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CICLO XXXII

EXPLORING MECHANISMS OF BIOLOGICAL AGING IN SUSCEPTIBLE SUBJECTS

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ABSTRACT

The aim of the PhD project is to explore molecular mechanisms that characterize the process of biological aging with the main focus on the two most prominent early hallmarks of biological aging such as telomere length (TL) and epigenetic age, also defined as DNA methylation age (DNAmAge), in order to answer the following main questions:

1) If environmental and occupational exposures accelerate the biological aging in healthy subjects and in subjects affected by age-relate disease.

2) If, on the other hand, correct lifestyle including an intensive meditation/relaxing training in healthy subjects and patients with cardiovascular diseases, and heart transplantation in patients suffering from end-stage heart failure, slow down biological aging.

To this aims we analyzed biological aging indicators in easily available human tissue (blood leucocytes) and in target organ (i.e., heart of donors and recipients and lung from induced sputum of Chronic Obstructive Pulmonary Disease (COPD) patients).

Study populations consist of:

1) HEALTHY SUBJECTS EXPOSED TO GERONTOGENIC ENVIRONMENTAL AND OCCUPATIONAL EXPOSURES
   a) 71 night-shift workers and 84 day workers as control exposed to circadian rhythm disruption.
   b) 585 individuals from general population living in North-East Italy exposed to everyday exposure to carcinogenic polycyclic aromatic hydrocarbons (PAHs), which are fundamental constituents of air pollution.
   c) A prospective cohort study of women and men aged 70 years and older from the area of Treviso, which is characterized by the highest longevity in Italy. TRELONG longitudinal study population comprises 576 subjects at baseline, 300 and 200 subjects at T1 and T2 respectively, exposed to lifestyle and occupational exposures.
2) SUBJECTS AFFECTED BY AGE-RELATE DISEASES
   a) Bladder cancer (BC), a chronic disease characterized by the interaction between environmental/occupational and genetic risk factors. Study population includes newly diagnosed, histologically confirmed BC patients, admitted to the Urology Departments of two large hospitals from 1997 to 2000. Controls are 94 non-neoplastic urological patients matched to cases by age, period and hospital of admission.
   b) Idiopathic pulmonary fibrosis (IPF) and Chronic Obstructive Pulmonary Disease (COPD) as the most common manifestations of aging-mediated diseases. Study population consists of n=101 IPF patients (ATS/ERS/JRS/ALAT guidelines) and n=18 moderate COPD patients

(GOLD 2019) all enrolled at the ambulatory of Pneumology and Respiratory Physiopathology Wards – Department of Cardiac, Thoracic and Vascular Sciences and Public Health, University of Padova.

3) HEALTHY SUBJECTS AND PATIENTS EXPOSED TO REJUVENATING FACTORS
   a) Patients after myocardial infarction (n=20) and healthy control individuals (n=10), age- and gender-matched, trained to meditation and relax for 60 days.
   b) 17 recipients receiving the heart from 17 donors in the period February 2018 - February 2019.

The local Ethics Committee - University of Padova, approved the study protocols (3843/AO/16 and 3054/AO/14).

Main finding stemming from our results revels that:

1) HEALTHY SUBJECTS EXPOSED TO GERONTOGENIC ENVIRONMENTAL AND OCCUPATIONAL EXPOSURES

- ✓ Night-shift work is associated with increased systemic inflammation as proved by higher C-reactive protein (CRP) levels among night shift workers. LTL is reduced by CRP, while is positively associated with long pentraxin 3 (PTX3) that, by orchestrating an efficient governance of inflammatory processes, may protect telomere from attrition. This would make nocturnal workers more susceptible to premature LTL shortening and aging.
- ✓ Certain preventable everyday life exposures to PAHs diminish LTL and even LmtDNAcn, in particular in males, acting through anti-B[a]PDE–DNA adduct formation. Our findings show that indoor activities and diet represent the primary determinants of PAHs exposure in increasing anti-B[a]PDE–DNA adduct levels that in turn decrease in presence of detoxifying GSTM1.
- ✓ In TRELONG population LTL significantly declines over the years, from baseline to follow up, also considering only n=161 subjects whit all measurements at three different time points.

2) SUBJECTS AFFECTED BY AGE-RELATE DISEASES

- ✓ LTL attrition is a critical event in BC. In particular, BC risk is increased directly by LTL attrition that depends on some preventable everyday life exposures genetically modulated. Furthermore, indirect effects on BC risk are evidenced via LTL reduction through age and genetic polymorphisms involved in modulating response to environmental exposure.
- ✓ IPF patients in follow up present an increase in LTL positively related to the duration of antifibrotic treatment, with both pirfenidone and nintedanib, and with a decrease in lung function decline. These results would suggest that telomere shortening in IPF patients treated with antifibrotic drugs may be reversed leading to an increase in LTL.

- ✓ In COPD patients: a) lung, i.e., pulmonary cells obtained from induced sputum, is biologically older than blood, as determined by TL and DNAmAge; b) blood age acceleration (AgeAcc) defined as the difference between DNAmAge and chronological age, but not TL, highly correlates with lung AgeAcc; c) blood AgeAcc significantly correlates with the main clinical features (CRP and FEV1) of COPD.

3) HEALTHY SUBJECTS AND PATIENTS EXPOSED TO REJUVENATING FACTORS

- ✓ In healthy subjects but not in patients, DNAmAge is reduced after an intensive relaxing training. Differently, LTL is preserved in healthy subjects, while in patients it continues to decrease. However, the correlation between LTL and chronological age becomes positive after training in both groups. These findings would suggest that intensive relaxing practices influence different aging molecular mechanisms, i.e., DNAmAge and LTL, with a rejuvenating effect.
- ✓ Biological donors' heart age is found to be younger than chronological age, suggesting that donors' cardiac tissues are biologically younger than chronologically measured. Furthermore, biological donors' left atrium age is 5 years younger than recipients' left atrium age. This would indicate that patients who underwent heart transplantation have received a younger heart.

Further results and comments are described in each work reported in the specific Chapters.

Our results contribute to reinforce the concept that biological aging may be modulated by a multiplicity of factors (environmental, occupational, lifestyle) making people more susceptible to premature aging or inducing an accelerated aging or, interestingly, eliciting a rejuvenation effect. In the era of the silver tsunami, identifying gerontogenic and rejuvenating factors is of paramount importance to develop anti-aging strategies for extending the number of healthy years of life.

ABBREVIATIONS

AAs: aromatic amines

AgeAcc: Age acceleration

B[a]PDE–DNA : anti- benzo[a]pyrene diolepoxide adducts

BC: Bladder cancer

BMI: body mass index

COPD: Chronic obstructive pulmonary disease

CpG: sites of cytosine-guanine dinucleotides

CRP: C-reactive protein

CVD or CD cardiovascular diseases

DNAmAge: DNA methylation Age

FEV1: forced expiratory volume in one second

FEV1% or FEV1%pred: FEV1 percentage of the predicted normal value

FVC: forced vital capacity

FVC% or FVC%pred: FVC percentage of the predicted normal value

ICS: combined inhaled corticosteroids

IPF: diopathic pulmonary fibrosis

LA: left atrium

LABA: along-acting β2 agonist

LAMA: long-acting muscarinic antagonist

LmtDNAcn: mitochondrial DNA copy number

LTL: leucocyte telomere length

PAHs: polycyclic aromatic hydrocarbons

RA: right atrium

RR: Relaxation Response

TL: Telomere length

TLC: total lung capacity

VC vital capacity

VR: residual volume

Chapter 1.

INTRODUCTION

The growing aging of the human population, often referred to as the "gray revolution" or “silver Tsunami”, is an increasingly serious health and socioeconomic concern for modern societies. Population aging is one of the four “mega-trends” that characterize the global population of today—population growth, population aging, urbanization and international migration [United Nations, Department of Economic and Social Affairs, Population Division (2020). World Population Aging 2019 (ST/ESA/SER.A/444)] - and the trend will continue. The percentage of persons aged 65 or over worldwide is projected to increase from 9 per cent of the total population in 2019 further to 16 per cent in 2050 (Figure 1) [United Nations, Department of Economic and Social Affairs, Population Division (2020). World Population Ageing 2019 (ST/ESA/SER.A/444)]. This demographic shift inescapably has led to a significant increase in the incidence of chronic conditions and age-related diseases [Suzman et al., 2015]. The rise in global burden of disease and disability affects various economic and social mechanisms with a negative impact on health care system and workforce, giving rise to need to reallocate financial/economic and social resources [United Nations, Department of Economic and Social Affairs, Population Division (2020). World Population Ageing 2019 (ST/ESA/SER.A/444); Shiels and Ritzau-Reid, 2015]. Exploring and understanding mechanisms and molecular basis of aging process are essential action to cope with effects of age-related diseases [Panagiotou et al., 2018] and to develop anti-aging strategies extending healthy years of life.

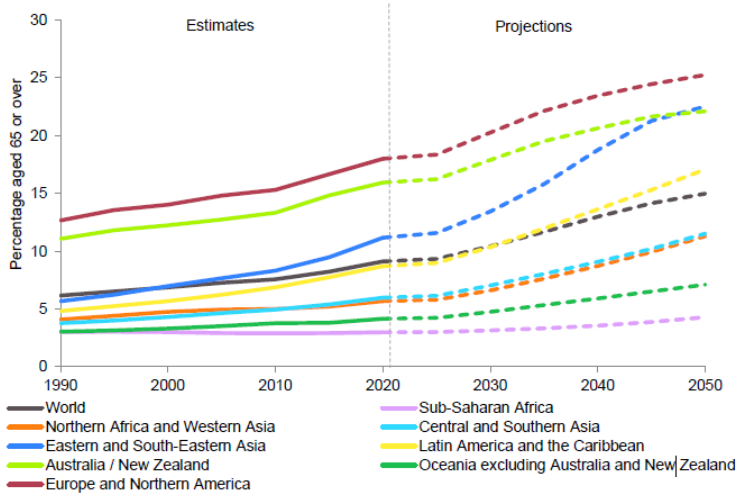

Figure 1. Share of total population aged 65 years or over, by region, 1990-2050 [Source: United Nations Department of Economic and Social Affairs, Population Division (2019). World Population Prospects 2019].

WHAT IS AGING AND BIOLOGICAL AGING?

The concept of aging may be broadly conceptualized as the by-product of the passage of time. This would define aging as a natural, ever-progressing "deterioration" of physiological functions; an increased susceptibility to certain diseases; and an intrinsic, age-related process of loss of viability and increase in vulnerability leading ultimately to death [Birren and Zarit, 1985; Strehler, 1985]. However, aging may also be defined more positively as the result of a time-dependent adaptation which ultimately becomes dysregulated, no longer obeying the principle of hormesis and leading to self-elimination [Calabrese, 2018], strongly suggesting that aging is random and purposeless. There are many types of aging such as the physiological, biological, molecular, functional, or even social. Just as life is hierarchically organized from molecules to cells to tissues, organs, systems, organisms, and populations, aging can also occur at multiple organizational levels, with consequences for the others. Here I will mostly discuss biological aging.

One of the most integrative definitions proposed so far states that biological aging [also defined "senescing" especially in relation to biological (cellular) phenomenon of "senescence"] is the process of change in the organism which over time decreases the probability of survival and reduces the physiological capacity for self-regulation, repair, and adaptation to environmental demands [Schroots and Birren, 1988]. This definition integrates all important aspects defining biological aging, including time, changes, decrease of reserves, dysregulation, and irreversibility of death. Such definition supports the classification of the aging process into **primary aging, which is postulated to reflect an intrinsic, presumably genetically determined limit on cellular (and organismal) longevity** (accounting for the relatively constant maximum lifespan observed in almost all animal species studied) and **in secondary aging, due to the accumulated effects of environmental insults, disease, and stress** (explaining most of the variability between individuals' aging trajectories within the species) [Anstey et al., 1993]. Primary and secondary aging can most likely influence each other *via* a positive feedback loop (Figure 2).

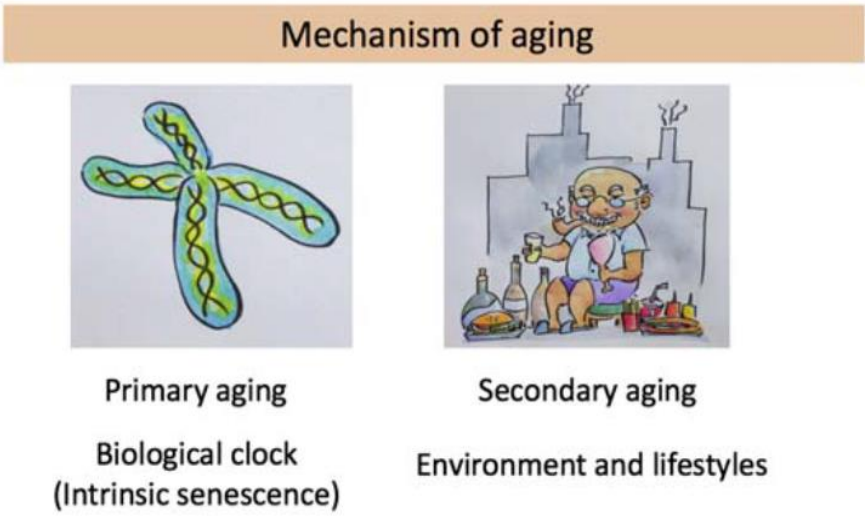

Figure 2. Mechanism of aging [Source: Wichansawakun and Buttar., 2018].

More recently, aging has been defined as an individual, natural, inexorable and very complex process characterized by a progressive deterioration of physiological integrity and in the body's ability to respond appropriately to internal and/or external stressors, with consequent increase in the susceptibility to illness and risk of death [López-Otín et al., 2013]. This physiological decline is the primary risk factor for major human pathologies and chronic diseases, including cancer, diabetes, cardiovascular disorders, and neurodegenerative diseases [López-Otín et al., 2013]. All these aspects have been summarized as the "nine hallmarks of aging" [López-Otín et al., 2013], that cover quite well the current knowledge of cellular and molecular mechanisms of aging (Section: Hallmarks of aging).

MECHANISMS OF BIOLOGICAL AGING: PROPOSED THEORIES

Over the years several theories of aging have been proposed. Most of these about what causes aging can be classified into two categories: program theories and damage or stochastic theories [daCosta et al., 2016]. **Programmed theories postulate that aging is a process genetically determined and organisms have an internal clock** that programs development, growth, maturity, and aging by sequentially switching genes on and off. Differently, **damage theories propose that aging is the result of the inescapable small random changes that accumulate over time and the failure of the body's repair mechanisms to fix the damage.** This accumulation of damage injures cells and tissues, contributing to the age-related declines in an organ's function [daCosta et al., 2016; Lipsky and King, 2015].

