# Serum mitochondrial DNA predicts the risk of acute exacerbation and progression of idiopathic pulmonary fibrosis

*To the Editor:*

Idiopathic pulmonary fibrosis (IPF) is a progressive, fatal interstitial lung disease with a median survival of 3–5 years [1]. Its disease course is highly variable, as some patients experience rapid deterioration in lung function while others experience more gradual decline [2]. The development of acute exacerbation of IPF (AE-IPF), a highly lethal complication of unknown aetiology, has been shown to accelerate disease progression [3]. Presently, there are no accepted biomarkers that predict clinical deterioration [4], thus indicating an important, unmet need in the management of this devastating disease.

The pathogenic role of innate immunity has been an emerging field of study, with recent work highlighting the endogenous innate immune agonist mitochondrial DNA (mtDNA), which acts as a danger associated molecular pattern for pathogen recognition receptors (PRRs). Elevated concentrations of extracellular mtDNA have been observed in multiple diseases, including IPF, where it was found to be predictive of poor survival [5]. Given that the role of mtDNA as a biomarker for disease progression has not been fully characterised, we hypothesised that elevated concentrations of serum mtDNA reflect the mechanisms underlying poor clinical outcomes in IPF, including the development of AE-IPF.

This study was approved by the Institutional Review Board at Tosei General Hospital and performed with written informed consent from participants. IPF diagnosis was made upon multidisciplinary discussion based on consensus guidelines [1]. Our IPF cohort included 70 subjects with a median age of 66 years (interquartile range (IQR) 63–71 years) that included 56 males (80.0%) and 46 ever-smokers (65.7%). Relevant clinical characteristics included 84% predicted forced vital capacity (FVC; IQR 69–95% pred), 58% pred diffusion capacity for carbon monoxide ($D_{LCO}$; IQR 42–73% pred), and 3 Gender-Age-Physiology (GAP) score 3 (IQR 2–4) [6]. Subjects were followed for a median of 52 months (Kaplan–Meier estimate), during which time 17 cases of AE-IPF (24.2%) and 30 deaths (42.9%) were observed. The diagnosis of AE-IPF was confirmed retrospectively according to published criteria [3]. Blood samples drawn at each subject's initial clinical encounter, prior to the initiation of anti-fibrotic therapy, were used in the analysis. Serum mtDNA concentrations were determined as previously described [7]; briefly, total cell-free DNA was extracted from 240 µL of serum, and mtDNA concentrations were expressed as copy numbers of the NADH dehydrogenase 1 gene per µL *via* quantitative PCR.

We first quantified serum mtDNA copy numbers in our IPF cohort, where the median concentration was 801 copies per µL. When subjects were stratified by this threshold value, subjects with serum mtDNA concentrations ⩾801 copies per µL exhibited higher incidence of AE-IPF (3.1 *versus* 14.5 per person-month; p=0.005 by Fisher's exact test), greater annual decline in FVC (−1.1 *versus* −7.5% pred; p=0.033 by Mann–Whitney U-test), and mortality (median survival; 36.2 *versus* 76.6 months; p=0.0001 by log-rank test). Moreover, no significant differences were observed in age, gender, smoking status or pulmonary function between the low (<801 copies per µL) and high (⩾801 copies per µL) mtDNA groups. These findings show that high serum mtDNA copy numbers are associated with poor outcomes in IPF.

To further characterise the role of serum mtDNA concentrations as a biomarker for the development of AE-IPF, we conducted time-dependent receiver operating characteristic curve analysis (figure 1a), which

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**Patients with IPF often suffer from acute exacerbation, an unpredictable and deadly complication. This study elucidated the potential of circulating mitochondrial DNA as a predictor of acute exacerbation and disease progression of IPF.** https://bit.ly/3i0OSza

