



Blood mitochondrial DNA as a biomarker of clinical outcomes in idiopathic pulmonary fibrosis

To the Editor:

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive fibrosing interstitial pneumonia of unknown aetiology [1] with ageing being one of the major risk factors [2]. Several ageing-related changes, such as the overproduction of mitochondrial reactive oxygen species, low adenosine triphosphate production, reduced mitochondrial biogenesis and inadequate mitochondrial (mt)DNA repair [3] have also been reported in the IPF lungs [4, 5] and in a bleomycin-induced mouse model [6]. Plasma cell-free DNA, including mtDNA, is released from dying cells into the circulatory system in response to injury [7]. Since circulating mtDNA contains CpG-rich sequences similar to bacterial DNA, disengaged mtDNA can activate the innate immune system by functioning as a damage-associated molecular pattern [8] and contribute to the stimulation of the profibrotic pathway [9].

Several studies have suggested the predictive value of plasma mtDNA in mortality associated with critical illness [10, 11], haemodialysis [12] and IPF [13]. RYU *et al.* [13] reported that the increase in plasma mtDNA levels was an effective predictor for all-cause mortality in patients with IPF ($n=81$; hazard ratio (HR) 3.79, 95% CI 1.53–9.43; $p=0.004$) after adjusting for age, sex, race and lung function (forced vital capacity (FVC) and diffusing capacity of the lung for carbon monoxide (D_{LCO})) or the Gender-Age-Physiology index [13]. However, the role of blood mtDNA in predicting clinical courses such as acute exacerbation (AE) and disease progression (DP) in IPF remains elusive. Here, we discuss the prognostic value of plasma mtDNA as a predictor of clinical outcomes in IPF.

Between January 2001 and December 2015, 1049 patients were diagnosed with IPF at the Asan Medical Center, Seoul, Republic of Korea. Of these, 320 patients (biopsy confirmed cases $n=119$) with blood samples available at the time of diagnosis were included in this study. All clinical parameters were recorded retrospectively within 1 month of sample collection. Data from follow-up visits usually every 3–6 months or hospitalisation were reviewed to assess the development of complications such as AE or DP. AE was defined according to the criteria proposed by COLLARD *et al.* [14], while DP was defined as $\geq 10\%$ absolute decline in FVC or $\geq 15\%$ decline in D_{LCO} within a year of diagnosis.

The plasma samples were stored at -80°C immediately after collection until analysed. For circulating mtDNA level measurement, DNA was isolated using a DNeasy Blood and Tissue Kit (#51306; Qiagen, Venlo, the Netherlands) from plasma samples according to the manufacturer's instructions. Real-time PCR assay was performed using the human nicotinamide adenine dinucleotide dehydrogenase 1 gene as a primer for mtDNA. As the mtDNA levels did not follow normal distribution, the values were subjected to natural logarithmic transformation (LN) to reduce skewness.

For the 320 patients, the mean age was 64.1 years, 78.4% were male, and the median (interquartile range) follow-up period was 37.4 (13.9–64.1) months. AE was reported in 84 (26.3%) patients during follow-up. Patients with AE had lower baseline lung function than those without AE (FVC 67.9% predicted *versus* 73.6% pred, respectively, $p=0.008$; and total lung capacity (TLC) 67.7% pred *versus* 73.3% pred, respectively, $p=0.002$); however, no significant differences were observed in age, sex, smoking status, body mass index (BMI), exercise capacity and treatment between the two groups. Among patients with serial lung function data available ($n=253$), 119 (47.0%) exhibited DP for 1 year after diagnosis. Patients with DP were younger than those without DP (62.7 *versus* 64.8 years, respectively, $p=0.043$); however, no