Currently, among the aforementioned theories, the oxidative stress theory (OST), is one of the most popular correlative theories of the aging process. OST explains aging at the molecular level and results from failure to maintain oxidative defenses, mitochondrial integrity, proteostasis, barrier structures, DNA repair, telomeres, immune function, metabolic regulation, and regenerative capacity (Figure 3) [Chandrasekaran et al., 2017].

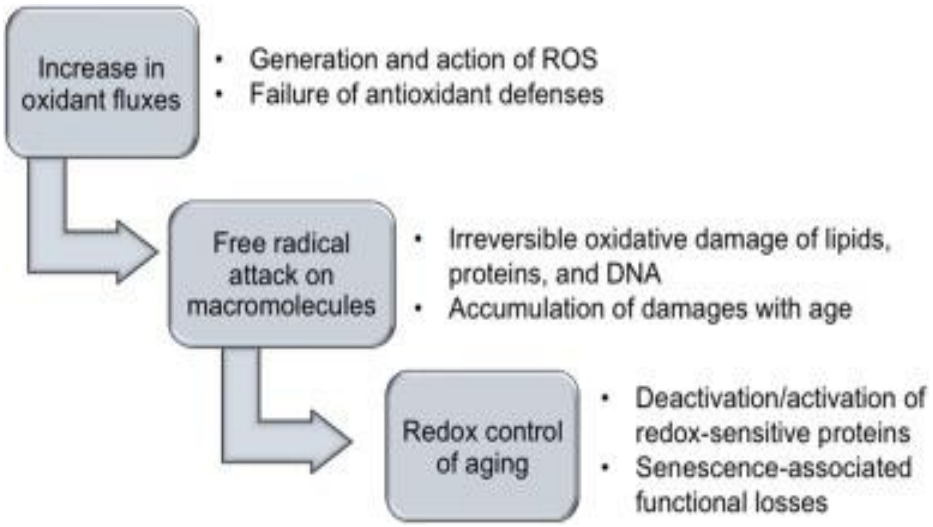

Figure 3. Schematic representation of the oxidative stress theory of aging. The key features of this hypothesis are that increase in oxidants and concomitant failure of antioxidant mechanisms cause structural damage to macromolecules which accumulates with age leading to corresponding decline or loss in function [Source: Chandrasekaran et al., 2017].

Oxidative stress occurs from the imbalance between the reactive oxygen and nitrogen species (RONS) production and their removal by antioxidant systems. RONS are free radical and non-radical reactive derivatives from oxygen and nitrogen, respectively. Free radicals are highly reactive atom or molecule containing one or more unpaired electrons in external shell, while non radical species can easily lead to free radical reactions [Genestra et al., 2007]. RONS are produced by all aerobic cells from both endogenous and exogenous sources. The endogenous sources of ROS include different cellular organelles such as mitochondria, peroxisomes and endoplasmic reticulum [Phaniendra et al., 2015]. Mitochondria represent one of the major sources of the intracellular ROS production. During the process of cellular respiration, the superoxide radicals are produced at two major sites in the electron transport chain, namely complex I (NADH dehydrogenase) and complex III (ubiquinone cytochrome c reductase) [Phaniendra et al., 2015]. The transfer of electrons from complex I or II to coenzyme Q or ubiquinone (Q) results in the formation of reduced form of coenzyme Q (QH2), which, in turn, regenerates coenzyme Q via an unstable intermediate semiquinone anion (•Q–) in the Q-cycle. This

intermediate immediately transfers electrons to molecular oxygen leading to the formation of superoxide radical [Phaniendra et al., 2015]. Furthermore, other mitochondrial components can contribute to the production of ROS [Phaniendra et al., 2015]. Additional endogenous sources of RONS include nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, myeloperoxidase (MPO), nitric oxide synthase (NOS) and lipoxygenase [Salisbury and Bronas, 2015; Liguori et al., 2018]. Exogenous sources of RONS include air and water pollution, tobacco, alcohol, heavy or transition metals, drugs, industrial solvents, cooking (eg, smoked meat, waste oil and fat), and radiation, which inside the body are metabolized into free radicals [Phaniendra et al., 2015]. When there is an imbalance between RONS production and antioxidant defenses, the RONS accumulation can damage nucleic acids, proteins, and lipids [Droge et al., 2002].

The OST of aging is based on the hypothesis that age-associated functional losses are due to the accumulation of RONS-induced damages to macromolecules (lipids, DNA, and proteins) [Beckman and Ames, 1998]. Even if the mechanism of oxidative stress-induced aging is still not clear, probably increased RONS levels lead to cellular senescence. Senescent cells acquire an irreversible senescence-associated secretory phenotype (SASP) involving secretion of soluble factors (interleukins, chemokines, and growth factors), degradative enzymes like matrix metalloproteases (MMPs), and insoluble proteins/extracellular matrix (ECM) components [Chandrasekaran et al., 2017; Pole et al., 2016]. Oxidative stress, cellular senescence and SASP factors, in particular associated with the inflammatory pathway, are involved in several acute and chronic pathological processes and to sarcopenia and frailty in elderly population [Liguori et al., 2018]. Considering the close relationship between oxidative stress, inflammation, and aging, the "oxidation-inflammatory theory of aging" or "oxi-inflamm-aging" has been proposed: aging is a loss of homeostasis due to a chronic oxidative stress that affects especially the regulatory systems, such as nervous, endocrine, and immune systems [Liguori et al., 2018]. The activation of the immune system induces an inflammatory state that creates a vicious circle in which chronic oxidative stress and inflammation feed each other leading to an increased age-related morbidity and mortality [De la Fuente and Miquel, 2009].

HALLMARKS OF BIOLOGICAL AGING

Aging may be described as a complex and rather interconnected gear mechanism [daCosta et al., 2016]. A comprehensive view of the molecular changes and mechanisms that characterize biological aging is not known because of the heterogeneity of the aging process, at an individual level too [Ahadi et al.,

2020]. López-Otín and co-workers [2013] identified and categorized nine cellular and molecular hallmarks of aging, that are generally considered to contribute to the aging process and together determine the aging phenotype. The nine hallmarks of aging include: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (Figure 4).

Figure 4. The Hallmarks of Aging [Source: López-Otín et al., 2013].

These interconnected hallmarks that co-occur during aging and are, have been classified in three categories: primary hallmarks, antagonistic hallmarks, and integrative hallmarks (Figure 5) [López-Otín et al, 2013]. The primary hallmarks are all unequivocally negative. It has been proposed that primary hallmarks are the initiating triggers whose damaging events progressively accumulate with time. The antagonistic hallmarks have beneficial or deleterious effects depending on their intensity. These hallmarks become progressively negative in a process that is partly promoted or accelerated by the primary hallmarks. The integrative hallmarks directly affect tissue homeostasis and function. They occur when the accumulated damage caused by the primary and antagonistic hallmarks, cannot be compensated by tissue homeostatic mechanisms [López-Otín et al, 2013].

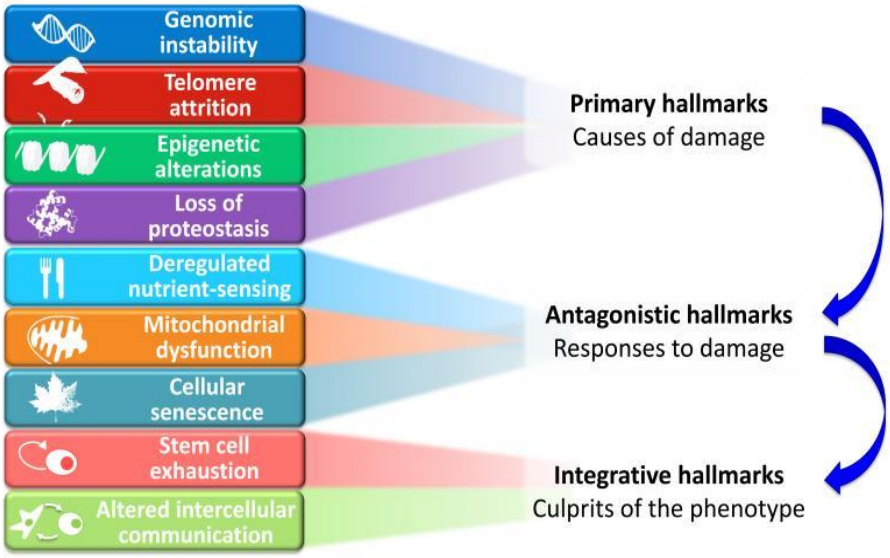

Figure 5. Functional Interconnections between the Hallmarks of Aging [Source: López-Otín et al., 2013].

HOW TO MEASURE BIOLOGICAL AGING: INDICATORS OF BIOLOGICAL AGING

It is known that there is a great heterogeneity in health outcomes and aging trajectories, also in people with the same age [Lowsky et al., 2014]. Not all individuals age at the same rate or in the same way [Fulop et al., 2010], consequently chronological age may not be a reliable indicator of physiological decline [Levine, 2013] but rather, just an estimation of the calendar time that has passed since birth [Horvath and Raj, 2018]. Indeed, primary and secondary aging may interact each other and take shape this heterogeneity in aging process. Furthermore, individual organ systems, cells, organelles, and molecules within individuals may age at significantly different rates [Shiels and Ritzau-Reid, 2015] revealing several 'ageotypes' defined as different types of aging patterns in different individuals [Ahadi et al., 2020].

Recently, the concept of biological age predictor has been defined as "a biomarker correlated with chronological age, which brings additive information in the risk assessments for age-related conditions on top of chronological age" (Figure 6). Among the six types of novel biological age estimators -DNA methylation age (DNAmAge), also referred to as the epigenetic clocks, telomere length, transcriptomic predictors, proteomic predictors, metabolomics-based predictors, and composite biomarker predictors- telomere length and DNAmAge/epigenetic clock are considered the two most prominent predictors and early hallmarks of biological aging [Jylhava et al., 2017; Marioni et al., 2018]; although, interestingly, they seem to be independently of each other [Marioni et al., 2018].

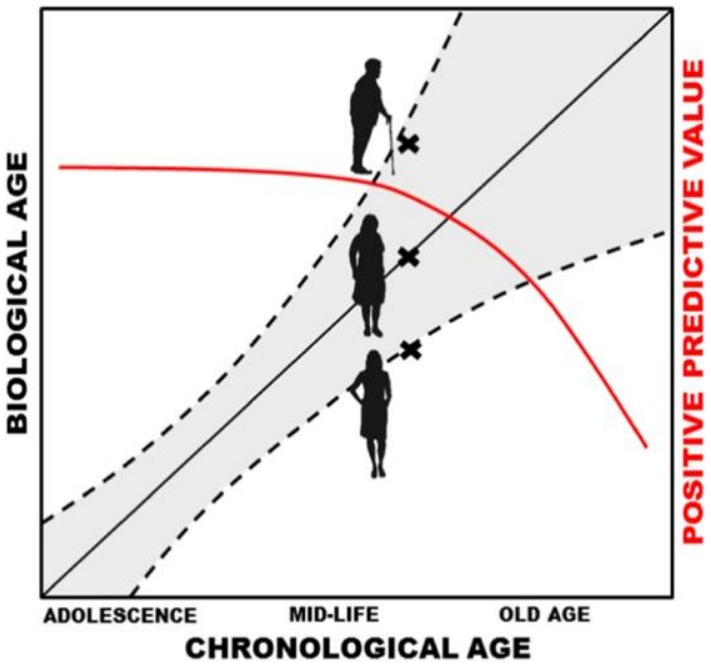

Figure 6. The concept of biological age predictors. A biological age predictor could be defined as a biomarker correlated with chronological age (black line), which brings additive information in the risk assessments for age-related conditions on top of chronological age. Hence, adult individuals of the same chronological age could possess different risks for age-associated diseases as judged from their biological ages (x's in figure). value (red line) of a biological age. Usually, the positive predictive value (red line) of a biological age predictor decreases from mid-life and onwards due to the increased biological heterogeneity at old age (confidence interval described by dashed lines increases at old age) [Source: Jylhava et al., 2017].

Telomere length (TL)

All cells of our body have a molecular clock in telomeres, the hexameric nucleotide (TTAGGG) n-protein repeats at the ends of chromosomes [Blackburn et al., 2006]. The structure of telomeres was first recognized in 1938 and thought to stabilize chromosome ends to prevent them from being recognized as DNA double-strand breaks [Montpetit et al., 2014]. Basically, telomeres protect chromosomes from recombination and end-to-end fusion maintaining chromosomal stability and preserving DNA integrity that in their absence would be gradually lost with each cell division [Blackburn et al., 2015]. Telomeres serve as molecular clocks that control the replicative capacity of human cells and their entry into senescence. In fact, telomeric repeats in normal somatic tissue shorten by 30 to 200 bp after each mitotic division eroding chromosomal terminations [Calado and Young, 2009] until a critical length leading to cellular senescence [Campisi and d'Adda di Fagagna, 2007]. A specific enzyme, telomerase, is involved in telomere synthesis, replenishing the lost DNA after mitosis.

Telomerase is an RNA-dependent DNA polymerase containing reverse transcriptase that adds telomeric repeat DNA to chromosome ends [Blackburn and Collins, 2011] but it is active only in progenitor cells and in certain diseases [Calado and Young, 2009]. Therefore, telomeres progressively shorten with each division of somatic cells and their length, measured in peripheral blood leukocytes (LTL), is considered an indicator of biological age [Frenck et al., 1998; Müezzinler et al., 2013] (Figure 7).

