Biological Age in COPD Patients Reveals an Accelerated Lung Aging

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Research Article

Biological age in COPD patients reveals an accelerated lung aging.

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Abstract

Chronic obstructive pulmonary disease (COPD), the third leading cause of death worldwide, shows striking clinical aging-associated features of lung. We tested the hypothesis that lung biological aging in COPD is accelerated. Biological aging was assessed by mitotic telomere length (TL) and non-mitotic age-dependent methylation changes in specific CpGs (DNAmAge) of lung cells, obtained from induced sputum, and peripheral blood leucocytes in 18 COPD patients (72.4±7.7 years; 50% males), clinically appraised by lung function and blood parameters. DNAmAge (67.4±5.80 vs 61.6±5.40 years; p=0.0003), AgeAcc (-4.5±5.02 vs -10.8±3.50 years; p=0.0003) and TL attrition (1.05±0.35 vs 1.48±0.21 T/S; p=0.0341) are higher in lung than blood in the same patients. Blood DNAmAge (r=0.927245; p=0.0026) and AgeAcc (r=0.916445; p=0.0037), but not TL, highly correlate with that of lung. Therefore, blood can be a proxy indicator of lung biological age. Multiple regression analyses show that both blood DNAmAge and AgeAcc decrease (i.e., younger) in patients with combined inhaled corticosteroids (ICS) therapy (p=0.0494 and p=0.0553) and FEV$_1$% enhancement (p=0.0254; p=0.0296). In conclusion, the new finding, that lung of COPD patients is remarkably biologically older than blood, opens new research challenge on novel therapeutic approaches to counteract this key aspect of the disease.

Keywords

Abbreviations

AgeAcc: Age acceleration; COPD: Chronic obstructive pulmonary disease; CRP: C-reactive protein DNAmAge: DNA methylation age; FEV$_1$: forced expiratory volume in one second; LTL: Leucocytes telomere length; TL: Telomere length; VC: vital capacity.

Introduction

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death worldwide [1] and one of the major causes of chronic morbidity [2]. This global burden is projected to rise because of aging of the population [3].

COPD shows striking lung aging-associated features including the reduction of function, pulmonary inflammation, increased gas trapping, loss of lung elastic recoil and enlargement of the distal air space [2]. These pathological signs are distinctive of COPD and, the progressive airway obstruction could represent an acceleration of the normal lung function decline with age [4, 5]. The understanding of age-related pathomechanisms associated with COPD is decisive also to uncover novel disease specific treatment strategies.

Smoking is the primary cause for COPD worldwide [6], but in developing countries COPD also arises as a result of exposure to household air pollution [6, 7]. The mechanisms linking tobacco smoke and other air pollutants with COPD have not yet been fully elucidated. One main working hypothesis is that air pollutants, especially the particulate matter (PM) of aerodynamic diameter of 2.5 μm or less, can deeply penetrate into the lung, deposit in the alveolar area, and locally trigger oxidative stress and inflammatory responses [8]. The pulmonary local oxidative-inflammatory reaction damages lung tissue leading to structural pathological changes such as lung parenchyma destruction (emphysema), fibrosis of peripheral airways and an increase in mucus-producing cells, with a consequent airflow limitation, that are all signs of an accelerated aging of the lung in COPD [8]. This local oxidative-inflammatory reaction leads to a subsequent systemic inflammation [9]. Several blood inflammatory markers, including C-reactive protein (CRP) [10], interleukin (IL)-6 [11], and white blood cells are all found altered in COPD patients [12].

Altered inflammatory responses and the induced oxidative stress are two key mechanisms accelerating biological aging that at cellular level may be followed by early signs including alterations in telomere length [13] and DNA methylation [14]. Telomeres act as a mitotic clock that by reducing itself at each cell division leads to cellular senescence (replicative senescence) or cell death. Shorter telomere length in blood leucocytes (LTL) has been described from patients with COPD [15] and in a meta-analysis of 14 studies [16]. A powerful emerging marker of non-mitotic cellular aging is the epigenetic age often referred to as DNA methylation age (DNAmAge) [17, 18]. DNAmAge in human [19 - 21] is assessed from methylation at a species-specific subset of cytosine-guanine dyads (CpGs), and is strongly correlated with chronological age [19 - 23]. Developing of epigenetic predictors has addressed to an "epigenetic clock" theory of aging according to which, the difference between DNAmAge and

chronological age defined as "age acceleration" (AgeAcc) [24] is indicative of altered biological functions [18] and elevated risk for morbidity and mortality [25]. One recent study from two independent longitudinal large cohort studies has found the non-mitotic cellular aging by DNAmAge in blood in relation to incident COPD and lung function [26]. However, no epigenetic age estimation was performed in the target organ of the disease, namely the lung.

