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Decreased serum sirtuin-1 in chronic obstructive pulmonary disease

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Author's contributions

SY conducted assay, carried out the data analysis and drafted the manuscript. JB conducted assay with primary epithelial cells. AIP, AP, CV, SL were involved in sample preparation and participated in the design of the original study. PB participated in the design of the study, and contributed substantially to preparation of manuscript. KI contributed to the data analysis, design of the study and the manuscript preparation.

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ABSTRACT

Background: The protein deacetylase sirtuin-1 (SIRT1) is an anti-aging molecule that is decreased in the lung from patients with chronic obstructive pulmonary disease (COPD). Recently, SIRT1 was reported to be detectable in serum, but serum SIRT1 levels have not yet been reported in patients with COPD.

Methods: Serum SIRT1 was measured by Western blotting, and relative ratio of band density in samples against that of a positive control were calculated.

Results: Several molecular sizes of SIRT1, including 120kDa (actual size) and fragments (102, 75kDa) were quantified by Western blotting. Among them, only the 120kDa serum SIRT1 (s120S) was significantly decreased in the patients with COPD compared to the control subjects without COPD (s120S ratio in healthy: 0.90±0.34, vs COPD: 0.68±0.24; p=0.014), and was positively correlated with airway obstruction (FEV_1/ FVC; r=0.31; p=0.020) and its severity measured by FEV_1 % predicted (r=0.29; p=0.029). Serum s120S also showed a positive correlation with body mass index (BMI; r=0.36; p=0.0077) and diffusing capacity of the lung per unit volume (K_{CO}%; r=0.32; p=0.025). It was also significantly decreased with increasing severity of lung emphysema (r=-0.40, p=0.027) and with a clinical history of frequent COPD exacerbations (infrequent: 0.76±0.20 *vs* frequent: 0.56±0.26; p=0.027). SIRT1 was not detected in supernatant of A549 and primary epithelial cells in normal culture condition.

Conclusions: Serum SIRT1 (s120S) was decreased in the patients with COPD, potentially as reflected by the reduced SIRT1 within cells as a result of oxidative stress, and might be a potential biomarkers for certain disease characteristics of COPD.

Abbreviation List:

$AaDO_2$: alveolar-arterial oxygen difference

BMI: body mass index

BODE: body mass index, airflow obstruction, dyspnea, and exercise capacity

COPD: chronic obstructive pulmonary disease

FEV_1: forced expiratory volume in one second

FVC: Forced vital capacity

MRC: Medical Research Council

SIRT1: silent information regulator 2 homolog 1

WB: Western blotting

6MWD: six minute walking distance

Introduction

Sirtuin-1(SIRT1) is the mammalian homolog of silent information regulator (Sir2) family, initially described in yeast,[1] and this highly preserved gene encodes nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylases.[2] Through modulating acetylating/de-acetylating balances of multiple substrate proteins, SIRT1 regulates various cellular responses such as apoptosis, cellular senescence, endocrine metabolism, glucose homeostasis and aging.[2–7] Although SIRT1 was originally described as a nuclear protein,[8,9] it has recently been shown that SIRT1 shuttles between the nucleus and cytoplasm,[10–12] where it may associate with different target proteins in responding to divergent extracellular stimuli.[13–15] Interestingly, SIRT1 has recently been measured in the serum,[16] although its precise origin is unknown. In previous reports, serum SIRT1 was consistently decreased with aging,[17] and there was an accelerated reduction of serum SIRT1 in neurological disorders, such as Alzheimer's disease,[16] frailty,[18] and in obesity [19,20]; all of which suggest that serum SIRT1 may be a potential biomarker for various aging-associated diseases. By contrast, an increase in serum SIRT1 has been reported in patients with asthma.[21]. However, the measurement of serum SIRT1 in other pulmonary diseases is not yet been elucidated.

Chronic obstructive pulmonary disease (COPD) is a major global health problem.[22,23] In contrast to asthma, COPD is mainly caused by noxious gases such as cigarette smoke,[24,25] and characterized by poorly irreversible small airways obstruction, emphysema and corticosteroid-insensitive inflammation.[26] COPD progresses slowly, and therefore most patients are elderly and there is increasing evidence that it reflects accelerated aging of lungs.[27–29] SIRT1 is decreased in the peripheral lung and peripheral blood mononuclear cells from patients with COPD.[30] In this report, we have measured the serum levels of SIRT1 by Western blotting in COPD patients and age-matched control subjects and examined how it

95 related to characteristics of the disease.

96

Materials and Methods

Reagents

Commercially available reagents were obtained as follows: RPMI medium 1640 (RPMI 1640) (#32404-014) and Dulbecco's Modified Eagle Medium (DMEM) (31053-028) were from Life Technologies (Carlsbad, CA, USA); fetal bovine serum (FBS), complete protease inhibitor cocktail (11836153001) and rabbit-derived anti-SIRT1 antibody (#5322) were from Sigma-Aldrich Co. LLC (St Louis, MA, USA); anti-β-actin antibody (ab6276) was from Abcam plc. (Cambridge, UK); goat-derived peroxidase-conjugated anti-mouse (P0447) or anti-rabbit (P0448) secondary antibodies were from Dako (Cambridge shire, UK).