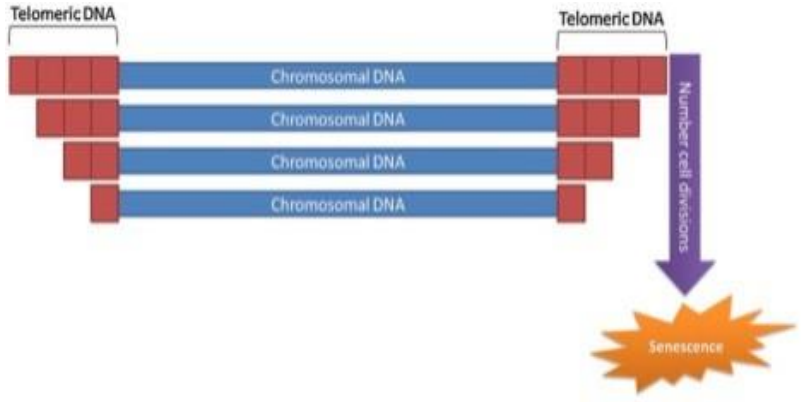

Figure 7. Model of telomere shortening on aging [Source: da Costa et al., 2016]**.**

DNA methylation Age (DNAmAge)

Epigenetic alterations in DNA methylation are associated with aging and age-related diseases [Calvanese et al., 2007; Fraga and Esteller, 2007; Gentilini et al., 2013] and may be considered an emerging robust biomarker of biological aging. Aging is specifically associated with both a genome-wide hypomethylation and a site-specific hypermethylation [Jones et al., 2015], i.e. the removal and addition of methyl groups from cellular DNA by DNA methyltransferases. The advent of epigenome-wide high-throughput sequencing analyses has led to a successful identification of a large number of genomic sites highly associated with age [Horvath, 2013]. DNA methylation levels at specific sites of cytosine-guanine dinucleotides (CpG) [Horvath, 2013; Hannum et al., 2013] have been used to create an "epigenetic clock" (Figure 8) able to estimate the epigenetic age, also defined DNA methylation age (DNAmAge), with the aim to build a predictive model of age evaluation in most human tissues [Horvath, 2013; Hannum et al., 2013]. Several multiple age-prediction statistical models, based on the age-dependent methylation changes in certain CpG loci, exist to determine the age of a person [for a review see Nwanaji-Enwerem et al., 2018] (Figure 9). Horvath's [2013] and Hannum's [2013] methods are considered the reference methods and most widely utilized. Developing of epigenetic predictors has led to an "epigenetic clock" theory of aging according to which acceleration in DNA methylation age is

indicative of altered intrinsic biological functions that make an individual susceptible to age-related diseases [Horvath and Raj, 2018]. Accelerated DNA methylation age is often commonly defined "age acceleration" or DNA methylation age acceleration (AgeAcc) and is used to denote the discrepancy between DNAmAge and chronological age [Dhingra et al., 2018]. However, for the few longitudinal studies, the term acceleration is used as deviation in DNA methylation age to indicate changes in the aging rate [Dhingra et al., 2018]. Anyway, age acceleration is of biological and clinical interest since it is considered a reliable clinical predictor of morbidity [Perna et al., 2016; Horvath et al., 2016; Roetker et al., 2018] and mortality [Chen et al., 2016; Lin et al., 2016; Fransquet et al., 2019].

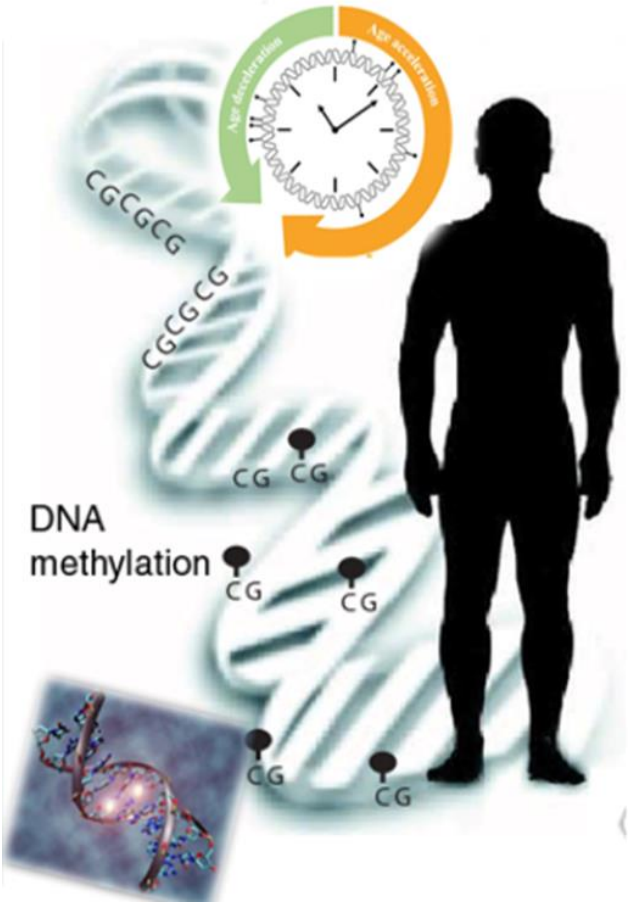

Figure 8. Epigenetic clock and DNA methylation age [Adapted from Zheng et al., 2016].

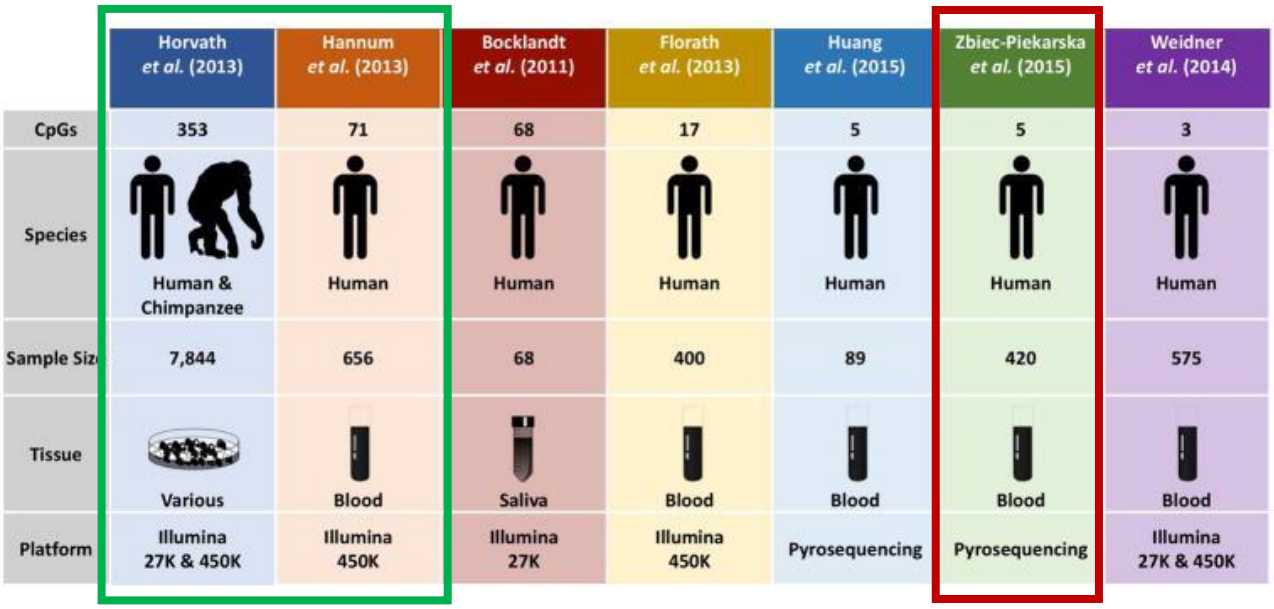

Figure 9. DNA methylation based predictors of chronological age. This figure presents seven of the known DNA methylation based predictors of chronological age, the number of CpGs used in each measure, and the species where the measure can be applied. The figure also presents the sample sizes, tissues, and platforms used to generate each measure [Source: Nwanaji-Enwerem et al., 2018].

DNAmAge- Model proposed by Zbieć-Piekarska

In this research project, we decided to apply the prediction model proposed by Zbieć-Piekarska et al. [2015] that was developed on methylation data from 5 CpG sites: ELOVL2, C1orf132, TRIM59, KLF14 and FHL2.

The gene ELOVL fatty acid elongase 2 (ELOVL2) is located on 6p24.2 and encodes for a 296-amino acid transmembrane enzyme protein involved in the synthesis of very long chain polyunsaturated fatty acids (PUFA). Elongase-2 (ELOVL2) is active with C20 and C22 PUFA [Gregory et al., 2011].

Chromosome 1 open reading frame 132 (C1orf132) located on 1q32.2 encodes long non-coding RNAs and seems to have a role in epigenetic regulation and to be important in human aging [Zbieć-Piekarska et al., 2015].

The gene TRIM59 on 3q25.33 encodes a 403-amino acid protein, which may be involved in regulation of immune signalling pathways [Kondo et al., 2012].

The gene KLF14 on 7q32.3 is intronless and encodes for a 323-amino acid protein belonging to the Kruppel-like family of transcription factors which is a zinc-finger family of transcription factors capable of linking to GC-rich sequences, and has appeared as a key controller of important functions in various organs [Swamynathan, 2010]. It especially regulates transcription cytokines and markers of

inflammation [Iwaya et al., 2018] and seems to be associated with coronary artery disease [Small et al., 2011].

The gene FHL2 encodes a multifunctional adaptor protein that is involved in the regulation of gene transcription, signal transduction, and cell proliferation and differentiation [Kadrmas et al., 2004]. Interestingly, FHL2 may act, depending on the tissue, as an oncoprotein or as a tumor suppressor [Verset et al., 2016]. It is highly expressed in human heart [Fimia et al., 2000] and it has been implicated in idiopatic dilated cardiomyopathy [Arimura et al., 2007].

The present method, generated with data from men and women aged between 2-75 years, shows high correlation between DNAmAge and chronological age (Figure 10) with an error of the estimate from the chronological age equivalent to those from Horvath [2013] and Hannum [2013], as reported in Table 1.

Table 1. Comparison between DNA methylation based predictors.

Model	CpG sites	Training set	Correlation with chronological age (R)	Model error
Horvath, 2013	353	3931	0.97	2.9 years *
Hannum et al., 2013	71	482	0.96	3.9 years †
Zbieć-Piekarska et al., 2015	**5**	**300**	**0.94**	**3.4 years** ††
Bocklandt et al., 2011	3	68	0.83	5.2 years †††

* Median absolute difference
† Root Mean Square Error (RMSE)
†† Mean absolute deviation (MAD)
†††Average absolute difference

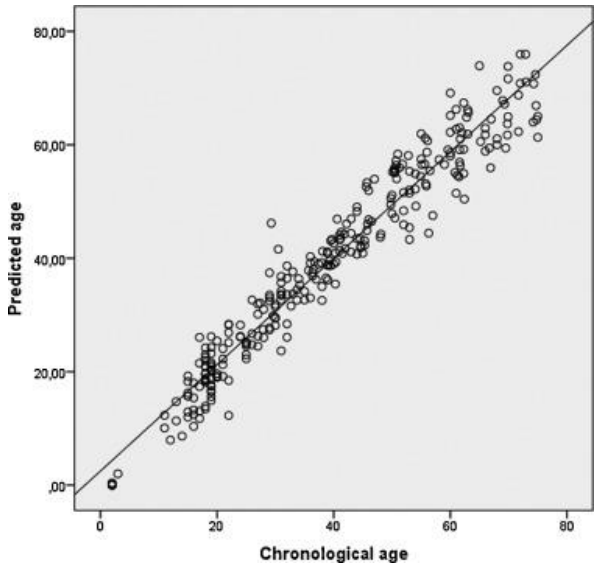

Figure 10. Predicted versus chronological age of the discovery set of 300 samples. Prediction made using the model including positions C7 in ELOVL2, C1 in C1orf132, C7 in TRIM59, C1 in KLF14 and C2 in FHL2. Negative prediction

values obtained for the 6 youngest individuals, 2 and 3 years old, were set at 0. $R^2 = 0.943$ [Source: Zbieć-Piekarska et al., 2015].

In brief, methylation level of 41 CpGs from Hannum's model was analyzed in a discovery set of 300 subjects in correlation with chronological age. Multivariate linear regression enabled simultaneous analysis of all the tested CpG sites. The prediction accuracy associated with individual CpGs and the developed prediction model was evaluated using the adjusted R^2 parameter, which is a measure of the proportion of age variance explained by particular predictors and their combined effect. This DNA methylation data obtained for 300 blood samples were then used to build a linear regression age prediction model, further validated using an additional set of 120 samples.

The linear regression equation corresponding to the developed age prediction model and implemented in the online age prediction calculator was as follows:

Predicted age (years) = 3.26847784751817 + 0.465445549010653 methC7-ELOVL2 – 0.355450171437202 methC1-C1orf132 + 0.306488541137007 methC7-TRIM59 + 0.832684435238792 methC1-KLF14 + 0.237081243617191 methC2-FHL2

The calculator is available at www.agecalculator.ies. krakow.pl. (Figure 11) [Zbieć-Piekarska et al., 2015].

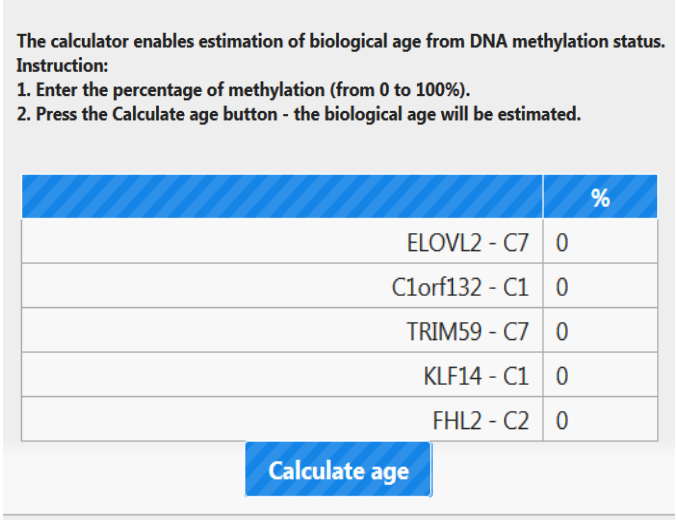

Figure 11. Age calculator.

This prediction model, based on methylation data from just 5 loci, combines technical simplicity using a method based on bisulfite conversion and pyrosequencing and high accuracy of prediction, increasing the practicability of test.

Upgrade of the analytical process in our Laboratory of Environmental Mutagenesis

Setting up of a new workflow from extraction of genomic material to data interpretation for evaluation of DNAmAge by using the model proposed by Zbieć-Piekarska [2015]. The entire workflow (Figure 12) was developed, with standard and custom protocols specifically designed, to perform the analyses, in a standardized way reducing the necessary time.