Sputum induction is a validated non-invasive method of airway sampling for analysis of lung cellular components and fluid-phase constituents [27]. It has been successfully applied for assessing disease severity and progression in COPD, producing reliable results comparable to biopsy and bronchoalveolar lavage [28].

In this study we explore the hypothesis that in COPD patients, biological aging, evaluated by mitotic TL and non-mitotic DNAmAge, proceeds at different rate in the target organ, i.e. lung, and at systemic level, i.e. blood, taking into consideration their demographic data, life style and occupational exposure, lung function, clinical and blood parameters.

Results

Characteristics of the study population

Characteristics of the study subjects are reported in Table 1 and 2. Interval variables (mean ± SD) of all COPD patients (n=18) with long-acting β2 agonist /long-acting muscarinic antagonist (LABA/LAMA) (n=9) and with combined inhaled corticosteroids (ICS) therapy with LABA/ LAMA administration (n=9), also defined as dual and triple therapy respectively, are showed in Table 1. The comparison of two groups (Mann Whitney U test) indicates that patients in LABA/LAMA therapy present higher values of FEV_1 (p=0.0003), FEV_1% (p<0.0001), FVC (p=0.0003), VC (p=0.0003) and TLC (p=0.0008), and also a lower systolic pressure (p=0.036), than those with ICS therapy and LABA/LAMA. No difference in the other parameters is observed. Table 2 shows the number and percentage of categorical variables in the same groups. All characteristics are equally distributed among the two groups with dual and triple therapy (Chi-square test p not significant).

Biological age of lung and blood by TL, DNAmAge and AgeAcc

Table 3 reports the results of TL analysis in lung and blood of COPD patients. Mean lung TL is shorter than blood TL of the same patient (n=8) (paired t test: mean 1.05±0.35 T/S vs mean 1.48±0.21 T/S; p=0.0341), as well as compared to blood TL of all patients (n=18) (Mann Whitney U test: mean 1.05±0.35 T/S vs mean 1.47±0.26 T/S; p=0.0133). On the same line, Table 4 shows that lung DNAmAge is older than blood DNAmAge of the same patient (paired t test mean 67.4±5.80 years vs

mean 61.6±5.40 years; p=0.0003). Lung AgeAcc is also extremely enhanced compared to that of blood of the same patient (paired t test mean -4.5±5.02 years vs mean -10.8±3.50 years; p=0.0003) as well as if compared with blood AgeAcc of all patients (n=18) (Mann Whitney U test: mean -4.5±5.02 years vs mean -10.3±3.50 years; p=0.0156).

Relationship between blood and lung biological age

Blood DNAmAge (Figure 1a: Simple linear regression, correlation coefficient (r) = 0.927245; Two sided p = 0.0026) and AgeAcc (Figure 1b: Simple linear regression, correlation coefficient (r) = 0.916445; Two sided p = 0.0037), but not TL (Figure S2: Simple linear regression, correlation coefficient (r) = -0.33554; Two sided p = 0.4165), highly correlate with those of lung.

Determinants of blood DNAmAge and AgeAcc

Multiple linear regression analyses (Table 5) show that the rise in blood DNAmAge correlates with age (p =0.0001), and both DNAmAge and AgeAcc increase with dual therapy without ICS (p =0.0494 and p=0.0553) and with decline in $FEV_1\%$ (p =0.0254; p=0.0296).

Discussion

In this study, we have determined the biological age of lung cells obtained from induced sputum and of peripheral blood leucocytes, by measuring the mitotic age (TL) and the non-mitotic epigenetic age (DNAmAge), in COPD patients.

The main findings stemming from this work reveal that:

a) lung is biologically older than blood as determined by DNAmAge and TL;

b) lung aging is more accelerated than blood as determined by AgeAcc;

c) lung DNAmAge and AgeAcc, but not TL, highly correlate with those of blood;

d) blood DNAmAge and AgeAcc significantly increased in patients with LABA/ LAMA administration without ICS therapy and with decline in lung function $FEV_1\%$.

To the best of our knowledge, this is the first time that DNAmAge and AgeAcc are determined in induced sputum and in the same time in blood of COPD patients, showing that lung in turn is biologically older than blood. Until now several studies have shown smoking-related methylation signatures in peripheral blood [29 - 31] and in induced sputum [32, 33], that also associated with the decrease in lung function in COPD patients. Furthermore, a genome-wide aberrant DNA methylation smoking-related was found in small airways of patients with COPD [34]. In our study, the higher DNAmAge and AgeAcc of lung cells from induced sputum would suggest that age-related methylation genes are the target of cigarette smoke injury too. In line with epigenetic age, we found that lung TL is