Patients and healthy volunteers for serum

This project was approved by the ethics committee of Sismanogleio General Hospital (approval number 5210-07/03/2012), and written informed consent was taken from patients and healthy volunteers. COPD was defined and categorized according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD).[22] Blood were taken from never smoker healthy subjects with normal lung function (NS, 12 subjects), smokers without COPD (SM, 19 subjects) and 26 patients with mild- to very severe-COPD (Stage 1-2, 13 subjects; Stage 3-4, 13 subjects Table 1). All COPD patients were considered to be clinically stable because none of them had required a change in their regular therapy during the 8 weeks preceding the sampling, nor had they been treated with systemic corticosteroids or antibiotics. Patients with asthma, pneumonia, or lung cancer were excluded from the study. The smoking history of each subject was represented from the mean number of pack-years of cigarette consumption by ex-smokers and current smokers. All COPD patients had history of smoking, but all patients were asked to refrain from smoking for three hours before the serum sampling.

Emphysema was characterized by high resolution computed tomography (HRCT).[31] The degree of emphysema was determined using a visual emphysema score as previously described [32] Briefly, emphysema was identified as areas of hypovascular low attenuation and was graded with a five-point scale based on the percentage of lung involved: 0: no emphysema; 1: up to 25% of the lung parenchyma involved; 2: between 26-50% of lung parenchyma involved; 3: between 51-75% of the lung parenchyma involved; and 4 between 76-100% of lung parenchyma involved. Grades of the axial images of each lung were added and divided by the number of images evaluated to yield emphysema scores that ranged from 0 to 4. COPD patients were characterized as frequent exacerbators if he has two or more severe exacerbations in one year.[33] The Medical Research Council (MRC) dyspnea scale,[34] Borg scale (dyspnea and fatigue),[35] six minute walking distance (6MWD),[36] BODE (body mass index, airflow obstruction, dyspnea, and exercise capacity) index [37] and Charlson index [38] were examined according to the original reports. We also examined the air trapping by RV/TLC, and oxygenation capacity of lung by PaO_2/FiO_2 or by alveolar-arterial oxygen difference ($AaDO_2$).

Blood sampling

Blood samples were collected in BD Vacutainer® Plus Plastic Serum and SST™ Tubes, which are coated with silicone and micronized silica particles to accelerate clotting. Then samples were centrifuged at 1500xg for 15 min at room temperature, and supernatants were aliquoted as serum samples, and immediately stored at -70 ^{o}C until measurement.

Pulmonary function tests

Pulmonary function tests were performed using MasterScreen (Erich Jaeger GmbH, Wurzburg, Germany) and included post-bronchodilator forced expiratory volume in one second (FEV_1), forced vital capacity (FVC), FEV_1/ FVC ratio, total lung capacity (TLC), residual volume (RV), inspiratory capacity (IC) and diffusing capacity for carbon monoxide (DL_{CO}). Diffusing capacity for carbon monoxide (DL_{CO}) and diffusing capacity for carbon monoxide adjusted for alveolar volume (DL_{CO}/V_A or K_{CO}) were assessed by the single breath method with the patient in the sitting position. Lung function measurements were expressed as percentage of predicted values. Tests were performed according to the the American Thoracic Society (ATS)/ European Respiratory Society (ERS) guidelines by the same technician in order to ensure consistency of results. All lung function data were shown in Table 1.

Serum SIRT1

Serum samples were diluted in the RIPA buffer (Sigma: 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0.) completed with protease inhibitor, as previously published,[39] separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane, and incubated with anti-SIRT1 antibody or with anti-β-actin antibody overnight. The membranes were then incubated with the appropriate peroxidase-conjugated secondary antibodies. The bound antibodies were visualized by chemiluminescence (ECL plus; GE healthcare, Buckingham, UK).

Cell culture

BEAS-2B cells (SV40-immortalized human airway bronchial epithelial cell line) and A549 cells (human lung adenocarcinoma epithelial cell line) were purchased from the American Culture of Tissue Collection (Manassas, VA, USA), and grown in complete growth medium (RPMI 1640 and DMEM supplemented with heat-inactivated 10% FBS and 1% L-glutamine, respectively) at 37 ^{o}C / 5% CO_2. Before use, cells were starved in minimum medium (RPMI 1640 or DMEM supplemented with 1 % FBS and 1 % L-glutamine), and cell culture supernatants were harvested at different time point. So as to eliminate the contamination of supernatant by free floating cells, the supernatant were centrifuged and upper half of the medium were taken as the samples.

Human primary bronchial epithelial cells obtained from x3 non-COPD and x3 COPD subjects were cultured as monolayers in LHC-9 media (Invitrogen, Paisley, UK) on collagen (1% w/v) coated plates as previously reported.[40] Cells were extracted from lung tissue from patients undergoing lung resection surgery at the Royal Brompton Hospital. All subjects gave informed written consent and the study was approved by the NRES London-Chelsea Research Ethics committee, study number 09/H0801/85. All cells were serum starved 16 h before stimulation. Cells were stimulated with 3% cigarette smoke condition media prepared as previously reported.[41]

Statistical analysis

Data from clinical samples were expressed as mean values ± SD. For the analysis of SIRT1, statistical significance was assessed by using non-parametric Kruskal–Wallis test with *Bonferroni* multiple comparison procedure to exclude possible interaction between various variables within subgroups (Statcel 2, OMS publishing Inc., Saitama, Japan). The analysis of correlation between each factors were performed by Spearman's correlation coefficient rank test. All reported *P* values are two-sided, and *P* values of less than 0.05 were considered to be

191 statistically significant.

