Title

Relating longitudinal change in collagen degradation biomarkers to outcome in idiopathic pulmonary fibrosis: an analysis from the prospective, multi-centre PROFILE study

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Short Title: MMP-degraded biomarkers in IPF

Key Words: matrix metalloproteinase, clinical trials, theragnostic, surrogate markers, C reactive protein

Word Count Abstract: 343 Body Text: 4234

ABSTRACT

Background

Idiopathic Pulmonary Fibrosis (IPF), a progressive and inevitably fatal disorder, has a highly variable clinical course. Biomarkers which reflect disease activity are urgently needed to inform patient management and as biomarkers of therapeutic response (theragnostic biomarkers) in clinical trials. We examined whether dynamic change in markers of extracellular matrix (ECM) turnover predicts IPF disease behaviour in the PROFILE study cohort.

Methods

Serum samples from 189 patients with incident IPF, prospectively collected at baseline, one, three and six months as part of the PROFILE study, were analysed for a panel of novel matrix metalloprotease (MMP)-degraded ECM proteins, by ELISA-based, neoepitope assay. Eleven neoepitopes were tested in a discovery cohort of 55 patients to identify biomarkers of sufficient rigor for more detailed analyses. Eight were then further assessed in a validation cohort of 134 patients with 50 age and gender matched controls. Changes in biomarker levels were related to subsequent progression of IPF (defined as death or decline in forced vital capacity >10% at 12 months following study enrolment).

Findings

In the discovery cohort mean levels of seven neoepitopes (BGM, C1M, C3M, C6M, CRPM, ELM2 and VICM) differed significantly between healthy controls and IPF subjects. Baseline levels of six neoepitopes (C1M, C3A, C3M, C6M, CRPM and VICM) were significantly higher in progressive versus stable disease. In the validation cohort, mean levels of C1M, C3M, C6M and CRPM at baseline were confirmed to be higher in IPF patients compared with healthy controls. When assessed longitudinally levels of six neoepitopes (BGM, C1M, C3A, C3M, C6M, CRPM) were significantly higher in progressive compared with stable patients by six months. Baseline levels of two neoepitopes (C1M: HR 1.62 95% CI 1.14–2.31, $p=0.007$ and C3A: HR 1.91 95% CI 1.06–3.46, $p=0.032$) were associated with increased mortality. The rate of change over three months of six neoepitopes (BGM, C1M, C3M, C5M, C6M and CRPM) was strongly predictive of overall survival, and furthermore the increased risk was proportional to the magnitude of change in neoepitope levels. The strongest relationship with three-month rate of change was observed for CRPM for which a $\Delta > 0$ ng/ml/month conferred a HR of 2.16 (95% CI 1.15–4.07) whilst a $\Delta > 1$ ng/ml/month resulted in an HR 4.08 (95% CI 2.14–7.8) and a $\Delta > 1.7$ ng/ml/month was associated with an HR 6.61 (95% CI 2.74–15.94).

Interpretation

Protein fragments generated by MMP activity are elevated in the serum of individuals with IPF compared with controls. Neoepitope levels increase with disease progression and the rate of this increase predicts survival. Serial measurements of neoepitopes have potential to be used as theragnostic biomarkers in clinical trials and to guide management in IPF.

Funding

GlaxoSmithKline R&D and MRC Industrial Collaborative Award with GlaxoSmithKline R&D

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive and inevitably fatal disease with a 5 year survival of only 20%¹. With the advent of specific anti-fibrotic medications there is now hope that disease-modification and improved survival may be realistic therapeutic goals^{2,3}. Advances in IPF management are, however, hampered by heterogeneity of disease behaviour, a lack of validated prognostic measures and the absence of any markers of treatment response⁴. Novel serum biomarkers of fibroproliferation are emerging across a range of non-pulmonary diseases but none have been prospectively validated in carefully phenotyped individuals with IPF.

A cardinal feature of IPF is the accumulation of disordered and architecturally disruptive extracellular matrix (ECM) within the lung. The ECM consists of a dynamic combination of structural proteins, the most abundant of which are collagens. Matrix turnover is determined by several factors including the rate of ECM synthesis by activated myofibroblasts, the extent of collagen cross-linking and enzymatic matrix degradation⁵. Biomarkers reflective of matrix turnover might therefore be indicative of disease activity in fibroproliferative disorders such as IPF.

The majority of extracellular collagen is degraded by a subset of matrix metalloproteinases (MMPs) including MMP-1, -3, -7, -8, -13, -14, -16 and -18⁶. Extracellular collagen fragments generated by these MMPs are then further digested by MMP-2 and -9⁶. In IPF markedly increased rates of ECM protein synthesis are, at least partially, offset by elevated MMP levels and activity⁷ and MMP-1, -3 and -7 have even been identified as putative prognostic serum biomarkers^{8,9}.