shorter than blood. The TL attrition we observed, in the specimens from target organ of the disease, agrees with previous studies that report shorter TL in parenchymal pulmonary cells of emphysematous COPD patients, associated with cell senescence and inflammation indicators [35, 36]. The TL shortening, we observed in lung of COPD patients, is coherent with the hypothesis that an elevated oxidative stress and increased release of pro-inflammatory cytokines derived from smoking and/or other noxious particles lead to TL attrition [35, 36]. Cigarette smoke carry an abundancy of well-known toxins including polycyclic aromatic hydrocarbons (PAHs), transition metals and N-nitrosamines that directly, as catalysts for ROS production, and indirectly, through their metabolism, are important sources for ROS generation [37] and trigger the activation of proinflammatory responses in cells of the airway mucosa [38]. These and our results disagree, however, with that of Saferali et al. [39] which reported longer TL in the DNA from lung biopsies compared to blood of cancer patients. Divergent results may be ascribed to the disease, i.e. cancer, considered in the Saferali's study. Differently, we analyzed lung cellular, obtained from induced sputum that reflects the whole respiratory tree of COPD patients; furthermore, COPD shows striking lung aging-associated features including the reduction of function, pulmonary inflammation and progressive airway obstruction. Furthermore, the advanced/accelerated aging of the lung in respect to the blood arising from our work, confirms that tissues and organs in our body may age at different rates within the same individuals [40], as we have already proved on donors' heart where DNAmAge is consistently younger than that of blood [23].

We found a close nexus between lung and blood DNAmAge and AgeAcc, advising that blood could be a surrogate tissue in lung aging studies. The correlation between lung and blood in the same subject is a strength point of the study because it may validate those studies carried out only on peripheral blood. For example our results would imply that findings of two recent large longitudinal studies from SAPALDIA and ECRHS cohorts [41] and KORA and NAS cohorts [26] on an increased AgeAcc, estimated using Horvath method [19]), in the blood of COPD patients [26] and general population of adults [41], would mirror what happens in the lung. Furthermore our results could allow us to translating, the investigation on biological aging aspect, linked to COPD, into the clinical practice through a simple blood sample. In a real clinical scenario blood samples may be easily and quickly acquired when visiting COPD patients and sent to the laboratory for biological age analysis. However, some caution is mandatory since, according to our findings, the difference between DNAmAge in lung and blood of COPD patients is almost 6 years. Further studies are therefore needed to optimize the use of blood as a surrogate indicator of lung biological age in clinical practice. Nevertheless, in contrast

with Saferali and co-workers [39], we didn't find this correlation for TL. The fact that we consider non-cancer patient may be the reason of this divergent result.

Furthermore, we discovered that blood DNAmAge and AgeAcc are related with the main clinical feature of COPD, i.e., altered lung function, measured in terms of FEV_1%. Only two studies have explored the association of epigenetic aging, evaluated by AgeAcc [26, 41] with lung function. In line with our results, the longitudinal data from SAPALDIA and ECRHS cohorts [41] and KORA and NAS cohorts [26] report the association between blood AgeAcc (estimated using Horvath method [2013]) and lung function decline evaluated by FEV_1 [41]. In our study the severity of disease measured by FEV_1% is associated with the speeding up of the DNAmAge and AgeAcc. FEV_1% punctually expresses lung function as a percentage of the predicted normal value according to reference values based on age, height, weight, sex, and race for each subject using the European Community for Steel and Coal as reference values [42].

Furthermore, we found that blood DNAmAge and AgeAcc are significantly reduced in patients with triple therapy combining ICS therapy with LABA/ LAMA. By exploring the effects of systemic corticosteroids exposure, Wan et al. [43] found site-specific differences in blood DNA methylation of COPD patients. ICS anti-inflammatory therapy reduces pro-inflammatory mediator secretion from COPD alveolar macrophages exposed to microbial or oxidative stress triggers [44, 45]. Since the inflammation represents a key aspect of aging, the anti-inflammatory role of ICS together with their ability to determine alterations in methylation profile, could explain the rejuvenating effect we found in COPD patients with ICS assumption in triple therapy. With these finding we strengthen the current literature that focuses on the role of age and aging-associated signaling pathways as well as their impact on current treatment strategies in the pathogenesis of COPD [46]. Thus, the understanding of age-related pathomechanisms associated with COPD is critical to uncover novel disease specific treatment strategies.

The strength of our study is the use of a validated non-invasive method of airway sampling for analysis of lung cellular components and fluid-phase constituents [27]. From our clinical experience this is a suitable and safe, non-invasive, and well-tolerated procedure producing reliable results comparable to biopsy and bronchoalveolar lavage [28] that could be applied in future studies to follow easily biological aging of the lung. Furthermore, comparing DNAmAge and TL in two different tissues in order to appraise the biological aging charge at the disease target organ, i.e. lung by a non-invasive method of sampling, and at systemic level, i.e. blood, is another strong point of the study. Determining the two most prominent biomarkers of biological age, DNAmAge and TL, with an almost totally

automated workflow, is also a strong point of our study. To assess DNAmAge, we applied the method proposed by Zbieć-Piekarska et al. [21] on data from five CpG sites using the locus-specific technology pyrosequencing with some modification as described by Pavanello et al. [22, 23], which makes the technical analysis achievable in few hours. It is noteworthy that pyrosequencing has the potential for multiplexing, which can simplify the protocol and reduce the cost of technical analysis. By using this process, we can perform the analyses in a standardized way while also reducing time and errors (see Supplementary Fig. S1 online).

