Biological age in COPD patients reveals an accelerated lung aging.

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Supplementary Material

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 [1, 2]. This assay measures relative TL in genomic DNA by determining the ratio of telomere repeat copy number (T) to single nuclear copy gene (S), i.e. the T/S ratio, in a given sample relative to reference DNA. The single-copy gene used was human (beta) globin (hbg). As reference DNA, we used a pool of DNA from the study population. Two different pool of DNA were made for TL analyses on DNA extracted from whole blood and induced sputum samples. A fresh seven points standard curve from the pool, ranging from 40 to 0.625 ng/µl (serial dilutions 1:2), was included in every “T” and “S” PCR run, against a negative control (water). In brief, Qiagility (QIAGEN, Milano, Italy) that enables a high-precision PCR set up, was used for transferring 10 µl of reaction mix and 5 µl of DNA (5 ng/µl) in a 96-well plate. In total, 25 ng of DNA sample was added to each reaction, and each sample was run in triplicate. All PCR reactions were performed on a SteponePlus Real-Time PCR System (Applied Biosystems). The average of the three T measurements was divided by the average of the three S measurements to calculate the average T:S ratio, i.e. the relative telomere length. A measure was considered acceptable if the standard deviation (SD) among triplicate measures was <0.25. The coefficient of variation for the average T:S ratio of samples analyzed over three consecutive days was 10%, which was similar to the reproducibility originally reported for this method [3].

DNAmAge analysis

DNAmAge was determined by analysis the methylation levels from selected markers using bisulfite conversion and Pyrosequencing® methodology as previously reported [4, 5]. 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. [6] with some modifications based on the fact that the method was almost completely automated using the PyroMark Q48 Autoprep (QIAGEN, Milano, Italy). Briefly, 1 µg DNA was submitted to bisulfite conversion: unmethylated cytosines in extracted DNA were converted to uracil using Epitect Fast® DNA Bisulfite Kit (QIAGEN, Milano, Italy) following the manufacturer's instructions. An aliquot of template DNA was used for PCR amplification of selected markers using PCR primers included in the AgePlex Mono kit (Biovectis, Warszawa, Poland). PCR reactions were performed in 25 µL, comprising 0.2 µM of each primers, 20 ng of template DNA, and PyroMark PCR Master Mix holding HotStarTaq DNA Polymerase, 1X PyroMark PCR Buffer and dNTPs. The amplification plan involved a preliminary denaturation step at 95 °C for 10 min, followed by 40–45 cycles of denaturation (94 °C for 30 s), annealing (56 °C for 60 s) and extension (72 °C for 90 s), and a final extension of 72 °C for 10 min. Each PCR amplification contained negative PCR controls. In total, 10 µL of PCR product was used for each pyrosequencing primer (2 µL) contained in AgePlex Mono kit (Biovectis, Warszawa, Poland) and loaded into a 48 well-plate (Pyromark Q48 Discs, QIAGEN, Milano, Italy). Details on PCR and sequencing primer sequences and the sequences analyzed are given in Table S1. Pyrosequencing was performed on a Pyromark Q48 Autoprep instrument (QIAGEN, Milano, Italy) using Pyromark Q48 Advanced Reagents (QIAGEN, Milano, Italy) according to the manufacturer's instructions. The resulting Pyrograms® generated by the instrument were automatically analyzed using Pyromark Q48 Autoprep Software (QIAGEN, Milano, Italy). The level of methylation was expressed as a percentage of methylated cytosines at the 5 CpG sites considered. The methylation percentages were inserted in an online calculator system accessible at *www.agecalculator.ies.krakow.pl*, for estimation of biological age from DNA methylation analysis. The equation corresponds to a previously developed age prediction model [6]. All samples were analyzed 3 times for each marker to verify the reproducibility

of our results, and their averages were utilized in the statistical testing. All samples were analyzed on two different days, and the coefficient of variation (CV) for replicate pyrosequencing runs was 0.5%.

