Development of a Multi-biomarker Assay for Serum Proteins by the Prognostic Lung Fibrosis Consortium (PROLIFIC)

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Rationale

Multiple peer-reviewed publications have consistently reported a reoccurring set of blood-based protein biomarkers linked to idiopathic pulmonary fibrosis (IPF) disease progression. Despite the strength of the evidence, no harmonized and validated panel has been available to the scientific community for this context of use. To address this unmet need, the Prognostic Lung Fibrosis Consortium (PROLIFIC) was formed to develop well-qualified assays suitable for use as exploratory, prognostic or predictive biomarkers within the context of clinical trials. (https://www.pulmonaryfibrosis.org/prolific).

Table 1. Markers selected for the PROLIFIC test

Category	Biomarker	Evidence of Prognostic or Pharmacodynamic Value (Ref)
Epithelial Damage	Cytokeratin 19 fragment (CYFRA 21-1)	Baseline CYFRA 21-1 was able to distinguish individuals at risk of 12-month disease progression (C-statistic 0.70 (95% CI 0.61 – 0.79), p < 0.0001) (Molyneaux 2022)
	Surfactant Protein-D (SP-D)	Significant improvement in the 1-year mortality prediction model when serum SP-A and SP-D (area under the receiving operator curve [AROC], 0.89) were added to the clinical predictors alone (AROC, 0.79; p = 0.03) (Kinder 2009)
	CA-19-9 (sialyl Lewis A)	Baseline of ≥22 U/mL was associated with a 3x increased risk of mortality (Maher 2017)
	CA-125 (MUC16)	Baseline of ≥12 U/mL was associated with a 3x increased risk of mortality (Maher 2017)
	KL-6 (MUC 1)	Serum baseline level >1000 U/mL is associated with worse prognosis (Yokoyama 2006) and >1300 U/mL with increased risk of acute exacerbation (Ohshimo 2014). KL-6 ≥ 1000 U/mL associated with disease progression (HR=2.761-2.845, p=0.040-0.045) (Chung 2022)
Fibrosis	Matrix Metalloproteinase 7 (MMP-7)	Higher levels (>3.5 ng/mL) lower transplant free survival (HR=2.3, p=0.016) (Richards 2012) Higher baseline (≥3.8 ng/mL) had higher risk of worsening (HR=2.2, p=0.001) (Bauer 2017)
	Tenascin C (TN-C)	Change from baseline Tenascin correlated with change from baseline FVC (van der Velden 2016)
	Periostin (POSTN)	Prognostic for FVC in the test cohort (Effect size=-3.6, p<0.001) and replication cohort (Effect size=-2.5, p=0.186) (Neighbors 2018)
Inflammation	CCL18 (PARC)	Prognostic for FVC in the test cohort (Effect size=-3.1, p=0.032) and replication cohort (Effect size=3.6, p=0.004) (Neighbors 2018)
	CXCL13 (BLC)	6-mo survival in the highest quartile of plasma CXCL13 was 65% versus 93% in the others (H= 5.5, P = 0.0008) (Vuga 2014). >62.1 pg/mL shorter survival (DePianto 2015)
	sICAM-1	High level (>202.5 ng/ml) associated with lower transplant-free survival (Richards 2012)
Thrombosis	Plasminogen Activator Inhibitor 1 (PAI-1)	Stable IPF =45 ng/mL vs AEx =70 ng/mL (p=0.0004), predicts survival (p=0.14) (Collard 2010)

Methods

Assay Development

Twelve protein biomarkers were selected based on evidence for their prognostic and mechanistic value in IPF (Table 1), including markers of epithelial damage (cytokeratin 19 fragment [CYFRA 21-1], surfactant protein D [SP-D], cancer antigen 125 [CA-125], cancer antigen 19-9 [CA-19-9], and Krebs von den Lungen 6 [KL-6]), fibrosis (matrix metalloproteinase 7 [MMP-7], tenascin C [TNC], and periostin [POSTN]), inflammation (pulmonary and activation-regulated chemokine [PARC or CCL18], B lymphocyte chemoattractant [BLC or CXCL13], and soluble intercellular adhesion molecule 1 [sICAM-1]), and thrombosis (plasminogen activator inhibitor 1 [PAI-1]). All 12 immunoassays were developed at Rules Based Medicine facility in Austin TX as either in single-plex or multiplex format, utilized the Luminex® xMAP® platform and consisted of antigen-specific antibodies optimized in a capture-sandwich format. The 12 assays were optimized into 3 multiplex panels and 2 singleplex panels. The assays were analytically validated for serum and EDTA plasma (MMP-7 for serum only) under formal protocols with design controls and pre-defined acceptance criteria with respect to Limit of Detection, Sensitivity, Accuracy, Precision, Parallelism, Matrix Interference, Freeze/Thaw Stability, Short-term Analyte Stability, and Sample Reproducibility.