However, we recognize some weak points. The weakness is mainly related to the low number of patients enrolled in the study and the low yields of samples obtained from induced sputum technique although the results obtained were significant, as well as to the lack of an age-matched control group to which to refer. Future research on COPD is mandatory with the aim of increasing the number of patients also including a control group.

In conclusion, by detecting that lungs of COPD patients is remarkably older than blood, and its AgeAcc is faster, we demonstrates that lung cells have acquired molecular aging characteristics. The correlation of these aging hallmarks with the main clinical feature of COPD i.e., decreased in FEV1%, supports the hypothesis of a molecular age-related pathomechanism in COPD. Our finding on the possible rejuvenating effect of ICS therapy on epigenetic age, opens new research challenge on novel therapeutic approaches to counteract this key aspect of the disease. However, we recognize the fact that the low number of people analyzed in this study raises the question of chance findings. Studies in larger cohorts are therefore required to confirm our findings.

Materials and methods

Study design

The present study includes n=18 moderate COPD patients (GOLD 2019)[2] enrolled at the ambulatory of Respiratory Physiopathology Ward – Occupational Medicine, Department of Cardio-Vascular-Thoracic Science and Public Health, University of Padova. The local Ethics Committee - University of Padova, approved the study protocols (3843/AO/16 and 3054/AO/14). The recruiting of COPD patients was carried out between September 2018 and September 2019. All patients were informed of the purpose of the study by trained interviewers and asked to sign an informed consent form. The study was conducted in accordance with the Declaration of Helsinki. Participants were interviewed with structured questionnaires to collect information regarding demographic data (age, gender), age parents

at birth and educational level (years), smoking history and pack-years, alcohol intake in the last 12 months and habitual alcohol consumption measured as unit of drink/day (1 unit=10-12cg alcohol intake), environmental exposure (diet, indoor, home, traffic, outdoor), physical activity (IPAQ score), clinical determinants (e.g., leucocytes, blood red cells, hemoglobin, glycaemia, C-reactive protein (CRP), etc..), diseases medical history and therapy. All patients underwent to a physical examination and lung function was assessed by spirometry recording forced expiratory volume in one second (FEV_1), forced vital capacity (FVC), vital capacity (VC), total lung capacity (TLC), residual volume (RV) and FEV_1/VC ratio also defined as Tiffeneau index. The inclusion criteria for the study participation were post-bronchodilator FEV_1/VC ratio < 0.70 and no acute exacerbation for at least 6 weeks. For each patient blood samples were collected in vacutainers K3ETDA tubes and Paxgene tubes, for basic biochemistry, TL determination and DNAmAge assessment. A plasma sample was also collected and stored in freezer at − 80 °C for further investigations. During medical examination, the procedure of sputum induction was carried out for each patient to collect a sample arises from the disease' target organ on which analyze TL and DNAmAge, in comparison with the same measurement on peripheral blood. Induced sputum was obtained from 8 patients in agreement with the yields obtained so far in our Ambulatory on this type of patients.

Induced sputum procedure and spirometry

All lung function measurements were measured using a spirometer (Master Screen PFT, PRO, Viasys Sanità) according to the guidelines /recommendations of European Respiratory Society (ERS) of the American Thoracic Society/European Respiratory Society (ATS/ERS) [48]. FEV_1 was used as the primary variable of lung function that was measured both before and 10 minutes after the use of post-bronchodilator. FEV_1 was expressed as liters and as a percentage of the predicted normal value (FEV_1%) according to reference values based on age, height, weight, sex, and race for each subject using the European Community for Steel and Coal as reference values [42]. These values obtained are in turn used as reference standards/assessments for consecutive spirometries performed during induced sputum standard procedure [49]. Nebulized sterile saline solutions (hypertonic at 3 and 4 percent) were consecutive administered through a nebulizer (DeVILBISS, UltraNeb) with an output flow of ~1 mL•min in four sequential five-minute inhalation periods. Since saline inhalation may cause bronchoconstriction, after each inhalation period FEV_1 was measured for the detection and monitoring of lung function during the process, stopping the procedure when FEV_1 decreased over 20 percent compared with that of post-salbutamol baseline. During the procedure the patient was asked to cough