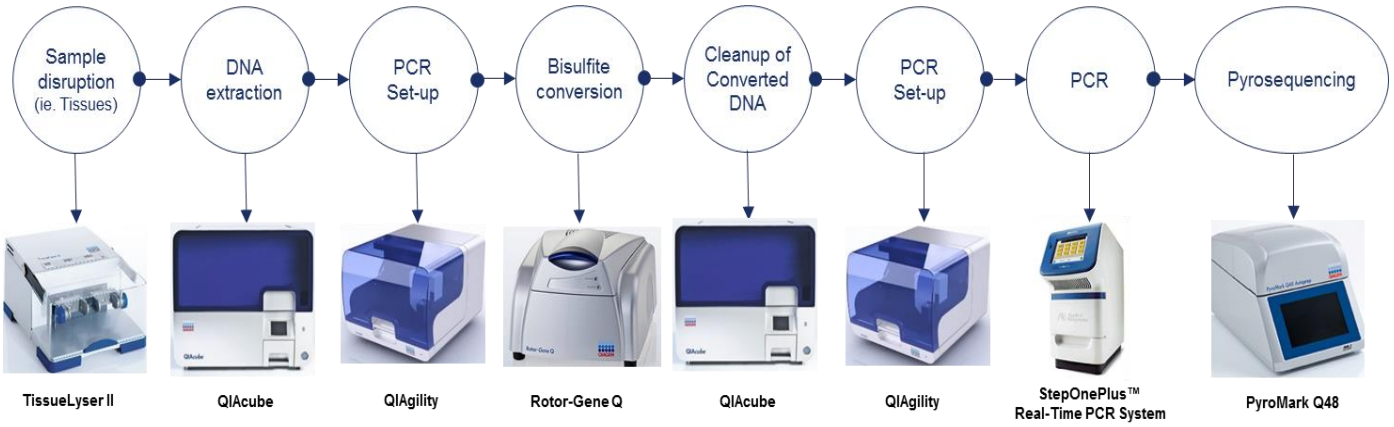

Figure 12. *Seamless Workflow from sample to data interpretation.* In brief, Tissue Lyser II (QIAGEN) is used to perform an efficient disruption of tissues followed by DNA extraction accomplished on QIAcube workstation (QIAGEN). The genomic DNA extracted is submitted to bisulfite conversion on Rotor-Gene 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 aliquote 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.

GERONTOGENS: ANOTHER FACE OF THE COIN

The concept that environmental exposures can promote aging is not new. Tom Perls noted that the rate of biological aging is determined largely (50–75%) by non-genetic factors [Perls and Puca, 2002]. Moreover, George Martin reasoned that there must be environmental agents that accelerate the rate of molecular aging, and coined the term **'gerontogen' for such age-promoting toxicants**. He presciently suggested that tobacco smoke might represent a gerontogen, based on its ability to promote disparate age-associated conditions (e.g., cancer, atherosclerosis, emphysema, and pulmonary fibrosis) [Martin, 1987]. Gerontogens, defined as an environmental stimulus, exposure or toxicant that accelerates the rate of molecular aging, can be classified as environmental agents (such as benzenes, ionizing radiation, etc.) or as exposures that occur intentionally (e.g. cigarette smoke, alterations in dietary intake) or as side-effects of a therapeutic treatment. Lastly, some evidence suggests psychological stress as a third class of potential gerontogens [Sorrentino et al., 2014]. More than ten years ago, the "exposome" concept was conceived by C. P. Wild as a way to represent the environmental, i.e., nongenetic, drivers of health and disease [Wild, 2005]. Given the increased recognition of the dominant role that nongenetic factors play in health and disease, and therefore in biological aging, the "exposome" concept strives to capture the exceptional diversity and dynamic nature of nongenetic factors and the range of exposures to synthetic chemicals, dietary constituents, psychosocial stressors, and physical factors, as well as their corresponding biological responses [Vermeulen et al., 2020]. Therefore, we can consider gerontogens as part of the exposome concept. According to this theory, differential exposure to such exceptional variety of gerontogens explains much of the non-genetic variation in the rates of human biological aging (Figure 13).

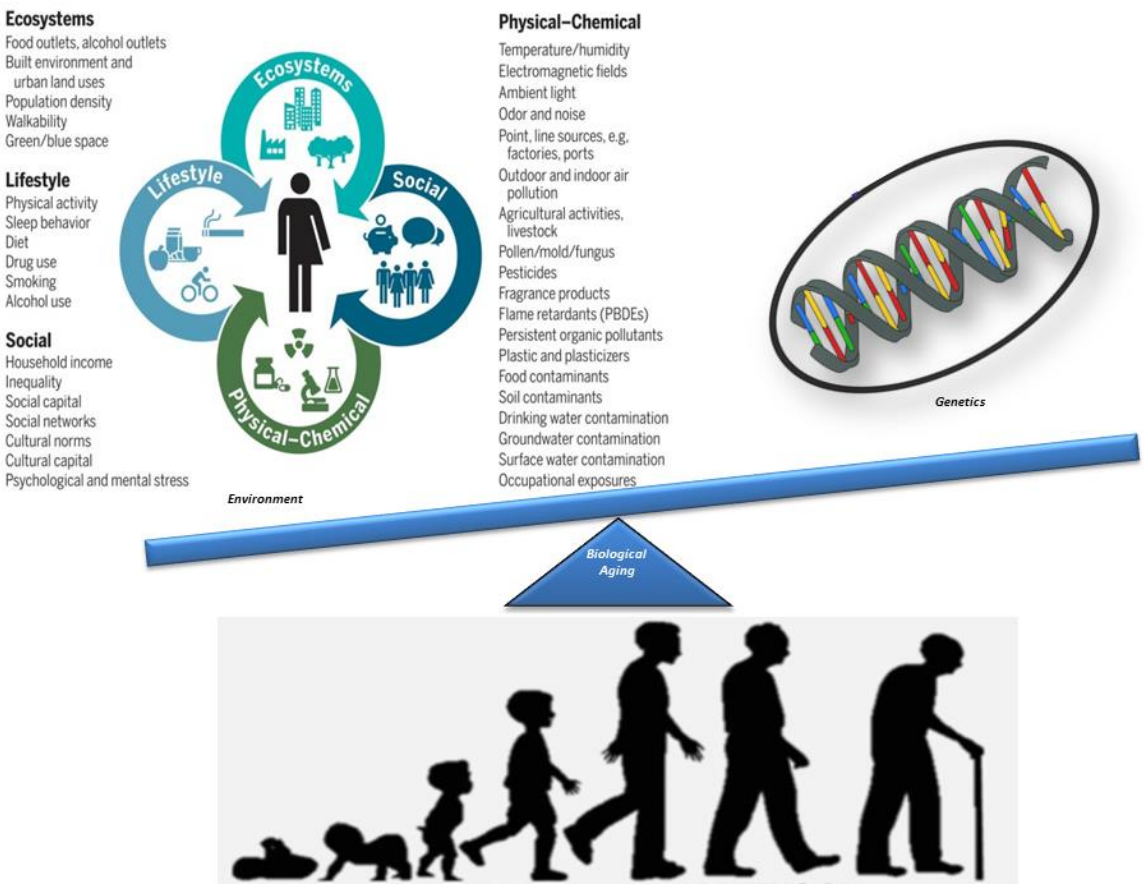

Figure 13. Proposed balance between environmental exposures and genetics on biological aging [adapted from Vermeulen et al., 2020].

Previous studies conducted in our Laboratory of Environmental Mutagenesis - Occupational Medicine (Department of Cardiac,Thoracic and Vascular Sciences and Public Health - University of Padova), have shown that biological aging is accelerated in environmental (smoke, alcohol, urban pollutants) and occupational exposures to polycyclic aromatic hydrocarbons (PAHs) [Pavanello and Lotti, 2012; Pavanello et al., 2009; 2010; 2011; 2013]. Available evidence supports the concept that a multiplicity of environmental factors may modulate telomere length [Louzona et al., 2019]. Exposure to environmental and occupational agents [Valdes et al., 2005; McGrath et al., 2007; Hoxha et al., 2009; Pavanello et al., 2010, 2011; Li et al., 2011, 2015; de Felice et al., 2012.; Lee et al., 2017; Lin et al., 2017], favoring the oxidative stress [Von Zglinicki, 2002; Passos et al., 2007], speeds up the physiological telomere erosion in leukocytes increasing the risk of age-related disease, i.e., chronic degenerative disease, including cancer [Kordinas et al., 2016]. Moreover, existing literature reports shorter telomeres in subjects with chronic psychological stress conditions [Epel et al., 2004; 2006;

Prather et al., 2011; Ahola et al., 2012; Okereke et al., 2012; Sibille et al., 2012, Gotlibt et al., 2015; Steptoe et al., 2017; Epel and Prather, 2018].

Over the last years, also DNAmAge has been widely used in literature to evaluate the impact of environmental exposures on biological aging. Some studies report the high responsivity of DNAmAge to the environmental exposures. Environmental factors that impact on age acceleration include pollution [Nwanaji-Enwerem et al., 2016; 2017; Ward-Caviness et al., 2016], dietary and lifestyle factors [Quach et al., 2017] and psychological factors such as cumulative lifetime stress [Zannas et al., 2015], traumatic and post-traumatic stress [Wolf et al., 2016; 2017; 2018; Boks et al., 2015].

To the best of our knowledge, there are no studies before ours [Pavanello et al., 2017; 2019] that have included disruption of circadian rhythm among possible gerontogens, by assessing its effect on both aging biomarkers and inflammation. Circadian rhythm disruption has become an emerging health issue being associated with premature aging and early onset of chronic conditions such as obesity, cardiovascular diseases, and metabolic diseases [Garaulet and Madrid, 2009; Kondratova and Kondratov, 2012]. By disrupting the human circadian rhythm, night-shift work has been associated with increased risk of several age-related disorders [Hublin et al., 2010; Leproult et al., 2014; Logan and McClung, 2019]. However, the mechanisms of this association are not well understood. Inflammation and the consequent biological aging, are plausible pathophysiological mechanisms through which night-shift work may influence the observed risk of diseases [McHill et al., 2014]. Moreover, it has been proposed that neurodegenerative diseases correlate clinically with disruption of sleep and circadian rhythm probably via oxidative stress or via dysregulation of metabolism [Mattis and Sehgal, 2016].

AGE-RELATED DISEASES

Bladder cancer

Bladder cancer (BC), the 4th most common cancer in men and the 11th in women, is a typical example of multifactorial disease, whose etiology is characterized by the interaction between environmental/ occupational and genetic risk factors [ACS, 2015; Grotenhuis et al., 2014].

Tobacco smoking and occupational exposure are major risk factors attributable (about 50% and 4-20%, respectively) to BC via exposure to aromatic amines (AAs) and polyaromatic compounds (PACs), which lead to increase oxidative stress and DNA damage [Hung et al., 2004]. In particular, occupational exposure to AAs (eg. dyestuffs industry and rubber textile and printing) and to PACs (eg.

metallurgy and engineering sectors, drivers and exposed to diesel emissions) has long time been associated with an increased incidence and mortality for BC [Grotenhuis et al., 2014]. A review and meta-analysis confirmed these associations even for the current occupational exposures [Cumberbatch et al., 2015]. On the other hand, increasing evidence suggests a significant influence of genetic predisposition, including that related to metabolism and DNA damaging repair, on BC incidence [Hung et al., 2004a; Hung et al., 2004b; Shen et al., 2003] accounting for almost 20% of the BC risk. In particular the application of genome-wide analyses (GWAS), beside confirming the involvement of genetic variants linked to detoxification pathways of bladder carcinogens (NAT2, UGT1A, GSTM1), has led to the identification of a number of variants, among which the most accredited are those involved in the telomere's biology, conferring an increased BC risk [Hung et al., 2004b; Cood et al., 2013]. Although it is widely recognized that dysfunction in telomere maintenance plays an important role in human carcinogenesis, the relationship between LTL and the risk of BC has not well been studied. Some retrospective case-control studies associated shorter LTL in patients with BC [Broberg et al., 2005; McGrath et al., 2007; Willeit et al., 2010; Russo et al., 2014]. Long LTL was instead strongly associated with an increased BC risk in Egyptian [Wang et al., 2015].

Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is a multidimensional long-term disease that causes inflammation in the lungs, damaged lung tissue and a narrowing of the airways, making breathing difficult [https://www.europeanlung.org/en/lung-disease-and-information/lung-diseases/copd]. It is currently the fourth cause of death in industrialized countries, but its prevalence is increasing in particular in the elderly. Since its high prevalence, morbidity and mortality, this pathology represents a remarkable challenge for health-care systems [López-Campos et al., 2016]. COPD is considered as result of the interaction of environmental agents, including tobacco smoking or exposure to biomass fuel, and inherited genetic factors [Vestbo et al., 2013.], although also occupational exposures are associated with disease [Melville et al., 2010]. COPD presents striking age-associated features as a result of continuous gerontogenic exposures and inflammation, and it has been proposed as a disease of accelerated aging [Ito et al., 2009; Maciewicz et al., 2009; Córdoba-Lanús et al., 2017]. Chronic inflammation and oxidative stress are among the generally accepted pathophysiological mechanisms [van Eeden and Sin, 2013] but there is growing evidence suggesting that the pathogenesis of COPD is linked to an accelerated aging process of the lung [Ito et al., 2009; Mercado et al., 2015] due to premature cellular senescence [Kuwano et al., 2016].

Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is an irreversible and usually fatal lung disease of elusive etiology, characterized by interstitial remodeling, leading to compromised lung function. The prevalence of IPF increases dramatically with age; IPF represents one of the syndromes associated with aging that is considered a strong risk factor for its development. Increasing evidence involves accelerated mechanisms of aging, including cellular senescence, in IPF pathogenesis [Faner et al., 2012; Schafer et al., 2017]. In fact, although IPF cause is still unknown, recent investigations have implicated telomere shortening in the pathogenesis of the disease [Stanley and Armanios, 2014]. LTL has been reported to be shorter in patients with IPF than in age-matched controls, suggesting that it might be a marker of increased disease susceptibility [Alder et al., 2008; Cronkhite et al., 2008]. Genetic predisposition, in particular telomere-related gene (TRG) mutations, rises the risk to disease development [Armanios et al., 2007; Allen et al., 2017]. Pulmonary fibrosis associated with TERT mutations (the catalytic component with reverse trascriptase activity of telomerase) is progressive and lethal with a mean survival of 3 years after diagnosis [Diaz de Leon et al., 2010]. LTL was also considered a predictor of survival in patients with IPF [Stuart et al., 2014]. Compared with the other disorders of telomere dysfunction caused by a single gene defect (such as dyskeratosis congenita, aplastic anaemia, and liver cirrhosis), IPF is the most common manifestation of telomere-mediated disease [Armanios, 2012].