192

193 **Results**

194 In a previous report, serum SIRT1 was found to be detectable by Western blotting,[18] which
195 showed an excellent correlation with enzyme-linked immunosorbent assay (ELISA). As
196 shown in Figure 1, anti-SIRT1 antibody used in this study detected different sizes of SIRT1,
197 including 75, 102 and 120kDa (the size originally reported) in BEAS-2B cells or A549 cells,
198 and therefore we determined these SIRT1 fractions in serum samples separately. Compared
199 to healthy subjects, the patients with COPD showed decreased levels of serum 120kDa SIRT1
200 (s120S) (SIRT1 ratio in healthy (NS+SM): 0.90±0.34 *vs* COPD: 0.68±0.24; p=0.014; Figure
201 2A), whereas SIRT1 with lower molecular weights (102kDa and 75kDa) did not (Figure 2B
202 and 2C, respectively, e-Figure 1 A, B and C). Serum s120S showed a significant positive
203 correlation with airway obstruction (FEV_1/ FVC ratio; r=0.31, p=0.020; Figure 2D, Table 2)
204 and also with the severity of airway obstruction, measured by FEV_1 % predicted (r=0.29,
205 p=0.029; Figure 2E); suggesting that s120S protein levels decrease with COPD progression
206 (Figure 2F).

207 In addition, s120S showed a negative correlation with the amount of cigarette
208 consumption (pack-year; r=-0.33, p=0.014) (Figure 3A). Patients with higher degree of
209 emphysema on HRCT had lower levels of s120S (r=-0.40, p=0.027; Figure 3B) when
210 analyzed in all subjects showing some degree of emphysema. A good correlation was also
211 observed in all subjects used (p=0.0048, Table 2) and COPD subjects only (p=0.091, Table 2)
212 In addition, patients with emphysema showed decreased level of s120S when compared with
213 the patients with normal lung (SIRT1 ratio in control: 0.92±0.37 *vs* emphysema: 0.71±0.24;
214 p=0.026; Appendix Figure 1D). This was confirmed by the significant positive correlation
215 between the s120S SIRT1 and K_{CO} % predicted (r=0.32, p=0.025; Figure 3C, Table 2). The
216 s120S was not correlated with the age, probably because of the elderly biased samples

included. In contrast, the BMI showed a significant positive correlation with s120S (r=0.36, p=0.0078; Figure 3D, Table 2). In addition, s120S decreased significantly as symptoms (MRC dysnea score) increased (Figure 3E). The severity of hypoxia (PaO_2 or de-saturation on movement), and oxygenation capacity of lung (PaO_2/FiO_2 or $AaDO_2$) did not show any correlation with s120S (Table 2); however s120S showed positive correlation with $PaO_2/PaCO_2$ ratio representing the combined effect on gas exchange [42] (r=0.28, p=0.034; Figure 3F), which suggested that the impairment of aerobic metabolism might contribute to the s120S protein level. Other patient background characteristics (Table 2) or subjects' co-morbidities (such as cardiovascular disease or diabetes mellitus, and Charlson index) did not show any association with the serum levels of SIRT1.

When we limited analysis to the COPD patients only, we identified two additional findings. Firstly, COPD patients with frequent exacerbations tend to have lower s120S levels, compared with those with stable disease (Figure 4A). Secondly, s120S had a positive correlation, not only with the FEV_1 % predicted (r=0.40, p=0.046; Figure 4B) but also with six-minute walk distance (6MWD, r=0.45, p=0.023; Figure 4C). This was also confirmed by the fact that s120S were negatively associated with the MRC dyspnea score (Figure 4D) and with the BODE index (Figure 4E), which is known to be the strong predictor of long-term prognosis in COPD.[37] We further analyzed the correlation with all parameters in GOLD stage 1-2 population only and 3-4 population only. We did not see any significant correlation except for IC% in stage 1+2 (e-Table 1).

In regard to the origin of serum SIRT1, we were unable to detect any active secretion from lung epithelial cell lines, such as BEAS-2B (airway) cells or A549 (parenchymal) cells (Figure 4F and 4G, respectively). In A549 cells, SIRT1 was detected in supernatant after 72 hours culture, but as the housekeeping protein β-actin was also detected, it was likely that this

241 may be due to increased cell permeability related to loss of function. Furthermore, we also
242 investigated SIRT1 protein release in supernatant from non-COPD (n=3) and COPD (n=3)
243 primary bronchial epithelial cells. We did not found original or degraded SIRT1 protein or β-
244 actin in supernatant in the absent or presence of 3cigarette smoke conditioned media (data not
245 shown). Thus, SIRT1 was unlikely excreted from bronchial epithelial cells.
246

Discussion

In the current study we have shown for the first time that the protein levels of serum SIRT1 in its 120kDa form (s120S) was significantly decreased in the patients with COPD. The s120S protein levels were positively correlated with the severity of airways obstruction, and showed a strong negative correlation with the amount of cigarette consumption, suggesting that oxidative stress may lead to the reduction in serum SIRT1. This is contrast to the case of bronchial asthma,[21] in which serum SIRT1 detected by ELISA, was increased. In addition, we found that serum SIRT1 was significantly correlated with the severity of emphysema (HRCT reading and K_{CO} %predicted) and functional disability represented by MRC dyspnea score, 6 minute walking distance and BODE score. These results suggest that serum SIRT1 may be a useful marker for assessing certain disease characteristics in the patients with COPD.

Among the seven sirtuin isozymes,[43] SIRT1 is the most widely studied in mammals from the viewpoint of regulation by oxidative stress, which is relevant to cellular senescence and chronic inflammation.[2–4] In fact, dysregulation of SIRT1 has been described not only in aging-associated diseases, but also in those associated with long-term cigarette smoking;[44,45] and all are characterized by oxidant/anti-oxidant imbalance.[46] We previously reported a reduction in SIRT1 in peripheral lung of patients with COPD.[30] Although reports of reduced SIRT1 relate to intracellular SIRT1 (mRNA or protein), Kumar and colleagues first reported that SIRT1 was detectable in the serum.[16] In this report, serum SIRT1 was measured by various methods, including Western blotting, ELISA and Surface Plasmon Resonance (SPR), with good correlation between each method, and confirming that SIRT1 is a serum protein. Interestingly, they also showed significant reduction of serum SIRT1 protein levels as dementia progressed, suggesting serum SIRT1 may be a useful biomarker for assessing the

cognitive disease. This report was surprising as SIRT1 had originally been described only as a nuclear protein.[8,9]. However, recent reports have demonstrated that SIRT1 can shuttle between the nucleus and cytoplasm,[10–12] therefore SIRT1 potentially being present in the extracellular component.