During ECM turnover, proteolytically cleaved matrix degradation fragments, or neoepitopes, are released into the systemic circulation¹⁰. Cleavage of each ECM protein by specific MMPs generates a unique neoepitope. These neoepitopes have been shown to be more accurate diagnostic and prognostic markers for individual fibroproliferative diseases than their protein of origin^{10,11}. Previous small point-of-diagnosis studies have suggested that matrix neoepitopes may be elevated in individuals with IPF compared with healthy controls¹²⁻¹⁴.

The PROFILE (Prospective Observation of Fibrosis in the Lung Clinical Endpoints) study is the largest prospective study of its kind undertaken to date. To our knowledge, this study enables, for the first time, sequential assessment of biomarkers over time in relation to clinical measures of disease activity and progression. Furthermore, the recruitment of incident IPF cases from multiple centres enhances the potential applicability of any findings to real-world clinical practice. This analysis of samples from the first 189 PROFILE subjects with IPF was undertaken to determine the prognostic value of longitudinal change in serum levels of proteolytically cleaved protein fragments, or neoepitopes.

METHODS

Participants

The PROFILE study is an ongoing prospective, multi-centre, observational cohort study of incident cases (within 6 months of presentation) of idiopathic fibrotic lung disease. For all subjects clinical, physiological and biochemical data were collected at baseline and at one, three, six, 12 months, 24 and 36 months. Participants with IPF or idiopathic NSIP were identified through two co-ordinating centres: Nottingham, UK and, Royal Brompton Hospital, London, UK. For the purpose of this analysis samples from confirmed IPF cases drawn from the first 214 subjects enrolled during the first 18 months of the study were examined. Mortality data were collected from the NHS registry with a date of censoring of 1st October 2013. Only subjects with a multidisciplinary team confirmed diagnosis of IPF made in accordance with current internationally accepted criteria¹⁵ were included in the current study.

Study Design

A two-stage study design was used to facilitate rational stop-go decision making in order to minimise unnecessary waste of patient samples. Initial analysis was performed on a discovery cohort of 55 individuals with IPF drawn from the first 100 subjects recruited into the study enriched for the mildest and most severe disease and compared with gender matched controls for benchmarking purposes. Analysis of data from the discovery cohort was undertaken prior to a complete detailed analysis of a validation cohort, consisting of the remaining 134 IPF patients. 70 physician-verified, control subjects without history of respiratory disease were recruited from primary care clinics and provided a single serum sample. 50 of these subjects, analysed alongside the validation cohort, were age and gender matched to the PROFILE study recruits. All individuals provided written informed consent. The PROFILE study is registered on clinicaltrials.gov (NCT01134822 and NCT01110694).

The discovery analysis was limited to assessment of baseline matrix neoepitope levels with comparison of subjects and controls and subjects with IPF dichotomised into those with progressive or stable disease. Neoepitopes were selected for testing in the validation cohort if the following conditions were met: the assay was sufficiently robust to have <1% of data falling outside the upper or lower limits of detection; and the difference between stable and progressive disease was significant at the 10% level.

Procedures

Serum samples collected from PROFILE study subjects at baseline, one, three and six months and single samples from control subjects were stored at –80 °C until assayed. Collection was standardised across centres by using agreed Standardised Operating Procedures. Blood was collected using anti-coagulant free, serum separation tubes (Becton Dickenson, Oxford, UK) which uses coated silica as the clot activator. All samples were processed within two hours of collection. Samples were allowed to clot at room temperature over 30 minutes. Serum was separated by centrifugation and aliquoted prior to freezing. A unique aliquot was used for neoepitope analyses to avoid repeated freeze-thaw. Serum levels of biglycan degraded by MMP-2/9 (BGM), collagen 1 degraded by MMP-2/9/13 (C1M), collagen 3 degraded by MMP-9 (C3M), collagen 3 degraded by ADAMTS-1/4/8 (C3A), collagen 5 degraded by MMP-2/9 (C5M), collagen 6 degraded by MMP-2/9 (C6M), C-reactive protein degraded by MMP-1/8 (CRPM), Elastin degraded by MMP-12 (ELM), elastin degraded by MMP-9/12 (ELM2), the synthetic marker for collagen 3 (P3NP) and citrullinated vimentin degraded by MMP-2/8 (VICM) were measured as previously described¹². The performance characteristics of the assays and non-standard abbreviations are shown in supplementary table 1.

Measurements of forced vital capacity (FVC) and DLco were recorded, at baseline, 6 and 12 months, as absolute and percent predicted values based on the European Coal and Steel Board reference equations using age and height at the baseline visit¹⁶.

Statistical analysis

Disease progression was defined as all-cause mortality or $\geq 10\%$ decline in FVC at 12 months. In cases where 12 month FVC data were absent, subjects were considered to have progressed if a $\geq 10\%$ decline in FVC was observed at 6 months. Where no lung function data were available beyond baseline, cases were adjudicated, following case note review, by the local principal investigator blinded to biomarker results. Missing lung function data were not imputed. In cases where biomarker data were below the lower limit of detection, values were imputed to be half the lower limit of detection. Values above the upper limit were conservatively imputed as the upper limit of detection. Except where stated the percentage of imputed data was below 1% of all measurements for each biomarker. Sensitivity analysis was performed demonstrating that data imputation did not alter the impact of the reported findings. For serial biomarker data, change from baseline for each subject is represented by a gradient on a linear model where the single explanatory variable is the number of months between the baseline visit and subsequent visits using the recorded visit date.