REJUVENATING FACTORS

Meditation and relax - Relaxation Response (RR)

Under physiological stress, the body ramps up its production of certain hormones, such as cortisol, and other biochemical factors. These compounds help to mediate an appropriate response to short-term stress, but when overproduced for months or years, they can alter gene expression. This abnormal stress results in prolonged cortisol activation and creates a persistent inflammatory state, with failure of the body to normalize these changes after the stressor is removed [Johnson et al., 2013]. The same factors can shorten telomeres in the case of cortisol, by reducing the activity of telomerase [Epel et al., 2004]. Meanwhile, both LTL attrition and stress have independently been associated with several common conditions, including cardiovascular diseases (CVD). The relaxation response (RR) is the counterpart of the stress response. Relaxing practices are able to counteract stress and stress-induced disorders,

through the activation of specific brain areas, evoking the RR [Lazar et al., 2000]. Millennia-old practices evoking the RR include meditation, yoga and repetitive prayer. The American Heart Association (AHA) recommends the potential benefits of relaxing techniques for primary and secondary prevention of CVD [Levine et al., 2017]. Although RR elicitation is an effective therapeutic intervention that interferes with the adverse clinical effects of stress in disorders, including hypertension, anxiety, and insomnia and aging too, the underlying molecular mechanisms that explain these clinical benefits remain undetermined. The RR reduces levels of stress hormones, inflammation and oxidative stress [Paul-Labrador et al., 2006; Kaliman et al., 2014; Black et al., 2016] that are molecular pathways involved in cellular aging processes [Epel et al., 2014]. Studies show that some forms of meditation, that help to mitigate psychological stress, have a favorable effect on telomeres by activating telomerase activity [Jacobs et al., 2011; Schutte et al., 2014]. The potential effect of intensive meditation training on telomere length elongation is suggested by two studies on healthy subjects [Thimmapuram et al., 2017; Conklin et al., 2018].

Organ transplantation and aging: a new heart a new life

Transplant represents the optimal solution for patients suffering from end-stage organ failure. However, the global shortage of human organs for transplantation is one of the most dramatic crises facing biomedicine today, while the number of patients that are waiting for a transplant is steadily increasing. As the elderly are increasingly, a current donor is likely to be older than 65y/o. Studies reporting the impact of donors' age on survival with regards to heart transplantation, are still controversial [Roig et al., 2015; Kush, 2018] and a critical re-examination of donor's criteria has been addressed, extending the age limit to older donors.

Considering cellular aging as a complex process regulated by a combination of cell-intrisic and environmental conditions, young and old cells may induce enhancing rejuvenation or accelerating aging respectively, with both local and systemic influences when placed into age-asynchronous environments [Lau et al., 2019]. Direct transfusion of blood or plasma in mice seems to be sufficient to transfer factors able to induce rejuvenation or aging [Villeda et al., 2014; Castellano, 2017]. Similarly, adult stem cells transplanted into recipients by differentiating into multiple tissues types may affect and contribute to recipient organ and tissue function [Kørbling et al., 2003]. Therefore, transplantation represents a setting in which young and old cells and tissues, donors cells and recipients environment interact each other with bidirectional effects leading to rejuvenation or else accelerated aging [Lau et

al., 2019.]. Three mechanisms have been proposed to explain transferring of rejuvenation or aging, including factor-mediated contributions, cell-mediated contributions and additional mechanisms involving extracellular vesicles (Figure 14) [Lau et al., 20191].

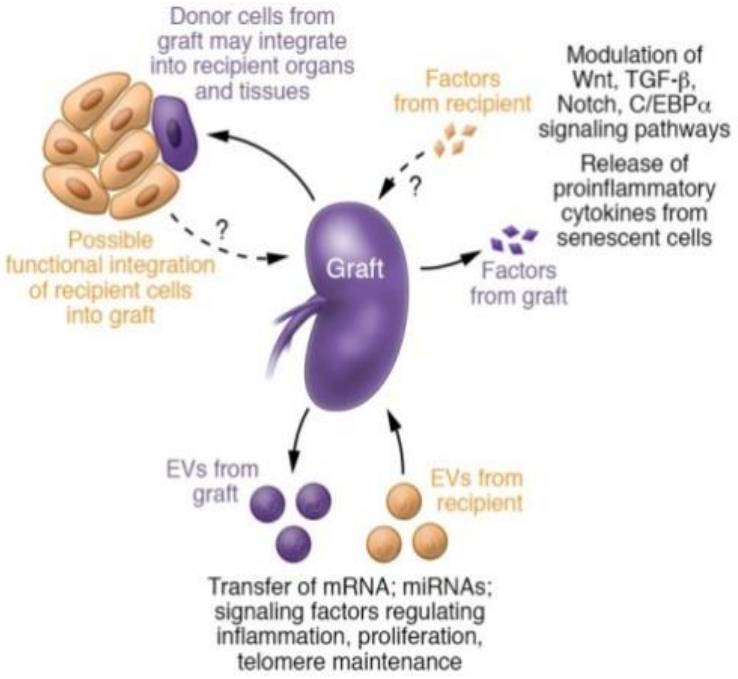

Figure 14. Mechanisms of transferring rejuvenation and aging. Several mediators of rejuvenation and aging may be transferred between young and old individuals. Donor cells may not only be capable of secreting factors that affect surrounding cells, but may also integrate into and contribute to tissue and organ function. Cells from the transplant recipient may also integrate into grafted tissue. Extracellular vesicles containing nucleic acids (predominantly mRNAs and miRNAs), proteins, and lipid products are capable of fusing with target cells to influence cellular behavior. Additionally, soluble factors secreted from cells can modulate signaling pathways implicated in the regulation of aging [Source: Lau et al., 2019].

This bidirectional effect of transferring rejuvenation and/or aging, not only in local site but potentially also at systemic level, is particularly relevant in solid organ transplantation considering the current re-examination of age-limit criteria and a clinical scenario of age-mismatched transplants.

Regarding the heart, Horvath reported a low DNAmAge of human heart tissues speculating that it could be explained through the recruitment of stem cells into cardiomyocytes for new cardiac muscle formation [2013]. Several studies claim the heart capacity of regeneration, although with various magnitudes over the lifespan [Zang et al., 2014.]. It is though there may be a persistent, but low, rate of new cardiac myocyte formation even in adult hearts, that could be originated from both a pool of persistent cardioblasts [Malliaras et al., 2014] and dedifferentiation of existing cardiac myocytes after

injury [Zhang et al., 2010]. Consequently, determining the biological age of the heart of donors could be paramount in order to define: a) the real biological age of the organ to be transplanted; b) whether balance between enhancing rejuvenation and accelerating aging is advantageous/beneficial for a recipient; and c) if heart transplant could be including amongst rejuvenating factors.

AIM OF THE STUDY

To explore molecular mechanisms that characterize the process of biological aging with the main focus on the two most prominent predictors and early hallmarks of biological aging such as telomere erosion and epigenetic age, also defined as DNA methylation age (DNAmAge), in order to answer the following main questions:

1) If environmental (i.e., exposure to incorrect lifestyle such as smoking, alcohol consumption, polycyclic aromatic hydrocarbons) and occupational exposures (i.e., exposure to Aromatic Amines, night-shift work) accelerate the biological aging in healthy subjects and in subjects affected by age-relate diseases, including idiopathic pulmonary fibrosis (IPF), obstructive pulmonary disease (COPD) and bladder cancer (BC).
2) If, on the other hand, correct lifestyle including an intensive meditation/relaxing training in healthy subjects and patients with cardiovascular diseases, and heart transplantation in patients suffering from end-stage heart failure, slow down biological aging.

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Chapter 2. BIOLOGICAL AGING IN HEALTHY SUBJECTS EXPOSED TO GERONTOGENIC FACTORS

INFLAMMATORY LONG PENTRAXIN 3 IS ASSOCIATED WITH LEUKOCYTE TELOMERE LENGTH IN NIGHT-SHIFT WORKERS.

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Inflammatory Long Pentraxin 3 is Associated with Leukocyte Telomere Length in Night-Shift Workers

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Aging is an emerging worldwide threat to public health, even in the workplace, as it links with risk of illness and death. Bewildered inflammatory responses and stressful conditions associate with age-related disorders. Additionally, circadian rhythm disruption, a critical health issue in night-shift workers, correlates with premature aging. We investigated the hypothesis of a link between altered inflammatory response, detected by plasmatic long pentraxin 3 (PTX3), and biological aging, measured by leukocyte telomere length (LTL), attrition, and possibly induced by night-shift work. Within the framework of a cross-sectional study, such possible relationships were appraised by simultaneous equation model (SEM) technique among day and night-shift hospital workers. PTX3 levels, modulated by several aging conditions [i.e., body mass index (BMI) (beta = −0.22; $p = 0.022$), C-reactive protein (CRP) (beta = −0.07; $p = 0.000$), and cardiovascular diseases with hypertension included (CVD) (beta = −0.12; $p = 0.000$)], positively associate with LTL (coefficient = 0.15; $p = 0.033$). LTL, in turn is reduced by CVD (beta = −0.15; $p = 0.000$), binge drinking (beta = −0.10; $p = 0.004$), and CRP (beta = −0.05; $p = 0.026$). On the other hand, night-shift work, found to be remarkably free from aging risk factors [i.e., age (beta = −0.13; $p = 0.017$), BMI (beta = −0.17; $p = 0.030$), CVD (beta = −0.14; $p = 0.000$), and binge drinking (beta = −0.13; $p = 0.000$)], does associate almost significantly with reversed PTX3 (coefficient = −0.09; $p = 0.089$) and even with CRP (beta = 0.17; $p = 0.000$). In conclusion, the SEM analysis indicates that PTX3 is positively linked to LTL. The finding suggests a possible new role of this long pentraxin that, by orchestrating an efficient governance of inflammatory processes, may protect telomere from attrition, ensuring therefore the genetic stability of cells. The higher CRP levels among night-shift workers suggest that night-shift work is associated with increased systemic inflammation. This would make nocturnal workers more susceptible to premature aging.

Keywords: inflammation, aging, premature, telomere, pentraxin 3, circadian rhythm, genetic instability, night-shift work

INTRODUCTION

Aging is an emerging worldwide threat to public health even in the workplace (1). The aging of the general population inescapably influences the composition of the workforce (CDC, http://www.cdc.gov/nchs/fastats/deaths.htm). In most industrialized countries, including Italy, the proportion of workers aged ≥45 years has increased by as much as 50% in the past 20 years and such increase is expected to continue in the next 20 years (2). As the population ages, the global burden of disease and disability is rising, with a negative impact on the "employability" of "elderly" workers. About 50% of those between 55 and 59 years, in fact, suffer from at least one chronic disease (2).

Aging is an individual, natural, inexorable, and biologically complex process during which a decrease in the body's ability progressively appears to respond appropriately to internal and/or external stressors. This results in an increased risk of illness and death (3). Some individuals have a more rapid physiological senescence than others (4). Consequently, age, when measured chronologically, may not be a reliable indicator of the body's rate of decline or physiological breakdown, but rather, serves only as an estimation of the rate of aging (5).

All the cells in our body have a biological aging clock in telomeres, the repetitive functional complexes of DNA/protein at the ends of chromosomes. Telomeres preserve DNA integrity that in their absence would be gradually lost with each cell division (6). A specific enzyme, telomerase is involved in telomere synthesis after mitosis, but is active only in progenitor cells and in certain diseases (7). The telomeres therefore shorten progressively with each division of somatic cells and their length measured in peripheral blood lymphocytes (LTL) is considered an indicator of biological age (8). Altered inflammatory responses (9) and exposure to environmental (10) and occupational agents (11), favoring the oxidative stress (12) can accelerate the physiological telomere erosion increasing the risk of age-related disease, i.e., chronic degenerative disease, including cancer (13).

Long pentraxin 3 (PTX3) is an acute phase protein that belongs to the same family of C-reactive protein (CRP), prototype of the short pentraxins (14). PTX3 is produced in response to primary inflammatory stimuli or microbial recognition and exerts non-redundant roles in innate immunity and in regulation of inflammation. Several observations point to a safeguarding role of PTX3 against cardiovascular disease (CD) (15) and tumor (16), acting through a functional/efficient tuning of the inflammation process and representing an extreme attempt of the body to limit an excessive inflammatory response (17).

Circadian rhythm disruption has become an emerging health issue being associated with premature aging and early onset of chronic conditions such as obesity, CD, and metabolic diseases (18, 19). By disrupting the human circadian rhythm, night-shift work is recognized as affecting several age-related disorders (20, 21). However, the mechanisms of this association are not well understood. With this respect, inflammation and the consequent biological aging is a plausible pathophysiological mechanism through which night-shift work may influence the observed risk of diseases (22). Biomarkers of biological aging, such as LTL, and of inflammation, such as PTX3, have never been examined in night-shift workers.

Aim of this study was, therefore, to explore the hypothesis of a link between inflammation, evaluated by PTX3 plasma levels, and biological aging, measured by LTL, in relation to night-shift work. This association was evaluated within the framework of a cross-sectional study conducted among day and night-shift workers of the five territorial hospitals in Ferrara, taking into account the occupational, physiological, pathological, and pharmacological history of each participant. Such complex interrelationships were appraised by the technique of simultaneous equation model (SEM). This estimation procedure uses a set of simultaneous regression equations and yields coefficient estimators more efficiently than single-equation estimators. It has recently been implemented in the program of simultaneous equation model of STATA (version 14).

MATERIALS AND METHODS

Study Design

A cross-sectional study was carried out involving the following steps: (a) nurses, working at the five territorial hospitals of the Ferrara area, were enrolled (between March 2015 and July 2015) during their periodic check-ups at the Preventive Medicine Surgery. Participants were identified by scrutinizing the hospital's occupational medicine service's computerized records. The inclusion criteria were: >18 and <65 years of age. (b) Subjects were informed of the purpose of the study by trained interviewers and asked to sign an informed consent form. (c) Study participants underwent a physical examination, an interview with structured questionnaires administered by trained interviewers and fasting blood sample collection for laboratory tests (i.e., basic biochemistry, CRP, telomere length, and PTX3 analyses) at the end of the shift work. The local Ethics Committee (School of Medicine, University of Ferrara) approved the study protocol (code number 140792). All subjects gave written informed consent and the study was conducted in accordance with the Declaration of Helsinki.

Subjects

Study population consisted of 84 day workers and 71 night-shift workers. A night-shift was defined as a work-shift schedule that included a night shift (from 8 p.m. to 6 a.m.). From a total of 342, 186 nurses gave their consent, but only 155 agreed to participate in the protocol definitively. Characteristics of the study subjects (physical examination, information acquired through questionnaires and laboratory tests) are shown in **Tables 1** and **2**.