The strength of our study is the selective determination of the fraction of full length SIRT1(120kDa) separately from other truncated SIRT1 proteins by Western blotting. This is in contrast to the previous reports which used ELISA for serum SIRT1 detection.[18–21] Despite its good quantitative capability, the ELISA assay does not appear to be specific for the full length functional fraction of SIRT1 because antibodies recognize several fractions with the target motif, irrespective of their function (as shown in Figure 1A). In previous reports several bands of SIRT1 protein (original and truncated proteins) have been described, indicating different molecular weights by Western blotting,[47,48] each of which may function differently, although this has not yet been elucidated. Therefore, our results are unique as we were able to analyze only the fully functional fraction of full length SIRT1 (120kDa SIRT1) that was separated by Western blotting. Thus, Western blotting should be used for serum SIRT1 detection.

In addition, this is the first report that serum SIRT1 is reduced in patients with COPD. Compared to healthy subjects, the patients with COPD showed decreased levels of s120S, which correlated not only with the airway obstruction but also with its severity, and with resultant lung emphysema and decreased diffusion capacity. These results were compatible with the previous reports that the SIRT1 protein level was decreased in peripheral lung or peripheral mononuclear cells from patients with COPD.[30] Furthermore, we could also detect the association of s120S protein levels with BMI, MRC dyspnea score and $PaO_2/PaCO_2$ imbalance; all of which suggested that s120S is not just an indicator of lung damage but a

surrogate marker for the oxygen metabolism and systemic metabolic status. Interestingly, our result appears to be opposite to that reported in asthmatic patients,[21] serum SIRT1 might be a potential biomarker to help to differentiate these two diseases. Future studies might be necessary for comparing the serum SIRT1 levels directly between the patients with asthma and COPD populations. Since reduced serum SIRT1 is also reported in association with frailty in elderly people, it may also be useful in understanding the multimorbidity associated with COPD. We further analyzed the correlation with all parameters in GOLD stage 1-2 population only and 3-4 population only. We did not see any significant correlation in all parameters except for IC %predicted in stage 1-2 population. However we observed nearly significant correlation in FEV_1/FVC (p=0.19), Emphysema score (p=0.080), K_{CO}% (p=0.17), RV% (p=0.090), 6MWD (p=0.11) in stage 1-2 population. We seemed not to have enough power to demonstrate the association in current study, but further big study will reveal usefulness of serum SIRT1 as potential biomarkers to determine early stage of COPD.

A limitation of the present study is that we have not identified the precise source of SIRT1 in serum. In A549 and BEAS-2B epithelial cells, we could not detect any fractions of SIRT1 in the cell culture supernatant, indicating that cellular leakage or active secretion are unlikely. In primary bronchial epithelial cells, we could not find full or degraded SIRT1 proteins or β-actin in supernatant in the presence or absence of cigarette smoke condition media, suggesting SIRT1 is unlikely secreted from bronchial epithelial cells. However, SIRT1 protein was detected in supernatant from A549 cells later stage, which was associated with an increase in β-actin expression. This suggests epithelial cells are still possible source of SIRT1 when cells are damaged. Considering that the SIRT1 in the patients with COPD has been reported to be decreased not only the lung[30] but also in endothelial progenitor cells[49] and circulating leukocytes [30,50], decreased serum SIRT1 might reflect the reduction of SIRT1 in

cells as a result of oxidative stress. PBMCs or alveolar macrophages might be potential sources of SIRT1. It might be necessary in the future study to identify the precise origin of serum SIRT1, and the factors that modulate serum SIRT1 levels in COPD and other chronic aging diseases. Secondary, even though there were statistical differences in SIRT1 levels between compared groups, there was significant overlap in the values of all groups. GOLD stage is defined by lung function (mainly by FEV_1 %predicted), but as shown above there was good correlation between SIRT1 and certain disease characteristics such as emphysema, MRC dyspnea score, 6MWD and BODE score. Therefore, SIRT1 level is influenced by several factors rather than lung function only. Current study is too small to evaluate further, but we believe that a big study with more patients in future will provide novel approach to classify disease stage based on SIRT1.

In summary, we report for the first time that the serum SIRT1 was reduced in the patients with COPD, and that this reduction was correlated with the extent of emphysema and reduced functional measurements that correlate with disease progression. Serum SIRT1 might be therefore serve as a potential biomarker for COPD.

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Author's contributions

SY conducted the assays, carried out the data analysis and drafted the manuscript. AIP, AP and CV were involved in sample preparation and participated in the design of the original study. KI contributed to the data analysis, design of the study and the manuscript preparation. PB and SL participated in the design of the study, and contributed substantially to preparation of manuscript.

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Figure Legends

Figure 1. SIRT1 protein in serum. Western blotting analysis of serum sample (S) was compared with whole cell extracts of BEAS-2B cells (B) and A549 cells (A).

Abbreviations: WCE: whole cell extract; SIRT1: silent information regulator 2 homolog 1.

Figure 2. Reduced levels of serum 120kDa SIRT1 (s120S) protein in COPD. (A) s120S protein level in serum from healthy subjects (NS+SM) and COPD patients (C1-4 disease stage). Protein levels of serum 102kDa (B) and 75kDa (C) SIRT1 with or without COPD. (D) Correlation between the s120S protein level and FEV_1 / FVC ratio. (E) Correlation between the s120S protein level and FEV_1 % predicted. (F) s120S protein levels of healthy subjects (NS+SM) and patients with COPD of different stages (C1-2, C3-4).