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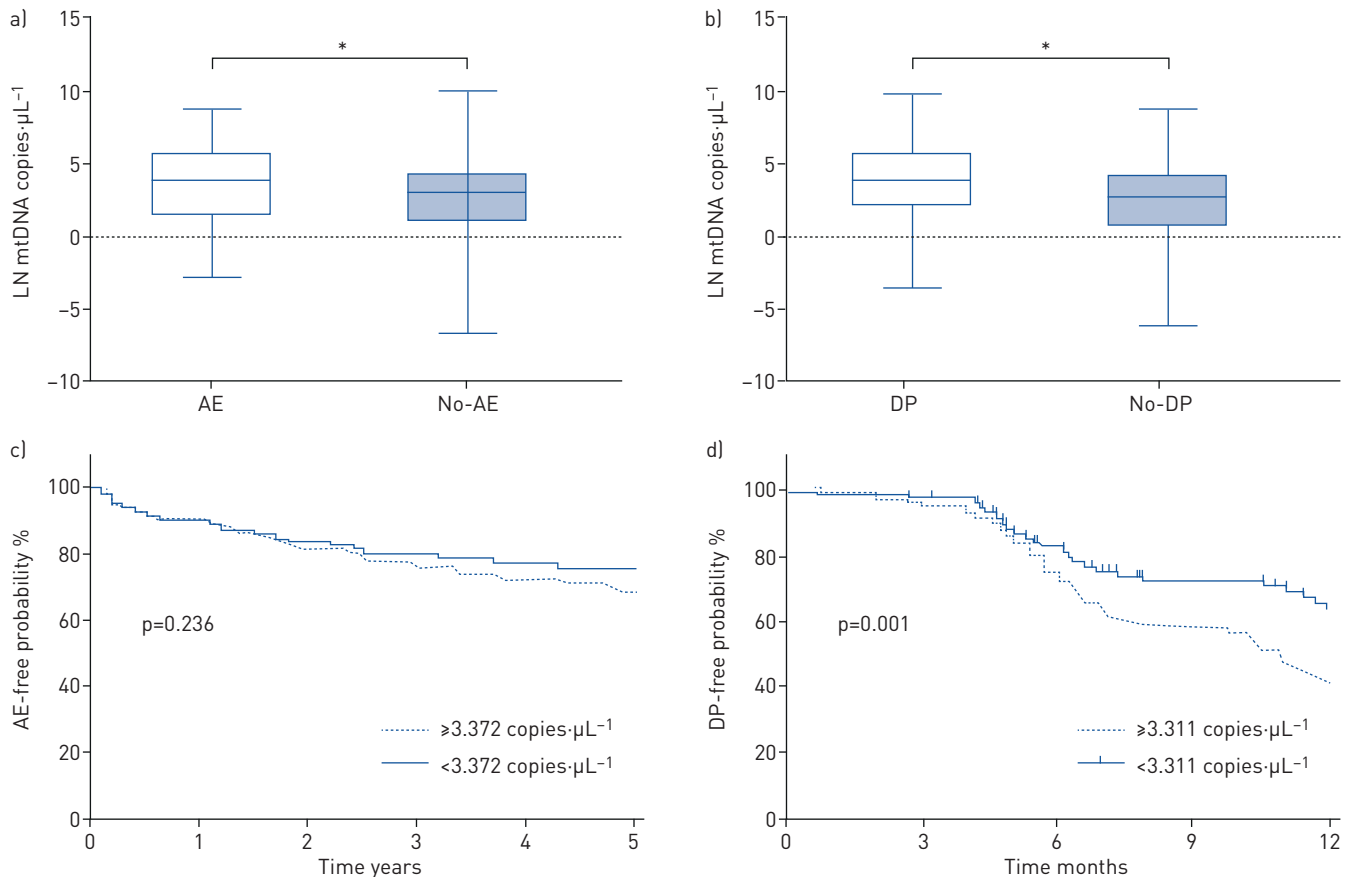


FIGURE 1 Comparison between plasma mitochondrial (mt)DNA levels at the time of diagnosis based on clinical outcomes. a) Comparison of plasma mtDNA levels at the time of diagnosis between patients with and without acute disease exacerbation (AE); b) comparison of plasma mtDNA levels at the time of diagnosis between patients with and without disease progression (DP); c) comparison of AE-free survival between patients with values above and below the cut-off values for mtDNA levels; d) comparison of DP-free survival between patients with values above and below the cut-off values for mtDNA levels. The box represents the interquartile range, the line within the box represents the median value, and the whiskers indicate the maximum and minimum values. AE- or DP-free survival was evaluated using Kaplan-Meier survival analysis and the log-rank test. LN: log-transformed. *: $p < 0.05$.

differences were observed in sex, smoking status, BMI, lung function, exercise capacity, and treatment between the two groups.