Prior to performing any association analysis, relevant variables were assessed for normality. Skewed data were transformed using the base 10 or base 2 logarithms. When comparing baseline biomarker values between control and IPF groups adjusted estimates of group means, differences and confidence intervals were obtained using a general linear model with age, gender and group as explanatory variables. The nature of the variation of biomarkers over time was evaluated in the validation population. A repeated-measures, mixed effects model was used with biomarker value as the dependent variable and progression status, visit and visit by progression status as explanatory variables. The covariance structure was constrained for measurements which were repeated within subject, with visit as a repeated effect and with an unstructured covariance matrix. Least Squares estimates were used to obtain the means and confidence intervals for each biomarker in each progression status and visit group and for the difference in biomarker means between progression and stable groups. Survival analysis was performed using a Cox proportional hazards model to assess

the association between continuous explanatory variables and overall survival. Multivariate analysis was undertaken with a stepwise selection method within a Cox regression model with entry criterion set to $p < 0.1$ and stay criterion set to $p < 0.2$. Survival analysis was performed for baseline biomarker and lung function measures and for biomarker gradients. Further analysis was performed to evaluate the impact of applying different thresholds to dichotomise explanatory variables in the survival analysis. Potential thresholds for biomarker gradients were selected by identifying unique values for each gradient, ordering by value, iteratively setting a threshold to each value and estimating the hazard ratio for subjects above the threshold compared with those below

Associations between explanatory variables and endpoints were declared significant at the 5% level. As the study comprises a biomarker discovery and validation cohort, there has been no adjustment for multiple testing within each cohort. All statistical analyses were performed using SAS version 9.2 and were validated by independent replication.

Role of the funding source

The PROFILE study was funded by the Medical Research Council (G0901226) and GlaxoSmithKline R&D (CRT114316), and was sponsored by Nottingham University and Royal Brompton and Harefield NHS Foundation Trust. Analysis of neoepitope biomarkers was conducted by Nordic Biosciences with funding from GlaxoSmithKline R&D. Investigators received no financial incentives from the funding sources. GlaxoSmithKline R&D participated in study design, co-ordination of biomarker analysis, and statistical analysis. TMM and RGJ were involved in all stages of study development and delivery, had full access to all data in the study and had final responsibility for the decision to submit for publication.

RESULTS

Of the 214 eligible subjects, recruited between September 2010 and March 2012, 189 had a confirmed diagnosis of IPF and were included in subsequent analyses (figure 1). These subjects (table 1 and supplementary table 2) were predominantly male (78.8%) with a mean age of 70.1 ± 8.3 years. Patients from the Royal Brompton Hospital were significantly younger than patients from Nottingham University Hospitals and had slightly, but significantly worse lung function (supplementary table 2). 184 participants had baseline FVC data. 148 of these individuals had available 12-month FVC data. Repeated measures of FVC were unavailable due to death in 23 cases. 16 of the remaining 18 subjects missing a 12-month FVC had sufficient available clinical data to assign a progression status.

11 neoepitopes were measured in the discovery cohort of 55 subjects and a group of 20 healthy gender-matched (80% male) controls with a mean age of 56.6 ± 4.6 years. Levels of seven of the matrix neoepitopes (BGM, C1M, C3M, C6M, CRPM, ELM2, and VICM) differed significantly between healthy controls and individuals with IPF (supplementary figure 2). To determine the relationship between baseline neoepitope levels and subsequent disease behaviour, subjects with IPF were dichotomised, based on 12-month outcome, in to those with stable ($n=23$) or progressive ($n=32$) disease. Levels of six neoepitopes (C1M, C3A, C3M, C6M, CRPM and VICM) were significantly higher at baseline in individuals with progressive disease compared with those with stable disease (figure 2). However, in an unadjusted analysis of the discovery cohort (Table 2) only baseline CRPM significantly associated with decreased survival time (HR 3.74 95% CI 1.46 – 9.58, $p = 0.006$). Baseline FVC (HR 0.09 95% CI 0.03 – 0.27, $p<0.001$) and DLco (HR 0.19 95% CI 0.08 – 0.42, $p<0.001$) were also both strongly associated with survival. In multivariable analysis only baseline DLco remained a significant independent predictor of survival.