Physical Examination

Waist circumference, height and weight, and the body mass index (BMI: body weight in kilograms divided by height in meters squared) were collected. Systolic and diastolic arterial pressures were the mean of three values measured at 10-min interval.

Information Acquired through Questionnaires

Structured questionnaires were administered during interviews to elicit information on: demographics (age, sex, marital status)

TABLE 1 | Interval variables in 84 day workers and 71 night-shift workers (mean ± SD) and *p*-values of the one-way ANOVA comparing the two groups.

Variables	Day workers	Night-shift workers	*p*-Value
Age (years)	48.9 ± 6.3	46.7 ± 5.3	**0.018**
Length of employment (years)	25.9 ± 7.4	22.6 ± 6.3	**0.004**
Length in the current job (years)	12.0 ± 8.8	22.0 ± 6.7	**0.001**
Work ability index (WAI score)	37.0 ± 4.8	38.3 ± 5.1	0.123
Education (years)	15.0 ± 1.6	15 ± 1.5	0.887
Body mass index (kg/m^2)	26.2 ± 5.2	25.5 ± 5.2	0.468
Waist (cm)	97.5 ± 12.0	96.3 ± 12.9	0.549
Systolic pressure (mm Hg)	117.5 ± 15.6	115.5 ± 13.2	0.398
Diastolic pressure (mm Hg)	75.3 ± 9.8	74.3 ± 9.8	0.529
Mother age (years)	27.4 ± 6.9	28.3 ± 5.8	0.385
Father age (years)	30.7 ± 6.3	31.3 ± 6.4	0.517
Pack-years [(cigarettes/20) × years]	7.5 ± 10.0	7.3 ± 10.6	0.886
Drinking (age at start, years)	13.4 ± 11.7	13.8 ± 11.3	0.835
Alcohol (daily intake last year)	0.2 ± 0.3	0.2 ± 0.4	0.502
Sport (IPAQ score)	2.7 ± 1.9	3.9 ± 3.3	**0.003**
Leukocytes (10^3/ml)	6.6 ± 1.7	7.2 ± 1.9	0.065
Blood red cells (10^3/ml)	4.6 ± 0.5	4.6 ± 0.4	0.423
Hemoglobin (g/dl)	13.2 ± 1.4	13.0 ± 1.4	0.498
Platelet count (10^3/ml)	275.8 ± 64.6	280.4 ± 68.5	0.670
Neutrophils (10^3/ml)	3.5 ± 1.3	3.6 ± 1.1	0.391
Lymphocytes (10^3/ml)	2.4 ± 0.6	2.7 ± 0.9	**0.020**
Monocytes (10^3/ml)	0.5 ± 0.1	0.6 ± 0.1	**0.009**
Eosinophils (10^3/ml)	0.2 ± 0.1	0.2 ± 0.1	0.943
Basophils (10^3/ml)	0.03 ± 0.01	0.03 ± 0.01	0.397
Glycemia (mg/dl)	85.1 ± 12.8	81.0 ± 14.5	0.067
Cholesterol (mg/dl)	204.8 ± 37.8	205.4 ± 42.5	0.925
Triglycerides (mg/dl)	91.3 ± 43.3	100.3 ± 53.6	0.254
Low-density lipoprotein (mg/dl)	118.8 ± 33.1	119.0 ± 38.0	0.968
High-density lipoprotein (mg/dl)	67.4 ± 17.2	68.5 ± 17.9	0.695
C-reactive protein (mg/ml)	0.3 ± 0.2	0.4 ± 0.2	0.144
Glycated hemoglobin (mmol/mol)	36.3 ± 6.6	35.8 ± 7.8	0.670
Pentraxin 3 (ng/ml)	3.9 ± 3.3	3.6 ± 1.7	0.412
Leukocyte telomere length (T/S)	1.1 ± 0.4	1.3 ± 0.5	**0.025**

Bold character is displayed only for significant values.

and other personal information (mother/father age at birth, years of education); occupation [job title; hospital department; total years worked; years spent in the current job; shift work (work was considered scheduled in day shift from 6 a.m. to 2 p.m., afternoon shift from 2 p.m. to 8 p.m. and night-shift from 8 p.m. to 6 a.m.); and frequency of night shifts/month; job energy requirement (expressed as metabolic equivalent) at work; work injury; work ability index (WAI)]. The latter has proved a valuable tool to identify any imbalances between what is required (performance requirements) and what you are able to give (individual potential) (23) and consisted of a seven-part self-assessment: current ability, work ability in relation to physical and mental demands of the job, reported diagnosed diseases, estimated impairment due to health status, sick leave over the previous 12 months, self-prognosis of work ability in the following 2 years, and mental resources of the individual. WAI ranged from 7 to 49 points; four categories have been suggested to describe WAI levels: poor (7–27), moderate (28–36), good (37–43), and excellent (44–49). Smoking history (current active smokers, former-smokers, never smokers) and pack-years (cumulative lifelong consumption of tobacco: cigarettes day/20× years) were also recorded. Habitual alcohol consumption was a dichotomous variable (yes/no), while alcohol intake was expressed in units of drink/day, each unit being approximately 10–12 g alcohol intake. Binge was defined as >4 drink-units/day (more than 40 g alcohol/day) (10). Alcohol intake in the last year was also calculated. Physical activity in leisure time was estimated according to the International Physical Activity Questionnaire (IPAQ score); finally, medically relevant complaints were arranged in groups to include cardiovascular disease (arterial hypertension included), musculoskeletal disorder, spinal disk hernia, gastrointestinal disease, endocrine disease, diabetes, respiratory disease, and tumors. The Charlson comorbidity index (24), a method of predicting mortality by classifying or weighting comorbid conditions (comorbidities), was calculated, excluding diabetes, tumors and/or respiratory diseases, and other inflammatory conditions.

Laboratory Tests

Basic Biochemistry

Data of basic biochemistry included number of blood red cells, platelets, lymphocytes, monocytes, neutrophils, basophils, eosinophils, and white cells, hematocrit, hemoglobin, glycated hemoglobin, blood glucose, triglycerides, cholesterol, low-density lipoprotein, high-density lipoprotein, and CRP.

LTL Analyses

Leukocyte telomere length was measured after DNA extraction from whole blood by Genomic DNA Purification Kit Protocol (Wizard®, Promega, Italy) and quantification, using the Quantus™ Fluorometer (Promega, Italy). DNA was available for all study subjects. LTL DNA was measured by the real-time quantitative PCR method developed by Cawthon (25) and described previously (10, 11). This method measures the relative TL in genomic DNA by determining the ratio of telomere repeat copy number (T) to single-copy gene (S) (T:S ratio) in experimental samples relative to the T/S ratio of a reference pooled sample (10, 11). The single-copy gene used in this study was human β-globin (hbg). A "seven-point" standard curve was generated from a serially diluted DNA pool (obtained from 50 DNA samples randomly selected from the DNA samples tested in the present study), ranging from 20 to 0.31 ng in each plate, in order to determine relative quantities of T and S (in nanograms). All samples and standards were run in triplicate and the average of the 3 T/S ratio measurements was used in the statistical analyses. The PCR runs were conducted in triplicate on a SteponePlus Real-Time PCR System (Applied Biosystems). After PCR amplification, the specificity of the product was confirmed by dissociation curve analysis. To test the reproducibility of telomere length measurements, we amplified telomere (T) and hbg (S) in 15 samples replicated 3 times on 3 different days. The within-sample CV for the average T/S ratio over the three consecutive days was 8.5%, which was similar to the CV reported for the original protocol (25).

PTX3 Analyses

Plasma levels of PTX3 were measured by in-house sandwich ELISA as previously described (26). Detection limit was 100 pg/ml. No cross-reaction with human CRP or serum amyloid P component was observed for antibodies used to detect PTX3. The concentration of PTX3 was determined by comparison with a

TABLE 2 | Distribution of categorical variables in 84 day workers and 71 night-shift workers with *p*-values of the chi-square test comparing the two groups.

Variables	Classes	Day workers		Night-shift workers		*p*-Value
		Number	Percent	Number	Percent	
Sex[a]	Males	7	8.3	8	11.3	0.538
Nights at work	0	84	100.0	0	0.0	**0.001**
	2–4			22	30.9	
	5+			49	69.0	
Work injury[a]	1 or more events	26	30.0	21	29.5	0.853
Smoking	Non-smokers	37	44.1	35	49.3	0.535
	Ex smokers	23	27.3	14	19.7	
	Smokers	24	28.5	22	30.9	
Drink[a]	Drinkers	53	63.1	46	64.7	0.827
Binge	None	75	89.2	67	94.3	0.439
	4	8	9.5	3	4.2	
	≥5	1	1.2	1	1.4	
Chronic disease[a]	1 or more events	74	88.1	48	67.6	**0.002**
Charlson index[b]	≥1	4	78.7	4	83.1	0.374
Musculoskeletal disease[a]	1 or more events	57	67.8	37	52.1	**0.046**
Spinal disk hernia[a]	1 or more events	49	58.3	29	40.8	**0.030**
Cardiovascular disease[c]	1 or more events	38	42.2	15	21.1	**0.002**
Gastrointestinal disease[a]	1 or more events	21	25.0	17	23.9	0.879
Endocrine disease[a]	1 or more events	24	28.5	18	25.3	0.653
Diabetes[a]	Yes	4	4.7	3	4.2	0.873
Respiratory[a]	Yes	8	9.5	7	9.8	0.944
Tumors[a]	Yes	6	7.1	5	7.0	0.981

Bold character is displayed only for significant values.

[a]*Dichotomous variable; table reports the individuals belonging to the class coded as 1; the remaining subjects may be obtained by subtraction.*

[b]*From the Charlson index were excluded conditions (i.e., in our study population specifically were diabetes, tumors, and/or respiratory diseases) that significantly impact on inflammatory response.*

[c]*Cardiovascular disease includes arterial hypertension disease.*

standard curve made with recombinant human PTX3 (range from 75 pg/ml to 2.4 ng/ml).

Statistical Analysis

One-way ANOVA and chi-square test were used to compare interval and categorical variables in the two groups of 84 day workers and 71 night-shift workers (**Tables 1** and **2**).

A hypothesis-driven analysis was performed with simultaneous equation modeling (SEM) analysis, where PTX3, LTL, and night-shift work were the Y variables (also termed "endogenous" variables) of three simultaneous regression equations. Since the same regressors (see below) were specified for each equation, the analysis was carried out using a simultaneous equation modeling program (SEM in Stata commands) rather than the Stata command which registers seemingly unrelated regression (sureg). SEM was used since it provides robust standard errors whereas sureg does not. Moreover, using the method "adf" (asymptotic distribution free) to obtain fitted parameters, SEM did not make assumptions on joint normality of all the variables and allowed using the variables (interval or categorical) as given. Finally, using the SEM option "stand," the effect of each predictor can be expressed as standardized (or beta) coefficients that make comparisons easily by ignoring the independent variable's scale of units. The other side of the coin is that it can be devilishly difficult for SEM to identify the best set of predictor variables. Often, SEM did not converge, reiterating while producing little improvement in the log-likelihood value. Since the error messages were generally not helpful, to solve the convergence problems, we simplified the model using a set of predictors (also called exogenous variables) not highly correlated to each other. Therefore, the regressors of the three equations above were the following: age, sex, BMI, waist, WAI, pack-years (PY), binge, history of cardiovascular diseases and arterial hypertension (CVD), protein-c-reactive, eosinophils, basophils, and monocytes. We used two SEM's goodness-of-fit statistics: (1) the chi-square test for "model versus saturated" (the saturated model is the model that fits the covariances perfectly) and (2) the stability index obtained from the analysis of simultaneous equation systems.

RESULTS

Baseline Characteristics of the Study Population

In **Table 1**, night-shift workers compared to day workers showed younger age ($p = 0.018$), lower length of total employment ($p = 0.004$), more years spent in the current job ($p = 0.001$), and they were physically more active according to the IPAQ ($p = 0.003$). All these characteristics are reflected by a significant longer mean LTL ($p = 0.025$), and a higher number of lymphocytes ($p = 0.020$) and monocytes ($p = 0.009$). WAI was quite a bit higher in night-shift workers but the difference was not significant. **Table 2** shows that in both groups more than 90% of nurses were females, a feature in line with this type of work. In particular, night shifts were never performed by day workers ($p = 0.001$).

Compared to the elder day workers, night-shift workers reported lesser percentages of chronic diseases ($p = 0.002$), musculoskeletal diseases ($p = 0.046$), spinal disk hernia ($p = 0.030$), and CVD ($p = 0.002$). All the other characteristics, including PTX3 plasma levels, smoking, and drinking history, were equally distributed between day and night-shift workers and were within the normal range for healthy subjects.

Outcomes

Table 3 displays the results of the simultaneous analysis of three regression equations with the Stata program SEM. The results are shown in three groups of columns and three groups of rows. Each group of column is an equation whose endogenous variable is PTX3, telomere length, or night-shift work, as shown in the column headings. The three groups of lines are the following:

- panel A (structural equations) that includes one exogenous variable per line with the corresponding beta coefficients, 95% confidence intervals, and *p*-values; the "minus" sign of beta coefficient shows an inverse relationship;
- panel B (variances) that gives the "error term" of each equation, which is used to estimate the percent of variation explained by the regression (=1−error)%;
- panel C (covariances) that measures the extent to which two equations are related through the correlation in their errors (see above). If, say, error 1 and error 2 have a contemporaneous cross-equation correlation they influence each other contemporaneously, then a SEM program is required to regress.

In the first equation of **Table 3**, PTX3 values appear to decrease with increasing BMI (beta = −0.22; $p = 0.022$) and CRP (beta = −0.07; $p = 0.000$). Since "sex" was 1 for males and "CVD" was 1 for subjects reporting such disease, therefore PTX3 mean was lower in males (beta = −0.13, $p = 0.000$) and CVD (beta = −0.12; $p = 0.000$). Conversely, PTX3 level slightly increased with increasing number of pack-years smoked (beta = 0.07; $p = 0.061$). Moreover, when comorbidities that could influence inflammatory response (i.e., diabetes, tumors, and/or respiratory diseases) were included in the Charlson's Index, PTX3 level associates positively with the Charlson's Index (beta = 0.15; $p = 0.005$, data not shown). The proportion to which SEM fitting accounts for the variation of data (variance explained) was as low as 8% (=1 − 0.92). Therefore, most variation of this outcome should be attributed to unknown predictors.

