorts

After a short description of the contents of a table of univariate results, all univariate results tables are presented. Each table occupies a single page and represents one outcome for one predictor for one cohort. For each predictor we computed quintiles, quartiles, and tertiles, and fit Cox proportional hazards (PH) models with four, three, and two degrees of freedom, respectively, to explore variation in the hazard ratio as a function of the predictor. We also fit a binary-split Cox PH model using profile likelihood, including a threshold parameter in the model and maximizing the profile (partial) likelihood. Each table has three sections separated by gray vertical lines. The left section lists numbers of patients at risk and the number who experience the event of interest during follow-up, along with hazard ratio estimates and 95% confidence intervals from Cox PH models. The center section describes the time-to-event distribution by category including Kaplan-Meier estimates of median time-to-event with Greenwood 95% confidence intervals. The right section lists model p-values from likelihood ratio and logrank (score) tests of the hypothesis that all the hazard ratios are unity. Finally, each table contains for each model a likelihood ratio test for deviations from linearity obtained by adding to a PH model with quantitative predictor a categorical version of the same predictor, with four, or three, or two, or one additional degree of freedom. This p-value is for a test of deviation from linearity in the predictor's effect on time-to-event.

After presenting all univariate results tables, we present plots of martingale residuals, for a further check on the linearity of predictors included in the Cox PH models. Each martingale residuals plot was obtained by fitting a univariate Cox PH model with a single continuous predictor and plotting the residuals versus the predictor, applying loess smoothing with automatic bandwidth selection to suggest the form of the predictor. There are two sets of martingale residuals plots for each cohort and outcome and predictor, one for clinical predictors and another for our biomarkers.

Description of Univariate Results Tables

Univariate results are presented by Cohort, Outcome, and Predictor, as outlined below with necessary definitions or clarifications added.

1) **Cohort:**
   a) **Derivation**
      Plasma samples from 140 patients with IPF, analyzed by Rules Based Medicine assay.
   b) **Validation**
      Plasma samples from 101 patients with IPF, analyzed by ELISA or Luminex.
2) **Outcome:**
   a) **Mortality**
      Event = Death;
      Censoring at lung transplant or end of follow-up (EOFu).
   b) **Transplant Free Survival**
      Event = Death or Lung transplant;
      Censoring at EOFu.
   c) **Progression Free Survival by FVC (Lung Transplant is Censored)**
      Event = Relative decline of at least 10% in FVC % Predicted in one year, or death;
      Censoring at lung transplant or EOFu.
   d) **Progression Free Survival by DLCO (Lung Transplant is Censored)**
      Event = Relative decline of at least 15% in DLco % Predicted in one year, or death;
      Censoring at lung transplant or EOFu.
   e) **Progression Free Survival by CPI (Lung Transplant is Censored)**
      Event = Relative increase of at least 5% in CPI in one year, or death;
      Censoring at lung transplant or EOFu.
   f) **Progression Free Survival by FVC (Lung Transplant counts as event)**
      Event = Relative decline of at least 10% in FVC % Predicted in one year, or lung transplant, or death;
      Censoring at EOFu.
   g) **Progression Free Survival by DLCO (Lung Transplant counts as event)**
      Event = Relative decline of at least 15% in DLco % Predicted in one year, or lung transplant, or death;
      Censoring at EOFu.
   h) **Progression Free Survival by CPI (Lung Transplant counts as event)**
      Event = Relative increase of at least 5% in CPI in one year, or lung transplant, or death;
      Censoring at EOFu.
3) **Predictor:**
   a) **Age (years)**
   b) **Gender**
   c) **Baseline (4-month) FVC % Predicted**
      FVC % Predicted nearest to blood draw, if performed within 4 months of blood draw.
   d) **Baseline (4-month) DLco % Predicted**
      DLco % Predicted nearest to blood draw, if performed within 4 months of blood draw.
   e) **Baseline (4-month) CPI**
      CPI nearest to blood draw, if available within 4 months of blood draw.
   f) **Baseline (3-month) FVC % Predicted**
      FVC % Predicted nearest to blood draw, if performed within 3 months of blood draw.
   g) **Baseline (3-month) DLco % Predicted**
      DLco % Predicted nearest to blood draw, if performed within 3 months of blood draw.

h) **Baseline (3-month) CPI**
CPI nearest to blood draw, if available within 3 months of blood draw.
i) **MMP7 (ng / mL)**
j) **ICAM1 (ng / mL)**
k) **IL8 (ng / mL)**
l) **VCAM1 (ng / mL)**
m) **S100A12 (ng / mL)**

The Univariate Results Tables are presented in the order outlined above, **Predictor** within **Outcome** within **Cohort**. Each table has the tri-partitite structure suggested by the gray vertical lines. The left side contains patient counts and hazard ratios (HRs) with lower and upper confidence limits (LCL and UCL, respectively) based on Cox proportional hazards (PH) models incorporating Quintiles, Quartiles, Tertiles, or binary split via profile likelihood. The middle section of each table describes survival distributions for each breakdown, including median survival with LCL and UCL. The right-hand section lists general model p-values. The individual columns in each table are described next.

Column	Description
Patients (N)	Number of patients initially at risk for the event, by category.
Events (n)	Number of patient experiencing the event during follow-up.
Events (%)	% of events during follow-up.
HR	Hazard ratio, relative to lowest category indicated by "----", based on Cox PH model.
HR 95% LCL	95% lower confidence limit on HR, from Cox PH model.
HR 95% UCL	95% upper confidence limit on HR, from Cox PH model.
Wald Pvalue	Tests single coefficient in the Cox PH model.
Median (yrs.)	Median time to event in each category.
Median 95% LCL	95% lower confidence limit on median time to event.
Median 95% UCL	95% upper confidence limit on median time to event, blank if infinite.
LR pvalue	Likelihood ratio test of Cox PH model; tests single parameter in binary-split model.
Logrank pvalue	Logrank test, of equality of time-to-event distributions across all categories.
DevLIN pvalue	Test for deviations from linearity: first fit univariate quantitative linear model, then perform LR test for significance of adding the same predictor categorized as Quintiles, Quartiles, Tertiles, or binary split via profile likelihood.