Following analysis of the discovery cohort, eight neoepitopes (BGM, C1M, C3A, C3M, C5M, C6M, CRPM and VICM) were measured in a validation cohort of 134 individuals with IPF and 50 age- and gender-matched healthy controls. In keeping with the results from the discovery cohort, baseline

levels of C1M, C3M, C6M and CRPM were significantly higher in IPF subjects than in healthy controls (supplementary figure 2). In contrast with the discovery cohort, at baseline, none of the neoepitopes showed a statistically significant difference between stable and progressive subjects. However, pooled analyses suggested five neoepitopes (C3A, C3M, C6M, CRPM, VICM) could discriminate between stable and progressive disease (supplementary figure 3). Analysis of serial samples demonstrated that by six months levels of BGM, C1M, C3A, C3M, C6M and CRPM were significantly higher in individuals with progressive IPF compared with those with stable disease (figure 3 and supplementary figure 4). Unadjusted analysis of the validation cohort demonstrated that baseline C1M (HR 1.62 95% CI 1.14–2.31, $p=0.007$) and C3A (HR 1.91 95% CI 1.06–3.46, $p=0.032$) together with FVC (HR 0.27 95% CI 0.10–0.70, $p=0.007$) and DLco (HR 0.18 95% CI 0.10–0.34, $p<0.001$) significantly associated with decreased survival (Table 2). Baseline CRPM, which showed an association in the discovery cohort, trended towards an association with survival in the validation cohort (HR 1.87 95% CI 0.98–3.56, $p=0.058$). Again, on multivariable analysis DLco remained the only significant baseline predictor of survival.

To understand the relevance of temporal variations in neoepitope values, the rate of biomarker change (Δ) over three months was calculated for each subject. An increasing rate of change of six neoepitopes (BGM; C1M; C3M; C5M; C6M; CRPM) indicated poorer overall survival (figure 4). After dichotomisation of subjects into those with rising levels of each neoepitope over 3 months ($\Delta>0$ ng/ml/month) or those with a stable or falling neoepitope level ($\Delta\leq 0$ ng/ml/month) the unadjusted mortality rate was significantly higher in subjects with rising levels of C1M, C5M, C6M and CRPM compared with stable or falling levels (figure 4). The greatest effect size was seen for CRPM (HR 2.16 CI 1.15–4.07, $p<0.001$). After adjustment for other covariates (age, gender, site, smoking status and baseline % predicted FVC and DLco), the individual rates of change over three months of C1M, C3M, C5M, C6M and CRPM were each significant independent predictors of overall survival (supplementary table 4). With the exception of CRPM, the relationship between temporal rate of change and subsequent outcome was less apparent after only a month, but was strengthened further by inclusion of six month neoepitope values (supplementary table 5).

To explore the effect of magnitude of the rate of change of neoepitope level on survival, the hazard ratio for overall survival was estimated at differing thresholds for rate of change at three months by comparing survival in patients with changes above and those below the threshold (figure 5). The most striking relationship with threshold was observed for CRPM for which a $\Delta > 0$ ng/ml/month conferred a HR of 2.16 (95% CI 1.15–4.07) whilst a $\Delta > 1$ ng/ml/month resulted in an HR 4.08 (95% CI 2.14-7.8) and a $\Delta > 1.7$ ng/ml/month was associated with an HR 6.61 (95%CI 2.74-15.94).

DISCUSSION

The PROFILE study is the largest prospective cohort study of incident idiopathic fibrotic lung disease conducted to date. It has enrolled patients with incident fibrotic lung disease of all severities, through two co-ordinating centres, with all subjects included in the current analysis being naïve for anti-fibrotic therapy. The slightly younger, but more severe, cohort from the Royal Brompton hospital probably reflects its role as a national tertiary referral centre, whereas the patients seen at regional hospitals, co-ordinated through Nottingham, represent truly unselected patients reflective of those seen in secondary care centres. This has generated a unique, prospective data set collected at pre-specified time intervals from incident cases of IPF. As such, to our knowledge, it is the first study to demonstrate that disease progression and subsequent outcome in IPF can be determined by detecting temporal change in the levels of circulating proteins. This analysis of the PROFILE dataset demonstrates proof of principle that serial longitudinal measurement of serum markers provides important information over and above single point in time measures, and furthermore may address the critical unmet need in the field of IPF research for prognostic biomarkers of molecular phenotype or therapeutic response (theragnostic biomarkers).

Serum biomarkers are unlikely to usurp high resolution computerised tomography (HRCT) in the diagnosis of IPF. Biomarker levels obtained at baseline may, however, facilitate management decisions in cases of unclassifiable, progressive fibrotic lung disease through identification of molecular phenotypes of disease predictive of response to anti-fibrotic therapies. It seems unlikely that baseline serum biomarker levels will outperform existing physiological measures of disease severity. Our data confirm the prognostic importance of baseline FVC and DLco^{17,18}. Whilst longitudinal change in these physiological measures of IPF disease severity associate with worsening fibrosis they do so only after a minimum of six or 12 months¹⁹⁻²¹. Furthermore, the value of serial DLco is hampered by measurement variability both within subjects (which has been shown to be as great as 15% in serial measures obtained in healthy individuals²²), and between lung function laboratories, and the

confounding effects of pulmonary hypertension. Additionally, unlike serum biomarkers, physiological biomarkers provide no insights in to disease mechanism and are thus unable to identify distinct molecular phenotypes of disease.