Statistical Analysis

The assays were used to measure biomarker levels in serum collected from IPF patients at the time of enrollment (baseline) into the Pulmonary Fibrosis Foundation Patient Registry (N=657) (Table 2). Statistical analyses were performed using a joint model for longitudinal and time-to-event outcomes with a random coefficients longitudinal sub-model for the decline in % predicted Forced Vital Capacity (FVC) (Hankinson 1999) and a Cox proportional hazards sub-model for transplant free survival at one year, adjusting for sex, age, BMI, anti-fibrotic medication, % predicted FVC, and % predicted DLCO.

Table 2. Patient characteristics of PFF Patient Registry serum samples (N=657)

IPF patients Baseline data	Total N=657 Mean ± SD
Age	70.69 ± 8.08 years
Male	n=489 (74.4%)
Asian	n=14 (2.1%)
Black	n=10 (1.5%)
White	n=617 (93.9%)
Smoking history, Yes	n=427 (65.0%)
Using anti-fibrotic meds	n=437 (66.5%)
Baseline FVC (at enrollment)	2.65 ± 0.78 liters
Outcome data	
≥10% relative decline in % pred FVC in 1 year	n=95 (14.5%)
Death in 1 year	n=62 (9.4%), time to death 0.52 ± 0.27 years
Lung transplant in 1 year	n=37 (5.6%), time to transplant 0.46 ± 0.25 years

Figure 1. Single-marker analysis for annual change in % predicted FVC associated with a one standard deviation difference in log-scale baseline biomarker concentration

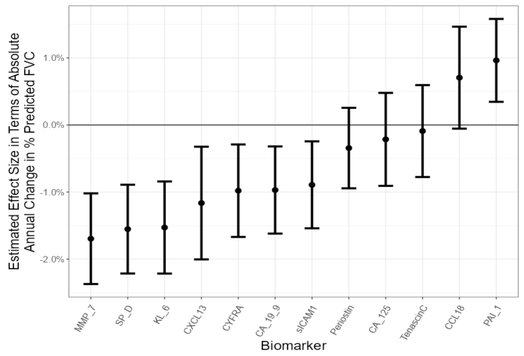


Table 3. Estimated longitudinal effects associated with a one standard deviation change in log-scale baseline biomarker concentration with standard error and p-values

Biomarker	Estimate	Std.Err	p-value
MMP-7	-1.70	0.344	< 0.001
SP-D	-1.55	0.338	< 0.001
KL-6	-1.53	0.351	< 0.001
PAI-1	0.96	0.315	0.002
CA-19-9	-0.97	0.332	0.003
CYFRA	-0.98	0.352	0.005
CXCL13	-1.16	0.429	0.007
sICAM1	-0.89	0.331	0.007
CCL18	0.71	0.388	0.069
Periostin	-0.34	0.306	0.26
CA-125	-0.21	0.354	0.54
TenascinC	-0.09	0.350	0.80

Figure 2. Estimated baseline biomarker effect on annual change in % predicted FVC

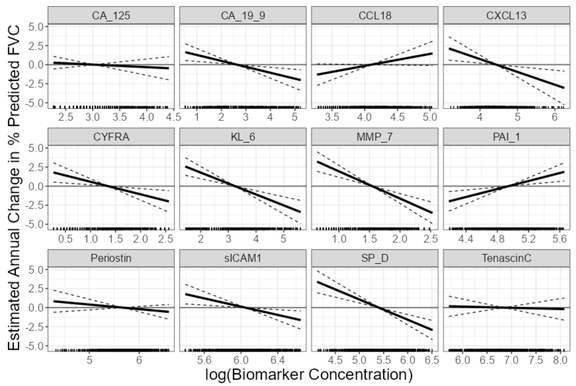


Figure 3. Transplant-free survival hazard ratios associated with a one standard deviation difference in log-scale baseline biomarker concentration