References

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4. Pavanello, S., Campisi, M., Tona, F., Dal Lin, C. & Iliceto, S. Exploring Epigenetic Age in Response to Intensive Relaxing Training: A Pilot Study to Slow Down Biological Age. *Int J Environ Res. Public. Health.* **16**, 3074 (2019).
5. Pavanello, S. et al. The biological age of the heart is consistently younger than chronological age. *Sci Rep* **10,** 10752 (2020).
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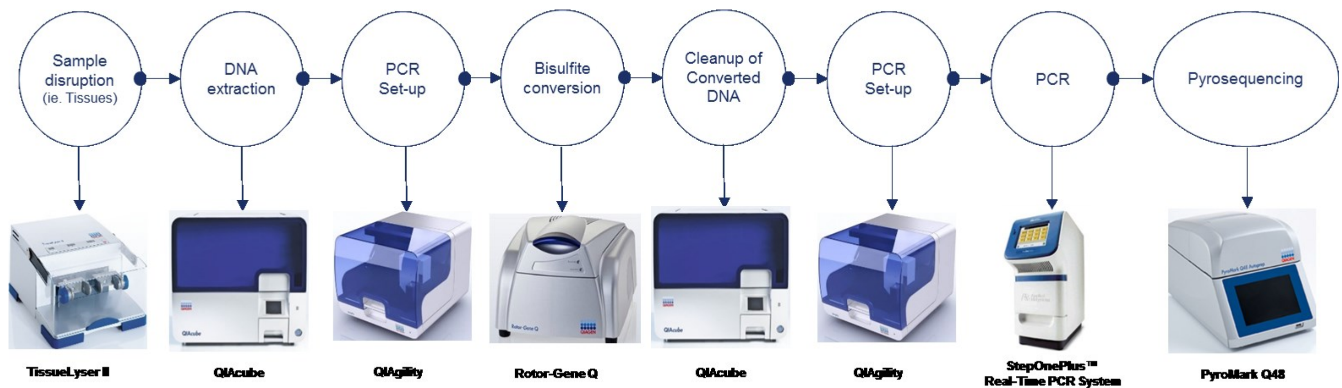
Table S1. PCR and Pyrosequencing primer sequences, and sequences to analyse.