A lack of short term measures of change in disease activity has hampered the development of early phase clinical trials in IPF²³. Importantly, therefore, we show that changes in the levels of six neoepitopes (BGM, C1M, C3M, C5M, C6M and CRPM) over time strongly associate with outcome. Moreover, the magnitude of change of the values of these neoepitopes associate strongly with subsequent survival. Vitally, for each neoepitope, change over only three months was predictive of subsequent outcome. The observed relationship between rate of change in serum neoepitope levels and outcome suggests that these markers may have the potential for use as surrogate clinical trial endpoints and could therefore transform early phase clinical trial design in IPF. It is also tempting to speculate that measurement of neoepitopes could aid clinical decision-making in IPF. With the approval of both pirfenidone and nintedanib in the US and Europe there is, for the first time, a choice of anti-fibrotic treatments. Both drugs have been shown to slow disease progression, but are not anticipated to halt or reverse IPF. Therefore, in a disease that displays variable rates of progression there is now a desperate need for theragnostic biomarkers to define, for individual patients, treatment 'success' and 'failure' to thus enable clinicians to make rational decisions regarding treatment initiation and discontinuation. Appropriately designed interventional trials, utilising either pirfenidone or nintedanib, will be required to determine whether serial measurement of neoepitopes predicts treatment response. The development of proven theragnostic markers for IPF has the potential to shorten clinical trials and to enable better delivery of patient care. Measuring matrix neoepitope levels could enable patients to avoid unnecessary adverse events and prevent irreversible lung function loss whilst reducing the economic cost of persevering with ineffective therapy.

Measurement of neoepitopes provides an indirect mechanism for determining the activity of degradative proteolytic enzymes¹⁰. In keeping with our data, Leeming *et al* have previously shown

C1M, C3M, and C6M to be elevated at diagnosis in serum from 30 individuals with IPF compared with 15 unmatched controls¹². The markers chosen for this study were selected specifically to identify metalloproteinase cleavage products (together with a single marker of collagen synthesis) with the intention of indirectly assessing ECM turnover in IPF. Several strands of evidence suggest that MMP activity is upregulated in IPF. MMPs -1, -2, -3, -7, -8 and -9 have been shown to be elevated in the serum, lung tissue and lavage fluid of subjects with IPF^{7, 8, 24, 25}. Furthermore, MMP-9, measured at the RNA level in whole lung homogenate and the protein level in BAL, discriminates between stable and progressive cases of IPF²⁵. Activation of MMPs is associated with increased fibroproliferation in both *in vitro* and *in vivo* studies^{7, 26}. Furthermore, neoepitope levels have been shown to track the evolution of fibrosis in tissue sections¹³. It is, therefore, tempting to speculate that increased MMP activity alone is responsible for the rising neoepitope levels observed in progressive IPF. However, whilst C1M, C3M, C5M, C6M, ELM, ELM2 and VICM are all degraded by MMP-2 or -9 we found that serum levels of these different neoepitopes vary in their relationship to IPF disease progression. A possible explanation for our observations are that increased activity of collagenases (MMP-1,-3,-7 and -8) generate increased substrate for the gelatinases (MMP-2 and -9). This supposition is supported by the strongest association being found with CRPM. Further experimental data are needed, however, to confirm whether high levels of collagen neoepitopes reflect a phenotype of enhanced collagenase activity.

Baseline assessment of a number of serum proteins (including MMP-7, CCL-18, SP-D and KL-6) have been shown to weakly predict subsequent disease behaviour in IPF^{8, 27-29}. None of these proteins has, however, been assessed serially to determine whether change over time maps to worsening of underlying fibrosis. The PROFILE study was designed with the explicit aim of longitudinally phenotyping a carefully described cohort of individuals with idiopathic fibrotic lung disease (both IPF and idiopathic NSIP) with the intention of identifying markers of disease behaviour. Any such markers can then be tested for their potential as therapeutic biomarkers. A major strength of the study is that all patients with an incident diagnosis of IPF, irrespective of disease severity, were included and therefore the outcomes observed in our cohort mirror the true clinical experience of IPF. Further

strengths of this study relate to the size of the cohort, the two-stage design, the longitudinal sampling performed and the fact that longitudinal clinical data were available to determine disease outcomes in all but two subjects. As previously described the PROFILE cohort is the largest prospective biomarker study undertaken in therapy naïve, incident cases of IPF of all severity ranges. With the US and European approvals of pirfenidone and nintedanib it is unlikely that such a study will be repeated and thus the PROFILE study represents a unique and valuable resource. Given the finite nature of the samples available, it was crucial that rational stop-go decision-making was used to assess the matrix neoepitopes. The discovery analysis was designed to maximise the chances of detecting plausible biomarkers. Whilst our study design increased the risk of false positive results, this was mitigated by utilisation of a validation cohort. Although we chose to undertake measurements in serum, matrix neoepitopes are considerably more stable than many other protein analytes and so, with the exception of VICM, they can also be measured in plasma and other biological fluids such as urine or bronchoalveolar lavage fluid^{30, 31}.