and expectorate. Once collected, induced sputum was processed according to a standard technique [49]. The weight of the selected sputum plugs was recorded and the sample was diluted with a volume of phosphate buffered solution (PBS) and 0.1% dithiotreitol (DTT) equal to 4:1 of selected plugs. After filtration with a nylon mesh (52-56 μm), the sample was centrifuged (3000 rpm for 3 min) to separate cells and supernatant. The cell pellet was resuspended in 1 mL of PBS. The cells were stained for viability assessment using an equal volume (10 μl) of both sample and tryptan blue. Cell concentration was adjusted to obtain a final concentration of ~300000 cells/mL. The cells were cytocentrifuged (Cytospin, Shandon Scientific) at 450 rpm for 6 min, onto glass slides treated with aptex (3-aminopropyltriethoxysilane) according to a standard method [50] and stained with Diff-Quik (Dade Behring). The induced-sputum differential cell count was measured counting 400 nucleated cells per each of two slides stained reporting the percentage of eosinophils, neutrophils, macrophages, lymphocytes and bronchial epithelial cells. The induced sputum sample is considered acceptable and adequate if the percentage of squamous cells is < 20 % of the total cells, warranted the reproducibility of cell counts.

DNA extraction from blood samples

DNA was extracted from whole blood using the QIAamp DNA Mini kit (QIAGEN, Milano, Italy) on a QIAcube System (QIAGEN, Milano, Italy) for automated high-throughput DNA purification, according to a customized protocol as previously described [23]. In particular, 400 μL of whole blood from each sample were processed for DNA extraction. DNA was quantified and checked for quality using QIAexpert Quantification System (QIAGEN, Milano, Italy).

DNA extraction from induced sputum samples

During processing of induced sputum sample, an aliquot of selected plugs was collected for DNA extraction. The weight of the selected sputum plugs was recorded and then diluted with a volume of phosphate buffered solution (PBS) and 0.1% dithiotreitol (DTT) equal to 4:1 of selected plugs and gently vortexed. After filtration with nylon mesh (52-56 μm), the sample was centrifuged (3000 rpm for 3 min) to separate cells and supernatant. The cell pellet was resuspended in 180 μL of PBS. DNA extraction was performed on automated QIAcube System (QIAGEN, Milano, Italy) using QIAamp DNA Mini kit (QIAGEN, Milano, Italy) according to a customized protocol developed for highly viscous samples. After extraction, DNA was quantified and checked for quality using QIAexpert Quantification System (QIAGEN, Milano, Italy).

TL analysis

TL was measured after DNA extraction from both whole blood and induced sputum samples, by using quantitative Real-Time PCR as previously described [51, 52]. This assay measures relative TL in genomic DNA by determining the ratio of telomere repeat copy number (T) to single nuclear copy gene (S) in experimental samples relative to the T/S ratio of a reference pooled sample. The single-copy gene used was human (beta) globin (hbg). The PCR runs were conducted in triplicate on a SteponePlus Real-Time PCR System (Applied Biosystems) and the average of the 3 T/S ratio measurements was used in the statistical analyses. Details of TL analysis are reported in the Supplementary Materials.

DNAmAge analysis and AgeAcc estimation

DNAmAge was determined by analysis the methylation levels from selected markers using bisulfite conversion and Pyrosequencing® methodology as previously reported [22, 23]. This method is based on determination of the methylation level of a set of five markers (ELOVL2, C1orf132, KLF14, TRIM59 and FHL2) in genomic DNA, as described by Zbieć-Piekarska et al. [21] with some modifications based on the fact that the method was almost completely automated using the PyroMark Q48 Autoprep (QIAGEN, Milano, Italy) [22, 23]. AgeAcc was calculated as the difference between the detected DNAmAge of lung cells and blood leucocytes and the chronological age of patients. Details of DNAmAge analysis are reported in the Supplementary Materials.

Statistical analysis

Statistical analyses were performed with StastDirects software. Data are expressed as mean ± SD or number and percentage. The diversity among the two groups of patients split per therapy (dual therapy and triple therapy with ICS assumption) was appraised with Mann-Whitney U Test and Chi-square test, respectively. Levels of TL, DNAmAge, and AgeAcc in lung, as derived from lung cells of induced sputum, and blood, of the same patient, were compared by (two-tailed) paired T-test. Comparison between all samples in the two groups was also made using Mann-Whitney U Test. The influence of age, gender, therapy, lymphocytes % and FEV_1% as indicator of lung function on DNAmAge and AgeAcc in blood was appraised by multiple linear regression analyses. Results were considered significant when a p value of <0.05 was obtained.

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Authors' Contributions

Conceived and designed the study: SP and MC. Patients enrollment: GG, FL, PM. Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): SP and MC. Provided samples: GG, MC and SP. Performed the samples' analysis: MC and SP. Analyzed the data: SP and MC. Wrote the paper: MC and SP.