Abbreviations: COPD: chronic obstructive pulmonary disease; NS: non-smoking subjects; SM: smokers without COPD; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity; PC: positive control from healthy subject.

Figure 3. Serum 120kDa SIRT1 (s120S) protein and patient characteristics. Relationship between the s120S protein level and cigarette smoke exposure in pack-years (A), with emphysema score in all subjects demonstrating emphysema (B), K_{CO} % predicted (C), BMI (D), MRC dyspnea score (E), and $PaO_2/PaCO_2$ ratio (F) in all subjects.

Abbreviations: Pack-year = (number of cigarettes smoked per day / 20)×duration of smoking in years; K_{CO}: transfer coefficient of carbon monoxide; BMI: body mass index; MRC: Medical Research Council dyspnea score; PaO_2: partial pressure of arterial oxygen; $PaCO_2$: partial pressure of carbon dioxide.

Figure 4. The s120S protein levels of COPD patients. (A) The s120S protein level in relation to the frequent exacerbations of COPD. Relationship between the s120S protein level and FEV_1 %predicted (B), 6MWD (C) and MRC score (D). (E) s120S protein levels COPD patients with different BODE index. (F, G) Time dependency of SIRT1 in cell culture supernatant from BEAS-2B cells (B2B) (F) or A549 cells (G).

Abbreviations: WCE: whole cell extract; 6MWD: 6 minute walking distance (meters); BODE: body mass, airway obstruction, dyspnea, 6MWD index.

Table 1. The characteristics of study subjects in the study

	Non-smoker subjects	**Smokers without COPD**	**COPD Stage 1-2**	**COPD Stage 3-4**
Number(M/F)	12 (3/9)	19 (11/8)	13 (10/3)	13 (11/2)
Age (years)	65.3±11.1	58.9±12.3	64.6±10.1	64.2±11.1
Pack-years	0	38.9±25.3	70.9±27.2 **	68.1±25.8**
Emphysema	1/12	6/19	12/13	13/13
Emphysema score	0.29±1.01	0.39±0.74	1.33±1.17	2.25±1.20$^{\#\#,}$ **
MRC dyspnea score	0.33±0.89	0.68±0.95	1.46±0.78$^{\#}$	2.38±1.12$^{\#\#,}$ **
Charlson Index	0.58±1.00	0.95±1.08	1.54±1.27	1.92±1.12$^{\#}$
PaO_2 (mmTorr)	81.2±7.2	77.7±5.8	73.6±6.9	66.7±9.0$^{\#\#,}$ **
$PaCO_2$ (mmTorr)	38.3±2.4	39.5±1.7	39.6±2.7	39.8±7.4
PaO_2 / FiO_2 (mmTorr)	386.7±34.4	370.2±27.8	350.5±33.0	307.7±55.5$^{\#\#,}$ **
$AaDO_2$	20.6±9.4	22.6±6.3	25.4±7.2	41.0±23.1$^{\#\#,}$ **
$PaO_2/PaCO_2$	2.12±0.15	1.97±0.17	1.82±0.19$^{\#\#}$	1.72±0.33$^{\#\#,}$ *
BMI (kg/m^2)	25.8±3.2	29.0±7.5	24.8±3.6	23.0±3.5*
FEV_1 % predicted (%)	89.4±12.6	89.7±14.4	73.1±14.6$^{\#,}$ **	32.2±7.8$^{\#\#,}$ **
FVC % predicted (%)	84.2±11.3	87.2±14.9	90.3±18.9	57.2±11.7$^{\#\#,}$ **
FEV_1/FVC	84.4±5.6	81.8±9.2	61.4±7.2$^{\#\#,}$ **	45.9±7.8$^{\#\#,}$ **
DLCO % predicted (%)	78.9±19.7	77.3±17.3	63.5±20.8	43.0±13.7$^{\#\#,}$ **
K_{CO} % predicted (%)	85.1±16.9	89.2±10.9	67.8±19.5**	59.9±14.0$^{\#\#,}$ **
TLC % predicted (%)	83.7±9.4	86.0±25.6	94.6±13.4	102.7±36.2

FRC % predicted (%)	81.7±8.3	82.2±24.6	106.0±27.1	110.3±25.6$^{\#, *}$
RV % predicted (%)	82.8±8.6	80.2±20.7	107.9±23.9$^{\#, **}$	120.6±18.5$^{\#\#, **}$
RV/TLC	0.365±0.062	0.324±0.075	0.401±0.085	0.473±0.085$^{\#\#, **}$
IC % predicted (%)	84.8±10.1	86.6±23.8	85.5±16.3	56.7±16.5$^{\#\#, **}$
6MWD (m)	480.2±110.2	508.6±108.5	439.2±122.4	359.6±147.4**
DBOrgDyspena	0.92±2.27	0.68±1.70	2.00±1.73	3.15±1.91$^{\#, **}$
DBorgFatigue	0.92±1.68	0.68±1.11	1.23±1.17	2.38±1.66**
Dsat (%)	-1.2±4.4	-0.7±3.2	-2.5±4.6	-7.2±4.7$^{\#\#, **}$
DHR	30.6±8.3	21.3±9.5	32.5±23.1	22.3±15.4
LABA/ LAMA/ ICS	1/ 1/ 0	2/ 2/ 3	8/ 9/ 3	10/ 10/ 10
CVD/ DM	2/ 2	7/ 2	6/ 1	5/ 3