This study has some limitations. Because the discovery cohort was enriched for individuals with extremes of disease severity the validation cohort contains a disproportionate number of subjects with moderate disease. This may have reduced the power of our validation study to detect a relationship between neoepitopes and disease outcomes. Against this, however, mortality was similar in both the discovery and validation cohorts (supplementary figure 3) and, on association analysis, only BGM, C3M and ELM showed a relationship to baseline disease severity (data not shown). Furthermore, the observational nature of the PROFILE study precluded standardisation of therapy. However, sample collection occurred at a time when there were no approved IPF treatments routinely available in the UK and no patients were receiving pirfenidone for the duration of these analyses. Although many study subjects were on N-acetyl cysteine recent trial data suggest that this is unlikely to have influenced the natural history of the disease³². Whilst it would be reassuring to replicate our findings in a truly independent external validation cohort the unique nature of the PROFILE cohort means that this is unlikely to be possible. This is because other existing cohorts are considerably

smaller; most have not collected serial samples; commercial clinical trials do not include patients of all severities and most have failed to routinely collect samples. Furthermore, as noted, it will not be possible to collect a future cohort of patients naïve for anti-fibrotic therapy. The lack of age-matched controls is a limitation of the discovery analysis. However, in these analyses results are adjusted to avoid age as a confounding factor. More importantly, the data from control patients are not used to draw any conclusions rather they are presented to facilitate benchmarking and to provide context for the neoepitope values seen in IPF patients.

In conclusion, to our knowledge, this is the first IPF biomarker study to combine longitudinal clinical data collection with serial biological sample collection from incident cases of IPF, with disease of any severity and naïve to anti-fibrotic therapy. Although, further data relating to serum measures from this cohort will become available in the future, this primary analysis of the cohort demonstrates that protein fragments generated by metalloproteinase activity identify individuals with IPF from healthy controls and, more importantly, the rate of change of five of these protein fragments over three months associates with disease worsening and predicts reduced survival. We believe that these neoepitopes have the potential to address an urgent unmet need in the management of IPF. Rising levels of matrix neoepitopes may identify a phenotype of IPF characterised by rapid matrix turnover and poor prognosis which may be more amenable to anti-fibrotic therapy. Furthermore, the neoepitopes identified in this study have the potential to be used as theragnostic biomarkers. Whilst this hypothesis requires testing in an appropriately designed, prospective, therapeutic trial the predictive prognostic value of the 3-month change in these markers promises the potential to radically shorten future clinical trials and also to help clinicians determine treatment efficacy. This is an issue of heightened importance now that we are entering an era of multiple approved anti-fibrotic therapies³³.

Contributors

RGJ, TMM, JKS, PTL, RBH, TMc and RM participated in the design and planning of the study. AMR, GS, RB, AUW and PLM participated in recruitment of study patients and collected their data. DJL and MAK developed and undertook the neoepitope assays. AF, JHB, JKS, PTL, RGJ and TMM undertook the data analysis and prepared the manuscript for publication.

Declarations of Interest

TMM has received industry-academic funding from GlaxoSmithKline R&D, UCB and Novartis and has received consultancy or speakers fees from Astra Zeneca, Bayer, Biogen Idec, Boehringer Ingelheim, Cipla, GlaxoSmithKline R&D, Lanthio, InterMune, ProMetic, Roche, Sanofi-Aventis, Takeda and UCB. RGJ has received industry-academic funding from GlaxoSmithKline R&D, Novartis and Biogen Idec and has received consultancy or speakers fees from InterMune, MedImmune, Pulmatrix, Boehringer Ingelheim, PPD, Biogen Idec and GlaxoSmithKline R&D. AUW reports fees for work on scientific advisory boards for InterMune, Boehringer Ingelheim, Actelion, Gilead, Genentech, MedImmune, and Takeda; and lecturing fees from InterMune, Boehringer Ingelheim, and Actelion, outside of the submitted work. RH reports grants from Glaxo Smith Kline, and from the Medical Research Council, during the conduct of the study; . GS reports grants from Glaxo Smith Kline, and the Medical Research Council, during the conduct of the study; PTL, RPM, JHB, JKS and AF are all employees and shareholders of GlaxoSmithKline R&D. DJL is an employee of Nordic Biosciences and holds patents relating to neoepitopes. MAK is an employee and shareholder of Nordic Biosciences and holds patents relating to neoepitopes. The other authors declared no conflicts of interest.