Competing interests

The authors declare no conflict of interest.

Table 1. Interval variables in COPD patients with long-acting β2 agonist (LABA) /long-acting muscarinic antagonist (LAMA) and inhaled corticosteroid (ICS)/ LABA/ LAMA treatments (mean ± SD) and p-values of the Mann Whitney test comparing the two groups.

VARIABLES	All patients 18	LABA/ LAMA 9	ICS/ LABA/LAMA 9	p-Value
Age (years)	72.4 ± 7.7	71.1±9.0	73.7±6.4	0.502
Education (years)	9.9 ± 4.4	9.2±4.5	10.7±4.4	0.504
Body mass index (kg/m^2)	27.62 ± 4.5	27.1±5.1	28.0±4.0	0.561
Systolic pressure (mm Hg)	133.6 ± 12.5	128.3±12.7	138.9±10.2	**0.036**
Diastolic pressure (mm Hg)	81.4 ± 6.6	78.9±6.9	83.9±5.5	0.154
Mother age (years)	30.5 ± 5.9	29.4±7.5	31.4±4.6	0.319
Father age (years)	35.3 ± 6.8	32.8±6.3	37.5±6.8	0.236
Pack years ((cigarettes/20)×years)	33.4 ± 17.6	33.5±15.5	33.3±20.4	0.983
Drinking (age at start, years)	14.6 ± 10.4	15.3±10.5	14.1±11.0	0.910
Alcohol (daily intake last year)	0.6 ± 0.6	0.9±0.7	0.3±0.4	0.123
Sport (IPAQ score)	191.7 ± 376.8	363±483.4	20±42.4	0.077
Leucocytes (10^3/ml)	6.5 ± 1.9	5.9±1.5	7.0±2.3	0.385
Blood red cells (10^3/ml)	4.6 ± 0.4	4.6±0.5	4.6±0.4	0.983
Hemoglobin (g/dl)	13.2 ± 1.8	13.7±2.1	13.7±1.4	0.373
Platelet count (10^3/ml)	231.9 ± 49.5	231.2±40.2	232.5±59.9	0.843
Neutrophils (10^3/ml)	3.99 ± 1.4	3.4±0.9	4.5±1.6	0.094
Lymphocytes (10^3/ml)	1.6 ± 0.6	1.5±0.5	1.6±0.7	0.981
Monocytes (10^3/ml)	0.6 ± 0.2	0.5 ±0.1	0.6±0.2	0.351
Eosinophils (10^3/ml)	0.2 ± 0.3	0.3±0.4	0.1±0.1	0.979
Basophils (10^3/ml)	0.04 ± 0.04	0.03±0.02	0.05±0.05	0.493
Glycemia (mg/dl)	97.5 ± 35.9	110.3±32.6	80.9±35.3	0.238
C-reactive protein (mg/ml)	3.2 ± 3.2	2.7±1.9	3.7±4.4	0.983
FEV1 l/s	1.5±0.6	1.9±0.5	0.99±0.3	**0.0003**
FEV1%	63.1 ±16.6	77.1±9.1	49.1±7.9	**<0.0001**
FVC l/s	2.4 ± 0.9	3.2±0.8	1.7±0.4	**0.0003**
VC	2.5 ± 0.9	3.2±0.8	1.7±0.4	**0.0003**
TLC	4.9 ± 1.3	5.8±1.3	3.96±0.5	**0.0008**
RV	2.4 ±0.6	2.6±0.7	2.2±0.4	0.474
FEV1/VC%	58.6 ± 7	60.1±8.0	57.0±6.8	0.489

Bold character is displayed only for significant values.

Table 2. Distribution of categorical variables in COPD patients with p-values of the chi square test comparing the two groups.

VARIABLES	Classes	All patients	LABA/LAMA	ICS)/ LABA/LAMA	p-Value
		N(%)	N(%)	N(%)	
Sex [@]	Males	9(50)	6(67)	3(33)	0.202
	Non smokers	1(5)	0(0)	1(11)	0.500
Smoking	Ex smokers	15(83)	8(89)	7(78)	0.603
	Smokers	2(11)	1(11)	1(11)	0.999
Drink [@]	Drinkers	13(72)	7(78)	6(67)	0.750
Binge	None	0 (0)	0 (0)	0 (0)	NA
	≤ 1	1(5)	1(11)	0(0)	0.500
Charlson index	$\geq 2 \leq 4$	10(55)	4(44)	6(67)	0.395
	≥ 5	7(39)	4(44)	3(33)	0.667

Table 3. Telomere length of COPD patients' lung and blood.