The baseline plasma LN mtDNA levels were significantly higher in the AE group than in the non-AE group (3.5 ± 2.7 versus 2.4 ± 3.0 copies·μL⁻¹, respectively, $p = 0.003$) (figure 1a). In addition, the baseline plasma LN mtDNA levels were more elevated in the DP group than in the non-DP group (3.4 ± 2.8 versus 2.2 ± 3.0 copies·μL⁻¹, respectively, $p = 0.001$) (figure 1b).

The baseline plasma LN mtDNA levels showed an inverse correlation with changes in D_{LCO} for 6 months ($r = -0.133$, $p = 0.041$) or 12 months ($r = -0.161$, $p = 0.021$), while changes in FVC for 12 months showed a trend towards a negative correlation with plasma LN mtDNA levels ($r = -0.117$, $p = 0.092$).

In the unadjusted Cox proportional hazard analysis, higher LN mtDNA levels served as a significant predictor for AE occurrence in patients with IPF, along with lower FVC, D_{LCO} and TLC, shorter 6-min walk test (6MWT) distance, and lower initial and lowest oxygen saturation (S_{pO_2}) during 6MWT. In the multivariable analysis, LN mtDNA levels were an independent risk factor for AE (HR 1.117, 95% CI 1.025–1.217; $p = 0.012$) along with BMI, FVC and lowest S_{pO_2} during 6MWT. Analysis of the unadjusted Cox proportional hazard model implicated higher plasma LN mtDNA levels as a significant predictive factor for DP in patients with IPF, along with younger age and the lowest S_{pO_2} during 6MWT. In the multivariable model, the LN mtDNA levels were independently associated with DP (HR 1.134, 95% CI 1.051–1.123; $p = 0.001$) along with age and lowest S_{pO_2} during 6MWT.

In the receiver operating characteristic (ROC) curve analysis, the LN mtDNA levels could effectively predict AE occurrence (area under the ROC curve (AUC) 0.607, 95% CI 0.534–0.679; $p = 0.004$), with the best cut-off value of 3.372 copies·μL⁻¹ (sensitivity 53.6%, specificity 56.7%), and DP (AUC 0.633, 95% CI

0.564–0.702; $p < 0.001$), with the best cut-off value of 3.311 copies· μL^{-1} (sensitivity 61.3%, specificity 61.2%). Although patients with LN mtDNA levels above the cut-off value (3.372 copies· μL^{-1}) exhibited lower AE-free survival than those with levels below the cut-off value, no significant difference was observed ($p = 0.236$) (figure 1c). However, patients with high LN mtDNA levels (≥ 3.311 copies· μL^{-1}) had significantly lower DP-free survival (median survival period 24.4 months *versus* 30.8 months; $p = 0.001$) than those with low LN mtDNA levels (< 3.311 copies· μL^{-1}) (figure 1d).

There were certain limitations to this study. First, it was a retrospective observational study conducted at a single centre. The study subjects only included patients who agreed to provide blood samples. Therefore, the selected subjects may not accurately represent all patients with IPF. Second, although the relationship between the mtDNA levels and clinical outcomes was significant, the low statistical power posed a challenge. Replication of our results in a different cohort may help validate our findings. Third, the plasma mtDNA levels may vary in certain cases, such as intra-assay variation detected by real-time PCR. Therefore, we measured the mtDNA levels thrice per sample and used the average values for analysis to reduce experimental errors in real-time PCR. Last, we measured the mtDNA levels at a single time point; however, biomarker study in serial samples would be helpful. Despite these considerations, the large sample size from the longitudinal IPF cohort with a long follow-up period was the major advantage of this study.

Here, we demonstrated the potential of plasma mtDNA levels determined at diagnosis as a biomarker for AE and DP in patients with IPF and its correlation with the changes in lung functions, and highlighted its potential therapeutic applications.

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