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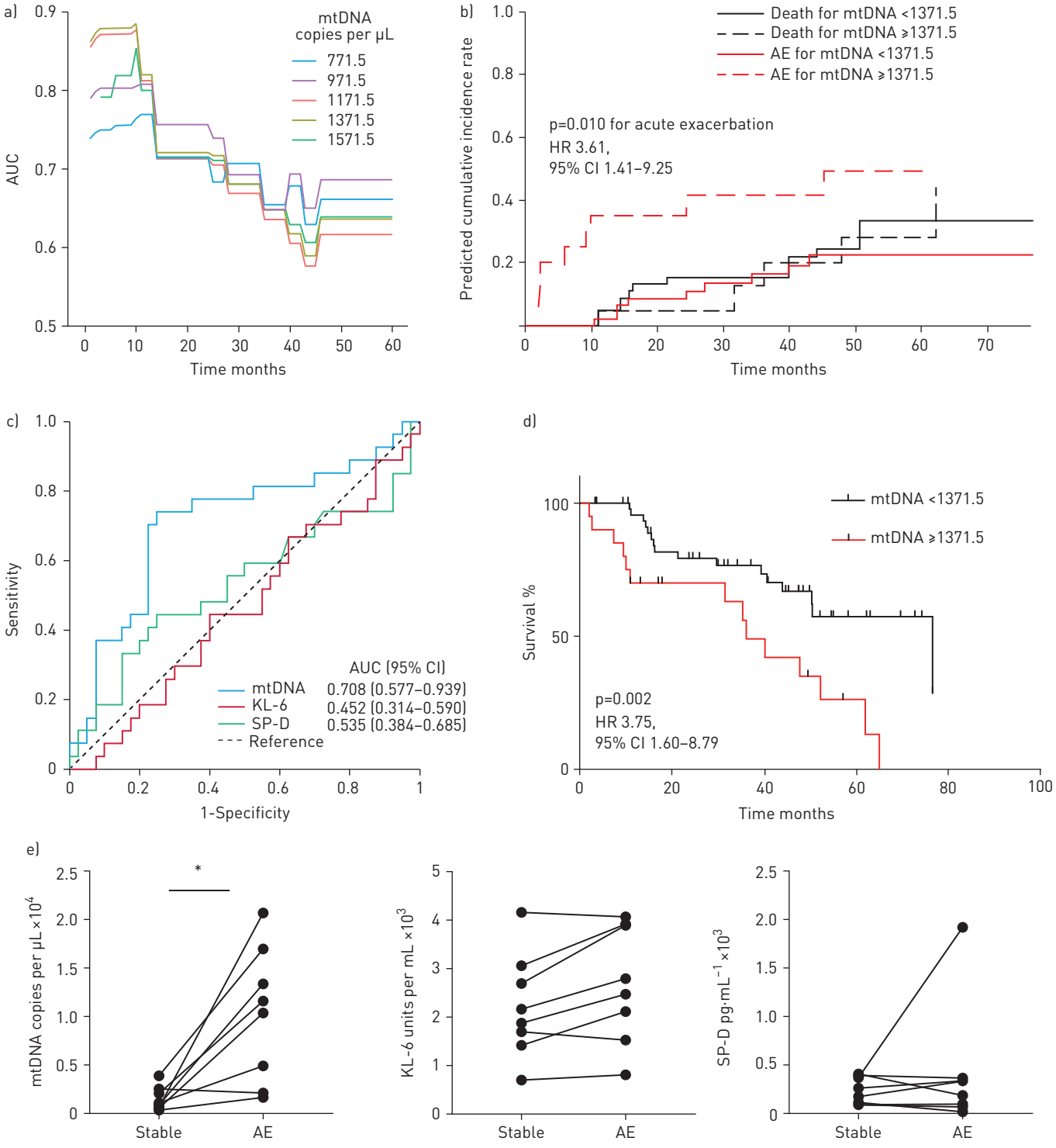


FIGURE 1 Assessment of mitochondrial DNA (mtDNA) levels in sera as a predictor of multiple clinical outcomes in idiopathic pulmonary fibrosis (IPF) patients. a) Time-dependent area under the curve (AUC) changes over time according to various cut-off mtDNA levels assessing predictive value for acute exacerbation of IPF. b) Cumulative incidence curves of acute exacerbation (red) and competing all-cause mortality (black) stratified by serum mtDNA level with the cut-off of 1371.5 copies per µL. In the comparison of the cumulative incidence rate of acute exacerbation (AE) between the low mtDNA group and high group, the p-value for Gray's test, as well as the hazard ratio (HR) and its 95% confidence interval for Cox regression analysis are shown. c) Receiver operating characteristic (ROC) curves for evaluating serum biomarkers as predicting composite outcome (>10% absolute decline of % predicted forced vital capacity (FVC), acute exacerbation or death) within a year. AUCs and their 95% confidence intervals for each biomarker are shown. d) Kaplan–Meier curve estimating the probability of overall survival in the cohort stratified by circulating mtDNA levels (cut-off 1371.5 copies per µL). p-value, hazard ratio and 95% confidence interval for log-rank test in the comparison of two groups are shown. e) Changes in levels of serum markers obtained from seven patients who developed acute exacerbation in their clinical course and subsequently died within 3 months. Changes of serum mtDNA, serum KL-6, and serum SP-D in each subject were depicted. *: p=0.031 by Wilcoxon rank test.