	Chronological age (years)	Telomere length (T/S)		
		Lung	Blood	Blood
N	18	8	8	18
Mean±SD	72±8	$1.05\pm0.35^{\S\dagger}$	$1.48\pm0.21^{\S}$	$1.47\pm0.26^{\dagger}$

§ Paired t tests on n=8: Lung versus blood P= 0.0341

†Mann-Whitney U test; Two sided P = 0.0133

Table 4. DNAmAge and AgeAcc of COPD patients' lung and blood.

	Chronological age (years)	DNAmAge(years)			AgeAcc (DNAmAge-chronological age)		
		Lung	Blood	Blood	Lung	Blood	Blood
N	16	7	7	16	7	7	16
Mean ±SD	74±7	67.4±5.80[§]	61.6±5.40[§]	63.3±5.60	-4.5±5.02[§†]	-10.8±3.50[§]	-10.3±3.63[†]

[§] DNAmAge Paired t tests on n=7: Lung versus blood P = 0.0003

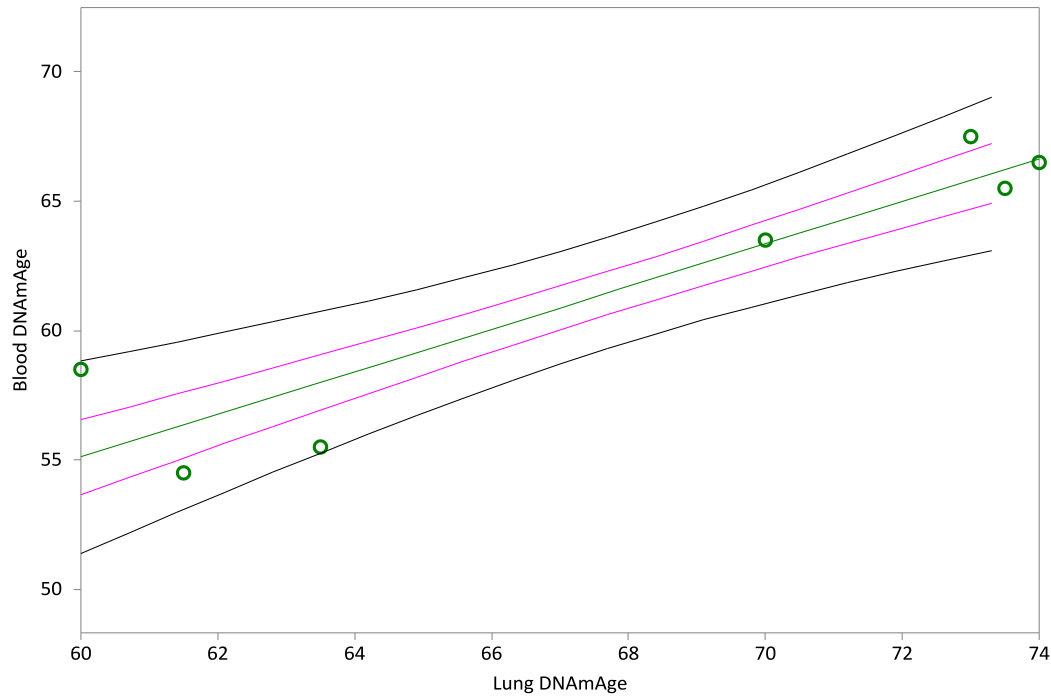[§] AgeAcc Paired t tests on n=7: Lung versus blood P = 0.0003

[†] AgeAcc Lung versus blood Mann-Whitney U test Two sided P = 0.0156

Table 5. Multiple regression analysis of the influence of age, gender, ICS therapy, lymphocytes % and FEV$_1$% on blood DNAmAge and AgeAcc.