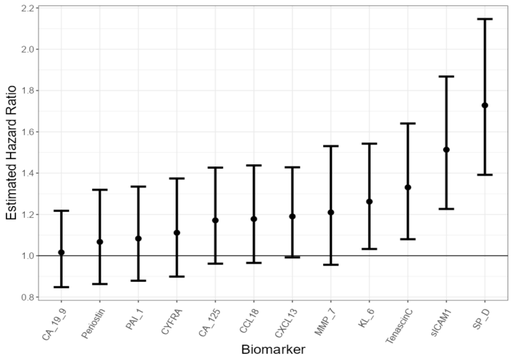
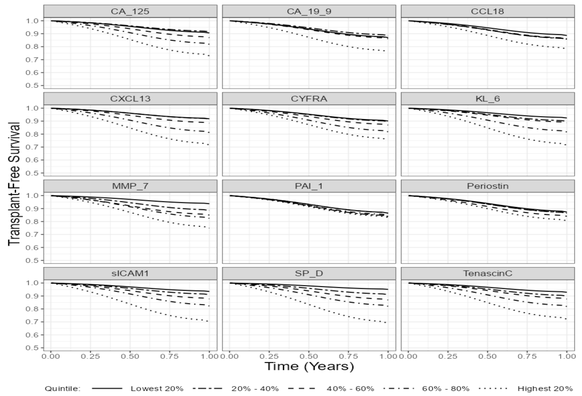


Table 4. Estimated hazard ratios associated with a one standard deviation difference in log-scale biomarker concentration with 95% confidence intervals and p-values.

Biomarker	Hazard Ratio	95% CI	p-value
SP-D	1.73	(1.39, 2.15)	< 0.001
sICAM1	1.51	(1.23, 1.87)	< 0.001
TenascinC	1.33	(1.08, 1.64)	0.007
KL-6	1.26	(1.03, 1.54)	0.023
CXCL13	1.19	(0.99, 1.43)	0.061
CCL18	1.18	(0.97, 1.44)	0.11
MMP-7	1.21	(0.96, 1.53)	0.11
CA-125	1.17	(0.96, 1.43)	0.12
CYFRA	1.11	(0.9, 1.37)	0.33
PAI-1	1.08	(0.88, 1.33)	0.45
Periostin	1.07	(0.86, 1.32)	0.55
CA-19-9	1.02	(0.85, 1.22)	0.86

Figure 4. Baseline biomarker effect on estimated transplant-free survival probability. Curves show estimated transplant-free survival (including non-biomarker effects) averaged across patients by biomarker concentration quintile.



Results

PFF Patient Registry Biomarker Results, Single Marker

All assays met pre-defined acceptance criteria.

The annual change in % predicted FVC was significantly associated with baseline MMP-7, SP-D, KL-6, PAI-1, CA-19-9, CYFRA 21-1, BLC/CXCL13, and sICAM-1 (Fig. 1, Table 3, Fig. 2).

Transplant-free survival was significantly associated with baseline SP-D, sICAM-1, TNC, and KL-6 (Fig. 3, Table 4, Fig. 5).

In a joint model combining the outcome measures, SP-D had the best model fit, followed by KL-6, sICAM-1, MMP-7, TNC, CA-125, PAI-1, CYFRA 21-1, PARC/CCL18, and CA-19-9..

Table 5. Ranking of biomarkers fit to joint model (change in FVC and transplant-free survival at one year) using Akaike Information Criterion (AIC) (A) without biomarker splines, (B) with biomarker splines*.

A

Biomarker	AIC Difference
SP_D	0.0
KL_6	27.0
sICAM1	29.3
MMP_7	31.5
TenascinC	35.1
CA_125	38.5
PAI_1	41.5
CYFRA	41.7
CCL18	44.7
CA_19_9	46.8
CXCL13	49.7
Null	49.8
Periostin	53.2

B

Biomarker	Long df	Surv df	Versus Overall Best
SP_D	1	1	0.0
MMP_7	4	1	5.8
KL_6	3	1	7.0
sICAM1	4	1	25.7
CYFRA	2	1	26.6
PAI_1	2	2	29.9
TenascinC	4	1	32.0
CXCL13	4	1	32.7
CA_19_9	3	3	34.3
CA_125	1	1	38.5
CCL18	3	3	39.1
Periostin	3	1	47.1

* For each biomarker, spline-biomarker models were fit wherein longitudinal and survival terms were modeled using natural cubic splines up to 4 degrees of freedom.

Conclusions

All biomarkers except POSTN, CCL18, and CA-125 were associated with the decline in % predicted FVC and/or transplant-free survival. These results indicate the assay is well-qualified to measure these prognostic biomarkers within the context of IPF clinical trials..

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Disclosures

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