COPD patients were categorized by definition of GOLD.[22] Emphysematous phenotype was characterized according to the presence of significant of emphysematous lesions (over 15 % of lung parenchyma) in high resolution computed tomography (HRCT).[31] COPD patient is characterized as frequent exacerbators if he has two or more exacerbations in one year.[33]

Abbreviations: COPD = chronic obstructive pulmonary disease; pack-year = (number of cigarettes smoked per day / 20) ×duration of smoking in years; MRC score = Medical Research Council dyspnea score; PaO_2 = partial pressure of arterial oxygen; $PaCO_2$ = partial pressure of carbon dioxide; FiO_2 = fraction of inspiratory oxygen; $AaDO_2$ = alveolar-arterial oxygen difference; BMI = body mass index; FEV_1 = forced expiratory volume in one second; FVC = forced vital capacity; DLCO = diffusing capacity or transfer factor of the lung for carbon monoxide; K_{CO} = transfer coefficient; TLC = total lung capacity; FRC = functional residual capacity; RV = residual volume; IC = inspiratory capacity; 6MWD = six-minute

walk distance; Dsat = De-saturation on movement; DHR = difference in heart rate during 6MWD; LABA = long acting beta-agonists; LAMA = long acting muscarinic agonists; ICS = inhaled corticosteroids; CVD = cardiovascular disease; DM = diabetes mellitus;

Significance of differences: $^{\#}$ p <0.05, $^{\#\#}$ p <0.01 vs. Non-Smoker subjects; * p <0.05, ** p <0.01 vs. Smokers without COPD; Data are expressed as mean values ± standard deviation.

Table 2. The Spearman's correlation coefficient rank test between the serum SIRT1 (120kDa) and patient characteristics

	All subjects		Normal Subjects		COPD only	
	r	p	r	p	r	p
BMI	0.36	0.0077	0.28	0.13	0.34	0.092
Pack-year	-0.33	0.014	-0.16	0.38	-0.13	0.51
FEV_1/FVC	0.31	0.021	-0.072	0.69	0.32	0.11
Emphysema Score	-0.38	0.0048	-0.082	0.33	-0.34	0.091
Kco % predicted	0.32	0.025	0.059	0.77	0.32	0.13
FEV_1 % predicted	0.29	0.032	-0.088	0.63	0.40	0.046
$PaO_2/PaCO_2$	0.28	0.034	0.22	0.22	0.058	0.77
RV % predicted	-0.27	0.054	0.11	0.57	-0.23	0.26
IC % predicted	0.28	0.064	0.26	0.20	0.25	0.28
DLCO % predicted	0.26	0.069	-0.043	0.83	0.33	0.10
PaO_2 / FiO_2	0.23	0.079	0.11	0.55	0.036	0.86
PaO_2	0.22	0.098	0.11	0.55	-0.026	0.90
6MWD	0.22	0.11	-0.097	0.60	0.45	0.023
Dsat	0.20	0.14	0.14	0.45	0.12	0.56
FVC % predicted	0.20	0.14	-0.059	0.75	0.27	0.18
$AaDO_2$	-0.18	0.17	-0.032	0.86	-0.052	0.80
FRC % predicted	-0.19	0.18	0.10	0.60	-0.083	0.69
RV/TLC	-0.16	0.24	-0.013	0.95	-0.025	0.90
$PaCO_2$	-0.06	0.65	-0.097	0.60	0.039	0.85
TLC % predicted	-0.06	0.66	0.21	0.30	-0.10	0.61