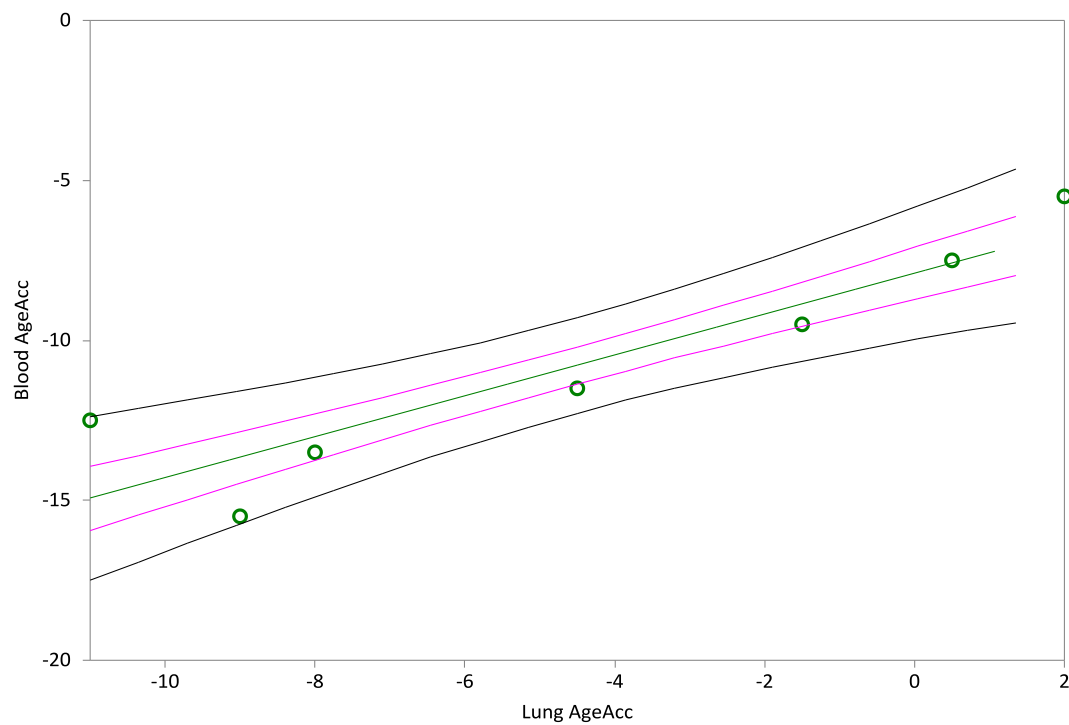|	Variables	b	r	t	p
DNAmAge	Age*	b1 = 0.706488	r = 0.886818	t = 6.068497	**P = 0.0001**
	Gender	b2 = 1.69824	r = 0.335629	t = 1.126708	P = 0.2862
	ICS therapy	b3 = -5.926199	r = -0.577206	t = -2.235228	**P = 0.0494**
	Lymphocytes %	b4 = -0.017714	r = -0.037777	t = -0.119548	P = 0.9072
	FEV$_1$%	b5 = -0.224904	r = -0.638578	t = -2.624059	**P = 0.0254**
	Variables	**b**	**r**	**t**	**p**
AgeAcc	Gender	b1 = 2.635837	r = 0.407475	t = 1.47987	P = 0.167
	ICS therapy	b2 = -6.860858	r = -0.542745	t = -2.143215	**P = 0.0553**
	Lymphocytes %	b3 = 0.166118	r = 0.303419	t = 1.056114	P = 0.3136
	FEV$_1$%	b4 = -0.258007	r = -0.601645	t = -2.498149	**P = 0.0296**

*The variable Age is not considered for AgeAcc because of its own definition.

Figure 1a. Simple linear regression between blood and lung DNAmAge
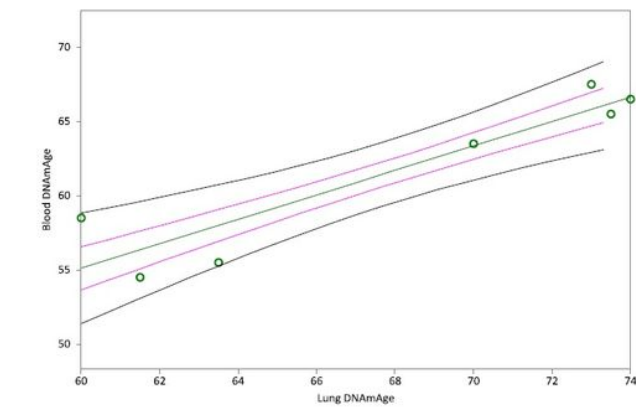

Correlation coefficient (r) = 0.927245; Two sided P = 0.0026

Figure 1b. Simple linear regression between blood and lung AgeAcc
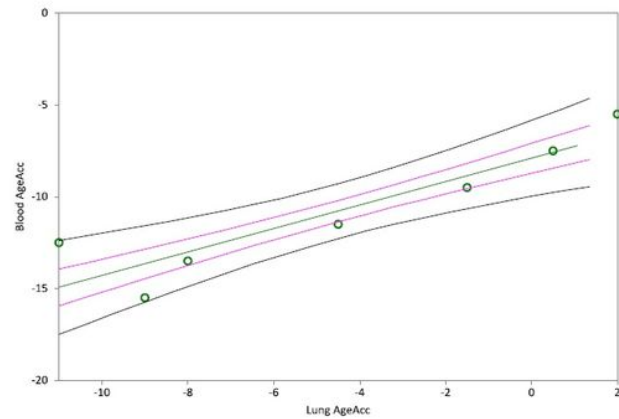

Correlation coefficient (r) = 0.916445; Two sided P = 0.0037

Figures

Correlation coefficient (r) = 0.927245; Two sided P = 0.0026

Correlation coefficient (r) = 0.916445; Two sided P = 0.0037

Figure 1

a. Simple linear regression between blood and lung DNAmAge b. Simple linear regression between blood and lung AgeAcc

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