Acknowledgements

Dr Toby Maher is supported by an NIHR Clinician Scientist Fellowship (NIHR Ref: CS-2013-13-017). We are grateful to all patients for their participation in the PROFILE study, and to the members of the Brompton Multi-Disciplinary Team. The work at the Brompton was supported by the National Institute for Health Research Respiratory Biomedical Research Unit at the Royal Brompton and

Harefield NHS Foundation Trust and Imperial College, who part fund the salary of AUW. Dr Gisli Jenkins received support from the MRC (MICA grant G0901226). We are also indebted to Simon Johnson, Rob Berg, Will Elston, Khalid Amsa, Nanda Uttam, Sanjay Agrawal, Ann Miller, Paul Beirne, Lisa Spencer and Moira Whyte for identifying and contributing patients to the Nottingham PROFILE cohort and to Dr Kate Pointon, Dr Maruti Kumaran and Dr Irshad Soomro for their support of the Nottingham Regional MDT meeting. Sam Garthside at GlaxoSmithKline R&D, provided invaluable support in the processing and quality control of clinical and biomarker data. Nordic Bioscience received funding from the Danish Research Foundation.

Research in Context

Systematic Review: We searched PubMed for reports before October 1 2014 with the search terms “idiopathic pulmonary fibrosis”, “biomarkers” and “prognosis”. No language restrictions were applied. At the time of our search, none of the published biomarker studies in IPF had assessed longitudinal change and only a small number had had been performed in similar sized cohorts (all of which were retrospectively assessed) to the one available through the PROFILE study.

Interpretation: These data are the first output from the largest and most detailed prospective observational cohort study in IPF. We demonstrate that a number of matrix-metalloproteinase degraded neoepitopes are elevated in the serum of individuals with IPF compared with age matched healthy controls. Importantly, three-month longitudinal change in six neoepitopes correlates with worsening disease and outcomes in subjects with IPF. Change in neoepitope levels predicts progression of fibrosis at an earlier stage than does decline in physiological parameters. Furthermore, magnitude of change relates to subsequent likelihood of disease worsening. Although

further studies are required, measurement of neoepitopes have the potential to be used as theragnostic biomarkers which could be of use in future trials and in clinical practice to define response to therapy.

Tables

	Discovery (n=55)	Validation (n=134)	All subjects (n=189)
Age (years)	68.5 (9.53)	70.7 (7.66)	70.1 (8.28)
Male gender	43 (78.2%)	106 (79.1%)	149 (78.8%)
Ethnicity			
European	51 (92.7%)	127 (94.8%)	178 (94.2%)
Asian	4 (7.3%)	6 (4.4%)	10 (5.3%)
Other	0	1 (0.7)	1 (0.5)
Ever smokers	40 (72.8%) n=54	94 (70.2%)	134 (70.9%) n=188
BMI (Kg/m²)	28.2 (4.84)	28.4 (4.20)	28.4 (4.39)
Baseline lung function			
Mean FVC (% predicted)	75.9 (23.46) n=53	78.1 (17.18) n=131	77.5 (19.16) n=184
FVC 60≤90 % predicted	24 (43.6%)	85 (63.5%)	109 (57.7%)
Mean DLco (% predicted)	44.4 (18.26) n=52	42.1 (13.51) n=124	42.8 (15.05) n=176
DLco 40≤60 % predicted	12(21.8%)	52 (38.8%)	64 (33.9%)
CPI	48.4 (16.42) n=52	49.7 (11.56) n=123	49.3 (13.15) n=175
Median follow up (range) days	792 (47 – 1071)	643.5 (24 – 1100)	673 (24 – 1100)
Data are Mean (SD) or number (%) unless otherwise stated. BMI – Body mass index. FVC – forced vital capacity, DLco – diffusion capacity for carbon monoxide. CPI – Composite Physiological Index. Moderate FVC defined as % predicted FVC between 60-90% inclusive. Moderate DLco defined as % predicted DLco between 40-60% inclusive.			

Table 1. Comparison of baseline clinical characteristics between subjects included in discovery and validation cohorts.

Group	Variable	Discovery Population		Validation Population	
		Hazard Ratio		Hazard Ratio	
		(95% CI)	P value	(95% CI)	P value
Nordic Markers	BGM	1.17 (0.53,2.58)	0.71	1.34 (0.92,1.97)	0.13
	C1M	1.21 (0.66,2.22)	0.55	1.62 (1.14,2.31)	0.01
	C3A	1.34 (0.95,1.88)	0.092	1.91 (1.06,3.46)	0.03
	C3M	2.18 (0.95,5.00)	0.066	1.56 (0.94,2.59)	0.09
	C5M	1.66 (0.95,2.91)	0.077	1.07 (0.66,1.72)	0.79
	C6M	1.49 (0.86,2.56)	0.16	1.39 (0.93,2.06)	0.11
	CRPM	3.74 (1.46,9.58)	0.006	1.87 (0.98,3.56)	0.06
	ELM	0.96 (0.75,1.24)	0.78	ND	ND
	ELM2	1.00 (0.68,1.45)	0.99	ND	ND
	P3NP	1.48 (0.67,3.27)	0.33	ND	ND
	VICM	1.11 (0.83,1.49)	0.50	1.15 (0.92,1.43)	0.24
Lung Function Measures	% pred FVC	0.09 (0.03,0.27)	<0.001	0.27 (0.10,0.70)	0.01
	% pred DLco	0.19 (0.08,0.42)	<0.001	0.18 (0.10,0.34)	<0.001