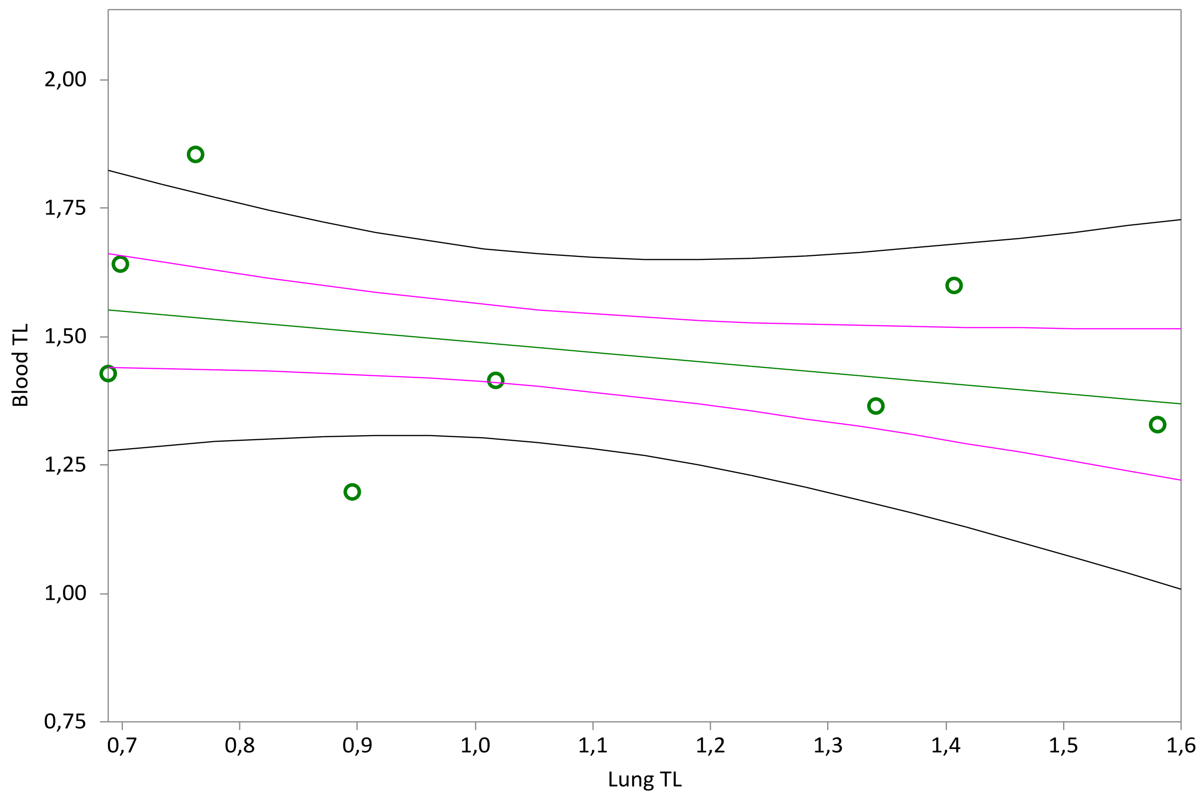
Marker	Primers	Primer sequence	Sequence to analyse
ELOVL2	Forward [B]	AGGGGAGTAGGGTAAGTGAGG	CCRTAAACRTTAAACCRCCRCRCRAAA CCRAC
	Reverse	AACAAAACCATTTCCCCCTAATAT	
	Sequencing	ACAACCAATAAATATTCCTAAAACT	
C1orf132	Forward [B]	GTAAATATATAAGTGGGGGAAGAAG GG	AAATCTACRCAAACRACRATAAATAAT CC
	Reverse	TTAATAAAACCAAATTCTAAAACATT C	
	Sequencing	CACCTTACCACCAAACCAAAATTT	
TRIM59	Forward	TATAGGTGGTTTGGGGGAGAG	GGTTTGGYGY GGGAYGAGGYGAAGYGTYGG TGGTYGAYGG TTTTTGAGGA ATTATTTTTT ATTT
	Reverse [B]	AAAAAACACTACCCTCCACAACATAA C	
	Sequencing	TTGGGGGAGAGGTTG	
KLF14	Forward	GGTTTTTAGGTTAAGTTATGTTTAATA GT	TYGYGTTTTTTTTTTGTYGGYGAGTTA GGTA ATGGTAATAGAG
	Reverse [B]	ACTACTACAACCCAAAAATTCC	
	Sequencing	ATAGTTTTAGAAATTATTTTGTTT	
FHL2	Forward	TGTTTTTAGGGTTTTGGGAGTATAG	AGTTATYGGG AGYGTYGTTT TYGGYGTGGG TTTTYGGGYG YGAGTTTYGG AYGAGGTTTG GG
	Reverse [B]	ACACCTCCTAAAACTTCTCCAATCTCC	
	Sequencing	GGTTTTGGGAGTATAGT	

Figure S1.



Seamless Workflow from sample to data interpretation. In brief, DNA extraction is accomplished on QIAcube workstation (QIAGEN). For DNAmAge analysis, the genomic DNA extracted is submitted to bisulfite conversion on Rotor-Gene Q (QIAGEN), a real-time PCR cycler, and all reactions are prepared on QIAgility (QIAGEN), which is an instrument that enables automated high-precision setup of PCR experiments. The clean-up of converted DNA is performed on the QIAcube (QIAGEN). An aliquot of template DNA is used for PCR amplification of selected markers on StepOnePlus™ Real-Time PCR System (Applied Biosystems™) and the PCR setup is automated performed on Qiagility workstation (QIAGEN). Pyrosequencing for DNA methylation analysis is carried out on PyroMark Q48 Autoprep (QIAGEN). The resulting Pyrograms® are automatically analyzed using PyroMark Q48 Autoprep Software. For TL analysis, an aliquot of genomic DNA extracted is used for Real-Time PCR on StepOnePlus™ Real-Time PCR System (Applied Biosystems™). All reactions and the PCR setup are performed on Qiagility (QIAGEN). The results are analyzed using StepOne Software 2.3.

Figure S2. Simple linear regression between blood and lung TL



Correlation coefficient (r) = -0.33554; Two sided P = 0.4165.