revealed that a serum mtDNA concentration of 1371.5 copies per µL can reliably stratify subjects at risk for developing AE-IPF within 1 year (sensitivity of 85.4%, specificity of 80.6%, area under the curve (AUC) 0.90). Following adjustments for mortality as a competing risk [8], we observed a substantially higher risk for developing AE-IPF (median incidence time 5.8 *versus* 24.3 months; p=0.010 by Gray's test; figure 1b). These data show that elevated circulating mtDNA levels can identify IPF subjects at high risk for developing AE-IPF.

Because current biomarkers poorly predict IPF disease progression [4], we then sought to determine if mtDNA copy numbers could provide insight into overall disease trajectory. Using serum mtDNA concentration as a continuous variable, we identified a significant association with composite IPF outcomes, which we defined as >10% absolute decline in FVC, acute exacerbation, or death within 1 year (AUC 0.708, 95% CI 0.577–0.939; figure 1c). More excitingly, serum mtDNA levels predicted this composite outcome better than other well-known IPF biomarkers, including KL-6 (AUC 0.452; figure 1c) and SP-D (AUC 0.535; figure 1c). Taken together, these findings suggest that serum mtDNA assessment in patients with stable IPF may predict future disease behaviour.

Because we also found a significant association between serum mtDNA concentration and mortality, we used the above serum mtDNA concentration cut-off value of 1371.5 copies per µL to confirm prior reports [5] showing that an elevated concentration of circulating mtDNA is associated with increased mortality (median survival 76.6 months *versus* 36.2 months; p=0.002 by log-rank test; figure 1d). Additionally, we performed Cox regression analysis with this serum mtDNA cut point and GAP stage, a validated clinical biomarker of IPF survival [6], to confirm that mtDNA levels (hazard ratio (HR) 2.970, 95% CI 1.418–6.218; p=0.004) and GAP stage (HR 1.821, 95% CI 0.822–4.033, p=0.140 for stage 2; HR 8.052, 95% CI 2.219–29.23, p=0.002 for stage 3, with stage 1 as a reference) are independently associated with mortality. These data further validate the role of circulating mtDNA as a prognostic biomarker in IPF.

Lastly, we performed exploratory longitudinal analysis with serum samples obtained at the time of AE-IPF diagnosis for seven subjects that died from this complication within 3 months following diagnosis. We found a substantial increase in their serum mtDNA concentrations relative to their respective baseline concentrations (p=0.031 by Wilcoxon rank test; figure 1e). Moreover, serum concentrations of KL-6 and SP-D exhibited only modest changes in these subjects (figure 1e). While this analysis is preliminary, these findings suggest that serial changes in mtDNA levels could function as an indicator of prognosis in AE-IPF.

Overall, our results robustly support the role of circulating mtDNA as a biomarker of poor clinical outcomes in IPF. Specifically, we found that elevated serum mtDNA levels were associated with the development of acute exacerbation, disease progression and worse survival. While additional work is needed to fully characterise the immunopathogenic role of extracellular mtDNA in IPF, studies show that differential expression of Toll-like receptor 9 [9] and cGAS-STING [10], PRRs that recognise mtDNA, is associated with IPF outcomes. This constellation of findings suggests that interactions between mtDNA and these PRRs determine individual susceptibility to the development of AE and rapid IPF disease progression.

Our work has several limitations. We have not determined the cell of origin of this extracellular mtDNA, which would require access to matched bronchoalveolar lavage and serum samples. In addition, our work is limited to a single cohort at one institution; validation studies, including determination of optimal threshold values through multicentre efforts, are needed. Nonetheless, our work shows that circulating mtDNA is a promising biomarker of poor prognosis in IPF. Further work in this area can lead to new approaches in the management of this poorly understood, fatal disease.

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