Table 2. Univariate analysis of overall survival by baseline measures in discovery and validation cohorts. FVC – forced vital capacity, DLco – diffusion capacity for carbon monoxide. Hazard ratios for the neoepitopes relate to the effect of a 1 unit increase in the variable whilst the hazard ratios for FVC and DLco relate to the effect of a two-fold increase in the explanatory variable.

Figure Legends

Figure 1: Study population. A total of 214 individuals were recruited into the study (between September 2010 and March 2012) of whom 25 had a diagnosis of NSIP and the remainder were confirmed to have IPF (n=189). A Discovery Cohort based on disease severity as determined by composite physiological index (38.5% mildest; 25% moderate; 36.5% severest) was drawn from the first 100 subjects and the Validation Cohort (n=134: 14.6% mild; 71.5% moderate; 13.8% severe using the criteria applied to the discovery cohort) consisted of the subsequent 114 subjects plus the remaining subjects from the first 100 patients. Three subjects were excluded from the validation cohort (one was missing a baseline serum sample and two were lost to follow-up).

Figure 2: Baseline comparison of neoepitope levels in healthy controls and IPF subjects with stable and progressive disease in the discovery cohort. Biomarker data from the Discovery Cohort is presented as means (ng/ml) and 95% confidence intervals adjusted for age and gender. Numbers in each group are; 20 healthy subjects (gender matched only) represented by '●'; 23 stable IPF represented by '■' and 32 progressive IPF represented by '▲'. Disease progression was defined as all-cause mortality or $\geq 10\%$ decline in FVC at 12 months. For ELM2 4% of values were imputed. For all other epitopes the imputation rate was $<1\%$. P values are provided where significant differences were observed between Healthy subjects and Stable IPF subjects ($\square$); between healthy subjects and Progressive IPF (Δ) and between stable IPF and Progressive IPF ($\diamond$).

Figure 3: Comparison of neoepitope levels in healthy controls and IPF subjects with stable and progressive disease at baseline and at 1, 3 and 6 months post baseline in the validation cohort. Subjects were recruited into the PROFILE study within six months of diagnosis (baseline) and serum samples stored for biomarker analysis at baseline and at one, three and six months post baseline. Numbers at baseline were 50 healthy subjects (●); 60 IPF subjects with stable disease (■) and 71 subjects with progressive IPF (▲). Biomarker data represent means (ng/ml) and 95% confidence intervals, adjusted for age and gender. Two subjects were excluded from analysis because of missing

longitudinal follow up and one subject because of missing baseline samples. For C5M 2% of values were imputed. For all other neoepitopes the imputation rate was <1%. P values are provided where differences were observed between stable and progressive disease at a particular time point (▼). Levels of BGM, C1M, C3A, C3M, C6M and CRPM increase over time in the subjects with progressive, but not stable, disease.

Figure 4: Effect of 3 month change in neoepitope levels on overall survival. For all individual participants and each individual neoepitope the three month change in neoepitope level (Δ) was calculated as the slope of the best fit line through baseline, one month and three month values. Subjects have then been dichotomised into those with rising ($\Delta > 0 \text{ ng/ml/month}$ shown in red) and those with stable or falling ($\Delta \leq 0 \text{ ng/ml/month}$ shown in blue) neoepitope levels. The number of deaths in each group is shown for each biomarker. The Hazard Ratio (HR) represents the mortality risk in subjects with rising neoepitope levels ($\Delta > 0 \text{ ng/ml/month}$) relative to subjects with stable or falling levels ($\Delta \leq 0 \text{ ng/ml/month}$). The p-values presented are those for association between the individual, dichotomous marker and survival. This association was significant for C1M, C5M, C6M and CRPM.

Figure 5: Effect of the magnitude of 3 month change in neoepitope levels on overall survival. The effect of different rates of neoepitope change was explored by using a range of threshold values of the biomarker gradient in order to dichotomizing the patients into a 'high' (gradient above threshold) or 'low' (gradient below threshold) group. The effect, on the hazard ratio, of dichotomising subjects at different available thresholds of 3-month neoepitope change is presented. The x-axis represents the threshold of the slope of the biomarker change that was used to dichotomise the patient population. The y-axis represents the hazard ratio plotted as base 2 log transformation of the unitary data. The data are expressed as mean hazard ratio with 95% confidence intervals for each threshold. For each threshold value on the x-axis, the hazard ratio plotted represents the risk for IPF subjects in "high" group vs "low" group. The upper and lower

extremes for each neoepitope have been limited to comparisons where there are at least 5 deaths in both the high and low groups.

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