Figure 1

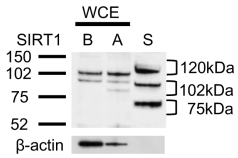


Figure 2

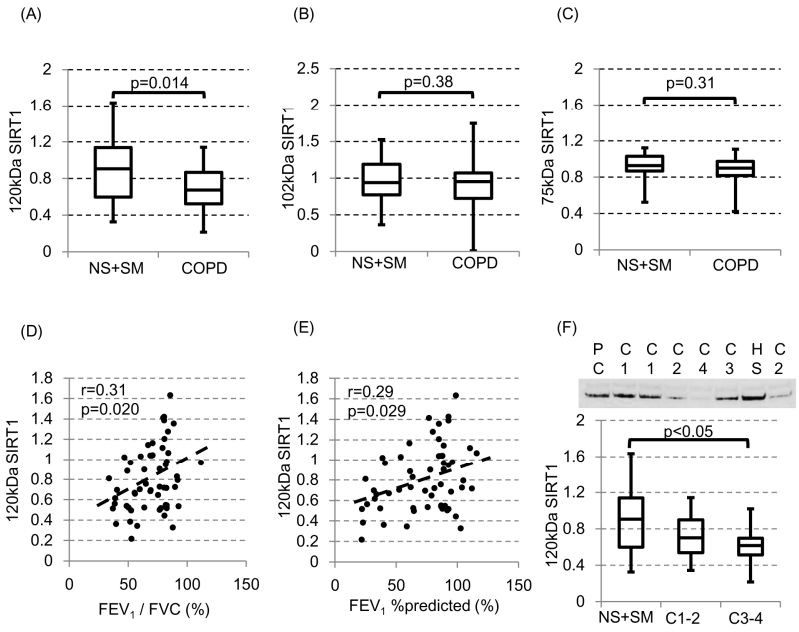


Figure 3

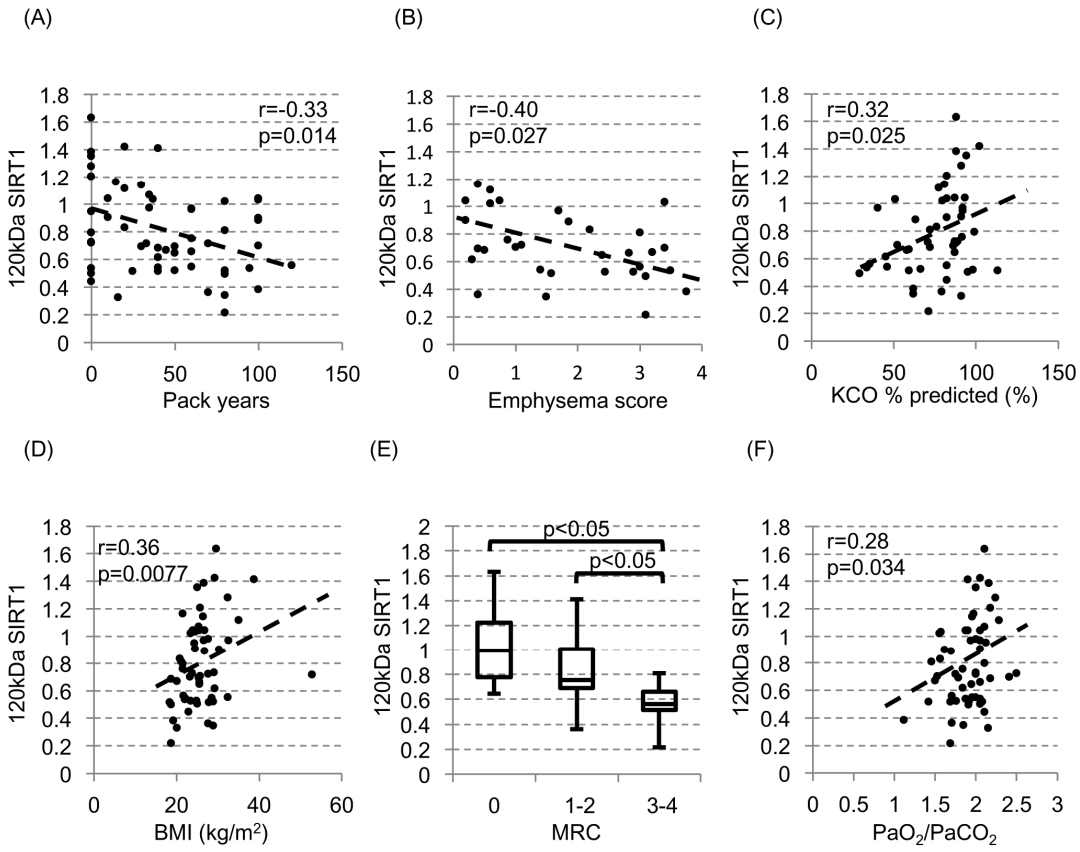
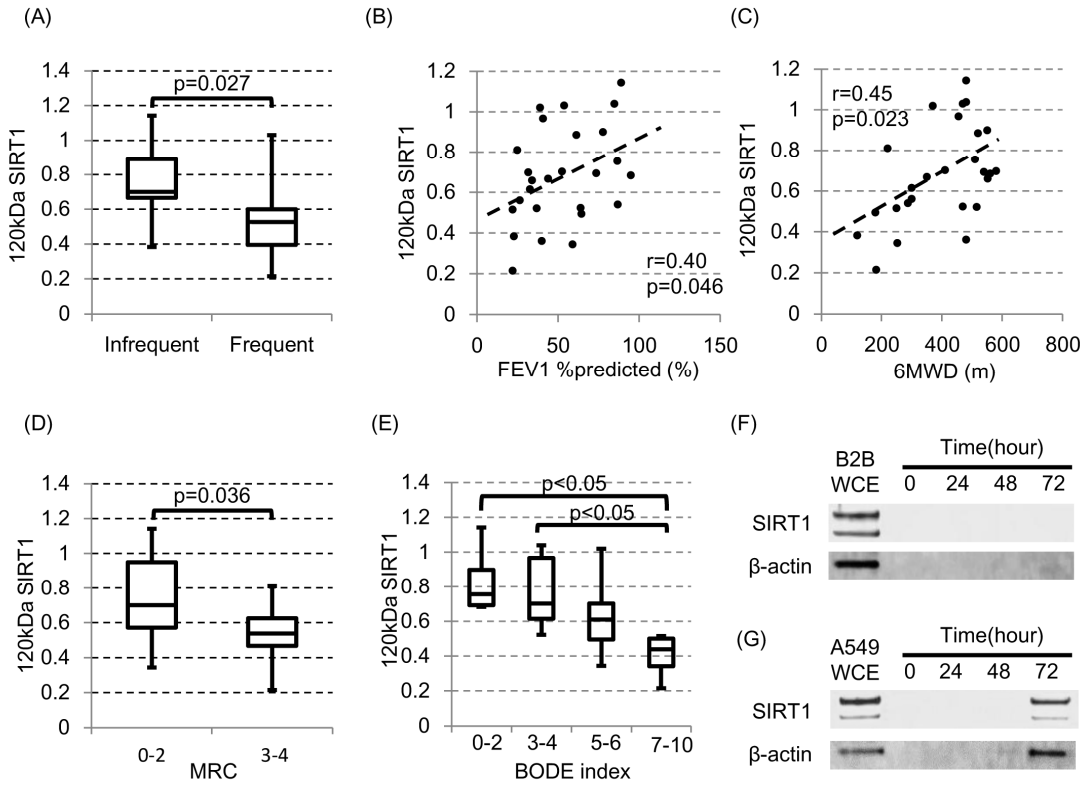