The second equation of **Table 3** shows that telomeres were shorter in subjects reporting a history of CVD (beta = −0.15; $p = 0.000$) and binge drinking (beta = −0.10; $p = 0.004$), and in those with higher values of CRP (beta = −0.05; $p = 0.026$) and peripheral eosinophils (beta = −0.21; $p = 0.000$). Conversely, the number of circulating monocytes associates with LTL (beta = 0.15; $p = 0.003$). The variance explained by the above fitting was 10% (=1 − 0.90).

Since the outcome of the third equation of **Table 3** was a dichotomous variable (day and night-shift workers), the beta coefficients should be interpreted as probabilities rather than means. Thus, the likelihood of employment as night-shift worker was found to decrease with increasing age (beta = −0.13; $p = 0.017$) and BMI (beta = −0.17; $p = 0.030$), in subjects with anamnesis of CVD (beta = −0.14; $p = 0.000$) and binge drinking (beta = −0.13; $p = 0.000$), and among those with a lower number of circulating eosinophils (beta = −0.09; $p = 0.008$). Conversely,

TABLE 3 | SEM results.

Exogenous \ Endogenous		PTX3		LTL		Night-shift work	
		Coef (95% CI)	*p*-Value	Coef (95% CI)	*p*-Value	Coef (95% CI)	*p*-Value
(A) Structural equations	Age	−0.10 (−0.21; 0.01)	0.087	−0.05 (−0.14; 0.04)	0.256	**−0.13 (−0.24; −0.02)**	**0.017**
	Sex	**−0.13 (−0.18; −0.07)**	**0.000**	0.01 (−0.04; 0.06)	0.726	**0.10 (0.01; 0.18)**	**0.021**
	BMI	**−0.22 (−0.40; −0.03)**	**0.022**	−0.02 (−0.17; 0.13)	0.790	**−0.17 (−0.33; −0.02)**	**0.030**
	Waist	0.10 (−0.10; 0.29)	0.321	0.03 (−0.13; 0.18)	0.737	0.11 (−0.06; 0.27)	0.200
	WAI	−0.06 (−0.14; 0.01)	0.098	0.06 (−0.03; 0.16)	0.197	**0.10 (0.00; 0.21)**	**0.046**
	Py	**0.07 (−0.00; 0.13)**	**0.061**	0.04 (−0.05; 0.13)	0.409	0.04 (−0.05; 0.13)	0.369
	Binge	−0.05 (−0.11; 0.02)	0.165	**−0.10 (−0.16; −0.03)**	**0.004**	**−0.13 (−0.20; −0.06)**	**0.000**
	CVD	**−0.12 (−0.17; −0.06)**	**0.000**	**−0.15 (−0.22; −0.08)**	**0.000**	**−0.14 (−0.22; −0.06)**	**0.000**
	CRP	**−0.07 (−0.11; −0.03)**	**0.000**	**−0.05 (−0.10; −0.01)**	**0.026**	**0.17 (0.12; 0.22)**	**0.000**
	Eosinoph	−0.01 (−0.10; 0.07)	0.756	**−0.21 (−0.30; −0.11)**	**0.000**	**−0.09 (−0.16; −0.02)**	**0.008**
	Basoph	−0.04 (−0.10; 0.02)	0.203	0.03 (−0.08; 0.14)	0.575	0.00 (−0.10:0.11)	0.915
	Monoc	−0.08 (−0.17; 0.01)	0.101	**0.15 (0.05; 0.24)**	**0.003**	**0.22 (0.14; 0.31)**	**0.000**
(B) Variances (error terms)		0.92 (0.89; 0.95)		0.90 (0.85; 0.95)		0.84 (0.79; 0.89)	
(C) Covariances		**e.PTX3, e.LTL**		e.LTL, e.night-shift work		e.PTX3, e.night-shift work	
		0.15 (0.01; 0.29)	**0.033**	0.09 (−0.02; 0.19)	0.100	−0.09 (−0.189; 0.013)	0.089

Bold character is displayed only for significant values.

Beta coefficients (Coef) with 95% confidence intervals (95%CI) and p-values for: (A) exogenous variables of three structural equations; (B) variances or error terms of three endogenous variables; (C) covariances from the pairwise comparison of three simultaneous equations.

Endogenous variables: long pentraxin 3 (PTX3: ng/ml); leukocyte telomere length (LTL: T/S); night-shift work (job done: variable yes/no). Error terms: e.PTX3; e.LTL; e.night-shift work.

Exogenous variables: age (years); sex (women = 0; men = 1); body mass index (BMI: kg/m²); waist (cm); work ability index (WAI, score: 7–49); pack-years (Py) of cigarettes; binge (≥4 UA/day); CVD (history of cardiovascular diseases hypertension included: yes = 1; no = 0); tumors (history of tumor: yes = 1; no = 0); C-reactive protein (CRP: mg/ml); Eosinoph (eosinophils: n × 10³/μl); Basoph [basophils (n × 10³/μl)]; Monoc (monocytes: n × 10³/μl).

the factors that appeared to increase with night-shift working were male sex (beta = 0.10; $p = 0.021$), increasing values of WAI (beta = 0.10; $p = 0.046$), CRP (beta = 0.17; $p = 0.000$), and peripheral monocytes (beta = 0.22; $p = 0.000$). The variance explained by the above fitting was about 16% (=1 − 0.84).

The last row of **Table 3** shows the three covariances resulting from the pairwise comparison of the three equations. The cross-equation correlation of error terms was significant (coefficient = 0.15; $p = 0.033$) comparing the first and second equations (headers: PTX3 and LTL) and in the comparison of first and third equations (headers: PTX3 and night-shift work) (coefficient = −0.09; $p = 0.089$); while it was not significant otherwise. This finding demonstrated that these variables were correlated to each other supporting the idea that increased PTX3 positively affects (protects) LTL, whereas PTX3 is slightly decreased in night-shift workers. Fitting of SEM model was evaluated with the Chi-square test (that was 0.00 with p-value equal to 1.00) and the stability index (that was 0.00), indicating that SEM model did not differ from a saturated model and satisfied stability condition (data not showed). The results of **Table 3** were displayed as path diagram in **Figure 1**, using the graphical interface of SEM.

DISCUSSION

Our study exploring the hypothesis of an association between inflammation and biological aging, evaluated by PTX3 plasma levels and LTL in relation to night-shift work, shows by SEM analysis (cross-equation term was significant in Panel C) that PTX3 levels are positively related (protect) with LTL, whereas PTX3 levels only to some extent associates negatively with night-shift work.

The long pentraxin PTX3 is an essential component of humoral innate immunity and plays a key role in the regulation of inflammation. The innate immune response is the first line of defense against invading microbes and tissue damage. PTX3, induced by primary proinflammatory cytokines, sensing microbes, and/or tissue injury, acts as pattern recognition molecule through interaction with molecular patterns and triggers a complex response that includes the regulation of leukocyte recruitment and the modulation of complement activation (27). By interacting with provisional matrix components, PTX3 contributes to the orchestration of wound healing and tissue repair/remodeling (28). PTX3 deficiency in model mice (ApoE mice) was associated with atherosclerosis development and increased macrophage accumulation within atherosclerotic plaques as well as with more pronounced inflammatory profiles in vascular walls (17). Relative PTX3 deficiency in humans is associated with increased inflammation, cardiac damage, and atherosclerosis, while its overexpression limits carotid restenosis after angioplasty (15). On the other hand, by modulating complement-driven inflammation, PTX3 acts even as an extrinsic oncosuppressor gene (16). Global PTX3 deficiency was associated with more pronounced cancer-related inflammation and with a higher number of tumor-infiltrating macrophages. PTX3-deficient tumors were also characterized by enhanced genetic instability features as indicated by increased p53 mutations, oxidative DNA damage, and expression of DNA damage markers, in line with the hypothesis that inflammation contributes to genetic events that can lead to the genetic instability observed in tumors (29). On the whole, certain observations point toward a protective role of PTX3 against CD (15) and tumor (16), acting through a better tuning of inflammatory response (17). Therefore, the positive association of PTX3 with LTL suggests that the presence

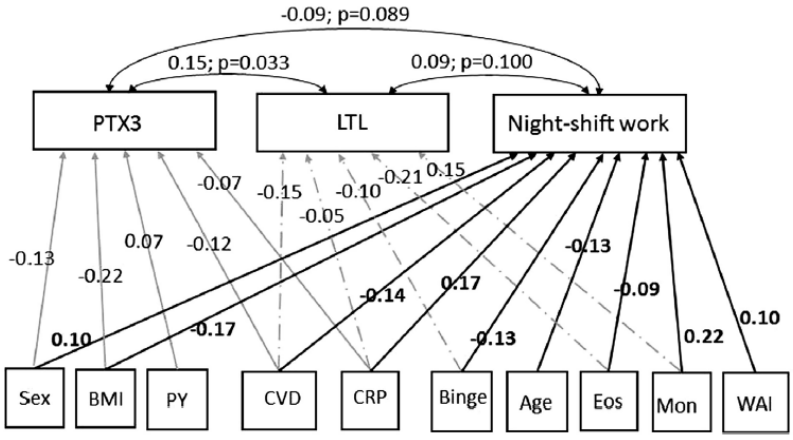

FIGURE 1 | Path diagram of the results shown in Table 3. Square boxes stand for: endogenous variables [long pentraxin 3 (PTX3: ng/ml); leukocyte telomere length (LTL: T/S); night-shift work (job done: variable yes/no)] and for exogenous variables [age (years); sex (women = 0; men = 1); body mass index (BMI: kg/m^2); pack-years (PY) of cigarettes; binge (≥4 UA/day); CVD (history of cardiovascular diseases hypertension included: yes = 1; no = 0); C-reactive protein (CRP: mg/ml); eosinophils (Eos: $n \times 10^3$/μl); monocytes (Mon: $n \times 10^3$/μl); work ability index (WAI, score: 7–49)]. Arrows specify the slightly significant ($p < 0.05$) causal link of variables with PTX3 (⟶), LTL (⟶ · ⟶), and night-shift work (→). The estimated beta coefficients appeared along the arrowed paths and the "minus" sign shows an inverse relationship. Curved routes display the cross-equation correlation of error terms resulting from the pairwise comparison of the three covariances. The estimated beta coefficients (with p-values) appear along the curved arrows, the "minus" sign indicates an inverse relationship.

of this long pentraxin, by orchestrating an efficient governance of inflammatory processes, may protect telomere from an accelerated attrition, ensuring therefore the genetic stability of cells. Conversely, a decrease in PTX3 would not provide this such protective function.

Inspecting our sample population, it can be seen that night-shift workers were younger than day workers, and consequently with lower length of total employment. Moreover, day workers never performed night-shifts with relatively more years spent in the current job, making day workers an appropriate control group. Compared to the elder day workers, night-shift workers were physically more active and reported lesser percentages of chronic diseases, musculoskeletal disease, spinal disk hernia, and cardiovascular disease. On the other hand, an overall reduction in number of lymphocytes and monocytes found in the elder group of day workers compared to the younger night-shift worker group can be accounted to age-associated changes/effects (30). All these characteristics are reflected on a significant longer mean LTL in night-shift workers, confirming LTL measure as a reliable marker of biological aging.

In the simultaneous analysis, the first of three regressions shows that PTX3 negatively associates with BMI, CRP, and history of CVD hypertension included, suggesting that PTX3 is modulated by several aging conditions and/or disorders. In particular, the negative association of PTX3 levels with BMI (31) corroborates some of the available scanty data from literature. On the other hand, the negative relationship with CRP and CVD is in contrast with other data available (32, 33), bringing out the dual antagonistic and complex role of PTX3 in the regulation of inflammation (17). Finally, we found PTX3 to be positively associated with number of pack-years smoked. This might support the idea that cigarette smoking (CS) can be an important determinant in PTX3 production, confirming an *in vivo* study on mice (34), and that PTX3 could be an indicator of this activation. In fact, by increasing levels of PTX3, CS elicits both immune and inflammatory responses (34). Thousands of ROS are produced in each puff of the burning cigarette and are not removed by cigarette butt filters (35). CS constituents (particularly ROS and trace of microbial cell components, including bacterial lipopolysaccharide) activate epithelial cell intracellular signaling cascades that lead to inflammatory activation [e.g., interleukin-8 and tumor necrosis factor-alpha (TNF-α)] (36, 37). During inflammation, different cell types produce large amounts of PTX3 and the level of circulating PTX3 increases in several pathological conditions (38), generally correlating with disease severity (39, 40). This is in agreement with the positive relationship between PTX3 and the Charlson's Index observed when conditions significantly impacting the inflammatory response, i.e., diabetes, tumors, and/or respiratory diseases, were included in the analysis (data not shown). The Charlson comorbidity index (24) has been widely utilized by health researchers to measure burden of disease and case mix. The index has been validated for its ability to predict mortality in various disease subgroups, including cancer, renal disease, stroke, intensive care, and liver disease (41). To our knowledge, no studies until now have investigated the relationships between PTX3 and Charlson's Index.

The second of three regression equations confirms literature available on shorter LTL in subjects reporting a history of CVD, hypertension included (42), binge drinking (10), and in those with higher values of the CRP, the systemic inflammation marker. Inflammation (43) and the ensuing oxidative stress (12, 43), two mechanisms that accelerate telomere shortening (44), have been linked with heavy alcohol consumption (45). Essential hypertension, the most common disorder of aging and one of the leading causes of CD, is linked to oxidative stress (46), and in turn with inflammation (47). These results support the hypothesis that telomere attrition may be related to diseases of aging, through mechanisms involving inflammation and exposure to risk factors of CD and cancer, including hypertension and heavy alcohol drinks. An increase in eosinophils in relation with shorter LTL, as well as with altered values of CRP, is in line with a study conducted on asthmatic subjects, in which chronic asthma (life-course-persistent asthma), via systemic eosinophilic inflammation, associated with LTL (48).

In the third regression, the likelihood of employment as night-shift worker was found to be associated with decreased age, BMI, CVD hypertension included, binge drinking, and number of circulating eosinophils. Conversely, night-shift work associates with male sex, increased values of WAI, CRP, and peripheral monocytes. These results fit well within the framework of the characteristics of our study population, wherein, among other things, night-shift workers were younger than day workers and physically more active. This is reflected in a decrease in BMI, blood pressure, eosinophils as marker of inflammation, and an increased WAI and peripheral monocytes, all features linked to younger age. On the other end, the higher CRP levels among night-shift workers, remarkably independent of the considered CD risk factors (such as smoking, BMI, and hypertension), beside confirming the results of a larger study on airline-company employes (n = 1,877, 60% man) (49), suggests that night-shift work is associated with increased systemic inflammation. Albeit most of our participants were female and it is known that immune/inflammatory parameters fluctuate during menstrual cycle, we missed information on menstrual cycle at the time of blood samples withdrawn. However, our female population was too heterogeneous to evaluate this issue correctly. Indeed, the number and complexity of the variables [age, phase of the menstrual cycle (follicular/luteal phase), recent pregnancies and breast-feeding, menopause, hormone medications, etc.] which influence the fluctuation of inflammatory parameters probably require an *ad hoc* study (50). Nevertheless, by SEM analysis, our work provides new evidence of inflammation as a possible pathway linking irregular nocturnal working hours and morbidity.