Figure 4



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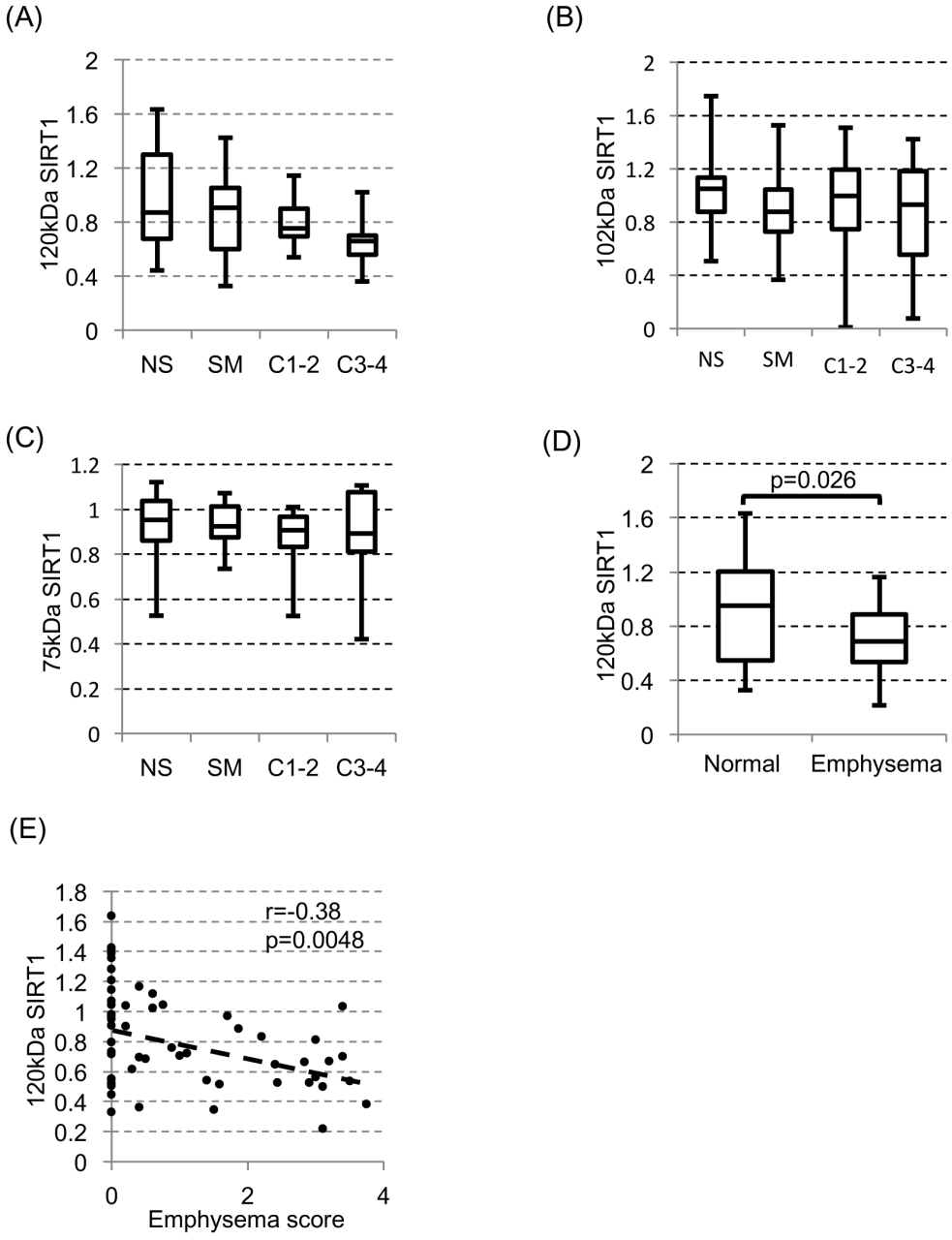
e-Figure 1



e-Figure 1. Reduced levels of serum 120kDa SIRT1 (s120S) protein in COPD and correlation with Emphysema. The levels of SIRT1 at 120 kDa (A), 102kDa (B) and 75kDa (C) in serum from healthy non-smoker subjects (NS), smokers without COPD (SM) and COPD patients (C1-2 or C3-4 disease stage). Comparison of SIRT1 120KDa between subjects with normal lung and with emphysema (D) and correlation between SIRT1 120KDa and emphysema score in all subjects (E).

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e-Table 1. The Spearman's correlation coefficient rank test between the serum SIRT1 (120kDa) and patient characteristics

	COPD Stage 1-2		COPD Stage 3-4	
	r	p	r	p
BMI	0.35	0.22	0.32	0.26
Pack-year	-0.070	0.81	-0.23	0.43
FEV_1/FVC	0.38	0.19	-0.0055	0.98
Emphysema Score	-0.51	0.080	-0.069	0.81
Kco % predicted	0.44	0.17	-0.14	0.62
FEV_1 % predicted	0.18	0.53	0.47	0.10
$PaO_2/PaCO_2$	-0.24	0.41	0.26	0.37
RV % predicted	-0.51	0.090	0.050	0.86
IC % predicted	-0.83	0.018	0.25	0.46
DLCO % predicted	0.27	0.38	0.19	0.52
PaO_2 / FiO_2	-0.25	0.40	0.094	0.74
PaO_2	-0.25	0.40	-0.072	0.80
6MWD	0.47	0.11	0.35	0.22
Dsat	-0.19	0.50	0.32	0.27
FVC % predicted	-0.099	0.73	0.41	0.16
$AaDO_2$	0.18	0.53	-0.14	0.62
FRC % predicted	-0.15	0.61	0.028	0.93
RV/TLC	-0.20	0.50	0.27	0.35
$PaCO_2$	0.16	0.59	-0.16	0.57
TLC % predicted	-0.12	0.68	-0.082	0.78

Abbreviations: r = correlation coefficient; p = probability value.