In conclusion, the relevant finding stemming from our work is that PTX3 positively correlates to LTL envisaging another new function of PTX3 that, by orchestrating an efficient control of the inflammatory process, may protect telomere from an accelerated attrition, ensuring therefore the genetic stability of cells. On the other end, the higher CRP levels among night-shift workers, remarkably independent of the considered CD risk factors (such as smoking, BMI, and hypertension), suggest that night-shift work is associated with increased systemic inflammation. This would make nocturnal workers more susceptible to premature aging.

ETHICS STATEMENT

The local Ethics Committee (School of Medicine, University of Ferrara) approved the study protocol (code number 140792). All subjects gave written informed consent and the study was conducted in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

All authors have a substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; drafting the work or revising it critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Moreover, MB, MN, PB and MS have a substantial contribution in subject enrollment, physical examination, interview with structured questionnaires, Ethics Committee application. SP and MC in laboratory analysis; GM in statistics; BB, AM, and RL in PTX3 analysis.

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Submitted to exposure and health

THE EFFECTS OF EVERYDAY-LIFE EXPOSURE TO POLYCYCLIC AROMATIC HYDROCARBONS ON BIOLOGICAL AGE INDICATORS

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PAH everyday exposure by B[a]PDE-DNA alters LTL and mtDNAcn.

ABSTRACT

Further knowledge on modifiable aging risk factors is required to mitigate the increasing burden of age-related diseases in a rapidly growing global demographic of elderly individuals. We explored the effect of everyday exposure to carcinogenic polycyclic aromatic hydrocarbons (PAHs), which are fundamental constituents of air pollution, on cellular biological aging. This was determined via

the analysis of leukocyte telomere length (LTL), mitochondrial DNA copy number (LmtDNAcn), and by the formation of anti- benzo[a]pyrene diolepoxide (B[a]PDE–DNA) adducts.

The study population consisted of 585 individuals living in North-East Italy. PAH exposure (diet, indoor activities, outdoor activities, traffic, and residential exposure) and smoking behavior were assessed by questionnaire and through measures of target dose [B[a]PDE–DNA]. LTL, LmtDNAcn and genetic polymorphisms [glutathione S-transferase M1 and T1 (GSTM1; GSTT1)] were measured by polymerase chain reaction methods. Structural equation modelling analysis evaluated these complex relationships.

In two models investigating LTL and LmtDNAcn, we found an ordered relationship between these measurements and PAH exposure (diet, indoor, outdoor, traffic, residential). Acting through anti-B[a]PDE–DNA adduct formation, we detected reduced LTL (p=0.028) and LmtDNAcn (p=0.018) levels, particularly in males (LTL p=0.006; LmtDNAcn p=0.0001) with PAH exposure. Active smoking only increased LTL (p=0.0001). Besides this, the most significant determinants of PAH exposure causing an increase in anti-B[a]PDE–DNA were “indoor” and “diet” (p=0.0001 for each) while the least significant was “outdoor”. Lastly, the presence of detoxifying GSTM1 decreased adduct levels.

New findings stemming from our study suggest that LTL and LmtDNAcn erosion depends on certain preventable everyday life exposure to PAHs. In particular, the clear association with indoor activities, diet, and gender opens new perspectives for tailored preventive measures in age-related diseases, including lung cancer.

Capsule:

Everyday life exposure to polycyclic aromatic hydrocarbons shortens leukocyte telomere length and mitochondrial DNA copy number through anti-B[a]PDE-DNA adduct formation.

Keywords

Polycyclic aromatic hydrocarbon; biological aging; telomere length; mitochondrial DNA copy number; DNA adduct; structural equation modelling.

1. INTRODUCTION

Continuous exposure to air pollution is one of the most important factors influencing adverse age-related outcomes, with lung cancer being the most strongly associated (Fajersztajn et al., 2013). Further understanding of other modifiable aging risk factors is needed to mitigate the increasing

burden of age-related morbidities, in particular cancer incidence, among a rapidly growing global demographic of elderly individuals (Calcinotto et al., 2019). In fact, the aging of the human population, referred to as the "gray" revolution, is a rising public health threat.

Aging cannot merely refer to the effect of chronological time, given that it is a personal and multifaceted biological process (López-Otín et al., 2013). Biological aging is assumed to mirror continuing modifications within a person, i.e. intrinsic physiological degeneration and the body's capability to respond to different stressors (such as genetic factors and exposure to environmental and occupational agents) (Belsky et al., 2015). On the cellular level, aging is related to a range of molecular, biochemical and metabolic alterations. Growing indications have shown that nDNA (e.g., telomere length attrition) and mtDNA alterations (e.g., mtDNAcn reduction) are considered early hallmarks of biological aging, and may be the primary cause of cellular dysfunction (López-Otín et al., 2013). Alterations of leukocyte telomere length (LTL) and mitochondrial DNA copy number (LmtDNAcn) are also implicated in age-related disorders, including lung cancer (Yuan et al., 2018; Chen et al., 2018). An emerging body of evidence has associated environmental factors (e.g. exposure to lung carcinogenic polycyclic aromatic hydrocarbons (PAHs)) to changes in LTL, whereas fewer studies have explored the impact of such pollutants on LmtDNAcn (Pavanello et al., 2013) (Figure 1).

PAHs are widespread contaminants and major constituents of air pollution, as they are shaped throughout incomplete combustion of organic materials like tobacco, wood, fossil fuels, petroleum products, and even through the cooking of food (Kim et al., 2013) (Figure 2). Since PAH exposure is pervasive and modifiable, it is an appropriate target for cancer prevention research studies. Benzo[a]pyrene (B[a]P), the key tracer of PAH mixtures, is the fundamental respiratory carcinogen of this complex mixture (Alexandrov et al., 2016). It has been suggested that B[a]P may both directly (by injuring DNA) and indirectly (by promoting the formation of oxidative damages and the onset of chronic inflammation) speed up the physiological aging process. This would accelerate the onset of chronic degenerative pathologies, lung cancer included. B[a]P has been extensively described for its ability to harm nuclear DNA (nDNA) when it forms the carcinogenic steady anti-B[a]P diolepoxide (anti-B[a]PDE)–DNA adduct at guanine exocyclic N2 (Alexandrov et al., 2016). Two additional molecular targets of B[a]P could be telomeres and mitochondrial DNA. Telomeres, as tandem triple-G-containing sequences repeated at each chromosomal end, keep the chromosome from eroding and fusing with neighboring chromosomes (Blackburn et al., 2015), and could represent a susceptible domain for injury by the genotoxic B[a]PDE. Additionally, B[a]P may even affect mitochondrial DNA (mtDNA), an independent DNA molecule with a central role in controlling oxidative balance and apoptosis, both of which are also related to lung cancer

carcinogenesis (Liu et al., 2017). B[a]P has been reported to possess greater damaging potential for mtDNA, with forty to ninety-fold higher affinity for mtDNA (Allen and Combs, 1980) than for nDNA. Furthermore, PAH (B[a]P) metabolic activation via aldo-keto reductase and/or manganese superoxide dismutase (Palackal et al., 2011) produces reactive oxygen species (ROS) that can produce high levels of oxidized guanine in both nDNA (Liu et al., 2006) and mtDNA (Raha and Robinson, 2000). Furthermore, mitochondria, with fewer protective histones and lower DNA repair capability compared with nDNA, are extremely liable to be broken (Allen and Combs, 1980) and would be another relevant target in B[a]P exposure.

Existing evidence suggests a possible negative association between long-term exposure to air pollution (PM2.5) and LTL, and a positive association between short-term exposure and LTL (Miri et al., 2019). However, for carcinogenic PAH exposure, the results are not conclusive. In our previous work we found a major impact of long-term exposure to high levels of PAHs in coke-oven workers on nDNA and LmtDNAcn, where exposure was evaluated by anti-B[a]PDE–DNA adducts and urinary 1-pyrenol, thus linking professional PAH exposure with cellular biological aging (Pavanello et al., 2010).

There is growing interest in structural equation modeling (SEM) as it represents a very important statistical tool for evaluating complex relations in several research areas (Victora et al., 1997). In epidemiology, the applications of SEM have been limited thus far. The attractiveness of SEM stems mainly from the fact that researchers have recognized the necessity of grasping the complex interrelations between multiple variables under study. Traditional statistical approaches apply solely to a limited number of variables, and thus fail to deal with emerging sophisticated theories. SEM analysis is a statistical technique that links observed data with qualitative causative assumptions and tests whether variables are interdependent, and if so, the details of their interactions. This is achieved through an estimation procedure (Victora et al., 1997), which uses a set of concurrent regression equations to yield coefficient estimators more efficiently than single-equation estimators. This methodology is appropriate for the investigation of complex interrelationships, as it tests causative relationships instead of mere correlations (Pavanello et al., 2017).

The aim of the current study is to investigate the extent to which long-term exposure to PAHs, together with genetic polymorphisms and anti-B[a]PDE–DNA adducts, could affect LTL and LmtDNAcn within the general population. These multifaceted relationships are evaluated using the analysis of SEM.

2. METHODS

2.1 Study design

The study population consisted of n = 585 participants listed within the LAINBIO project. Enrollment was done at the Preventive Medicine Service of the University of Padua, Italy, from October 2002 to July 2005, as previously described (Pavanello et al., 2006). All participants were notified of the purpose and strategies of the study and were requested to sign a consent form. The Ethics Panel of the School of Medicine, in accordance with principles of the Helsinki Declaration, approved the study (practice number 3843/AO/16). The admissibility criteria for participants were as follows: (1) older than eighteen years at registration, (2) not professionally exposed to PAHs, (3) inhabitant of the Veneto region at the time of the enrolment, and (4) willing to sign the consent form and provide blood and urine samples. Conditions for exclusion from the study included preceding diagnosis of cancer, cardiovascular disease, or stroke within the last year, as well as other chronic syndromes such as multiple sclerosis, Alzheimer's disease, Parkinson's disease, depression, bipolar disorder, schizophrenia, and epilepsy (Pavanello et al., 2006). Information on possible extracurricular PAH exposure (i.e. diet and indoor and outdoor exposure) as well as intake of fruit and vegetables were gathered by means of a structured questionnaire, as previously described (Pavanello et al., 2006). When subjects filled out the questionnaire, blood samples were drawn and conserved at −80°C until DNA was obtained by a Genomic DNA purification kit (Wizard, Promega, Italy), following the manufacturer's instructions. DNA was used for subsequent analyses of leukocyte DNA adducts, LTL and LmtDNAcn. All participants became anonymous after sample collection.

2.2 Estimation of PAH exposure from the questionnaire

Using a self-compiled questionnaire, we collected data on environmental exposure to PAH focusing on the following categories.

Diet. This is the number of times per year that PAH-rich meals were consumed, including grilled meat or pizza roasted in wood-burning ovens. In the statistical analysis, this was considered as a continuous data variable.

Indoor. Indoor exposure was the combination of a number of sources: presence of a coal- or wood-heater in the residence (used less than or more than 5 times per year = a score of 1 or 2, respectively), leisure activity with exposure to PAHs (a score of 1), and exposure to passive smoke (a score of 1). Participants were categorized as having no exposure (total score of 0), or low (total score of 1), intermediate (total score of 2) or elevated (total score of 3) indoor

exposure, the latter including one individual who had a score of 4. In the statistical analysis, this was taken into consideration as a continuous variable.

Home. Residential exposure was classified as urban/ peripheral or country areas, which was used as a categorical data variable with two levels in the statistical analysis i.e. 1 or 0, respectively.

Traffic. Traffic nearby the zone of habitation was accounted for as a categorical variable with two levels: intense (score = 1), or moderate or absent (score = 0).

Outdoor. Subjects with outdoor exposure to traffic pollution were individuals such as traffic police officers and gardeners. The variable was categorical in two levels: ≥4 hours/day (score = 1), or <4 hours/day (score = 0).

Smoking. Current smokers (including individuals who had quit smoking up to 4 weeks before participation to the study) were given a score as 1, while nonsmokers and former smokers were scored as 0.

2.3 Analysis of the *anti*-B[*a*]PDE–DNA adduct

Anti-B[*a*]PDE–DNA adduct was identified by high-performance-liquid-chromatography along with a fluorescence detector (Pavanello et al., 2006). The procedure was as previously described (Pavanello et al., 2005) with some minor changes, primarily concerning the mechanization of the HPLC assay. In this way the batch impact was abated (see complete description of anti-B[a]PDE–DNA adduct analysis in the Supplementary Material). In short, samples with non-measurable DNA adducts had a value of one-half the threshold of detection of the assay (LOD/2=0.125). Adduct levels were considered in the analyses both continuously and categorically (present or non-measurable). Individuals classified as having adducts present were those with a level of ≥0.5 adducts/10^8 nucleotides.

2.4 Leukocyte Telomere Length (LTL)

LTL was appraised by using quantitative Real-Time PCR as previously described (Pavanello et al., 2018). This test calculates LTL in genomic DNA by establishing the proportion of telomere replicate copy number (T) compared to copy number of a nuclear gene (S) in a specified sample relative to a reference DNA sample, i.e. the so called Telomere/Single gene (T/S) ratio. The single-copy gene was human (beta) globin (hbg). As reference DNA, we pooled DNA from 50 subjects randomly selected from the study population (500 ng for each sample). From this, a new standard curve ranging from 30 to 0.23 ng/µl (serial dilutions 1:2), was added in every "T" and "S" PCR run, versus a negative sample (water). In total 9 ng of DNA sample was incorporated in each analysis.

Each sample was threefold analyzed as reported in Pavanello et al. (2018). LTL was treated in the analyses both as categorical tl50 (higher or lower than median: 0 = below 0.896; 1 = equal/above 0.896) or as a continuous variable. See complete description of LTL analysis in the Supplementary Materials and Methods.

2.5 Leukocyte mtDNAcn (LmtDNAcn)

LmtDNAcn was determined in the same DNA of LTL testing by means of the real-time quantitative PCR (qRT-PCR) as previously described (Pavanello et al., 2013). This assay appraises mtDNAcn in experimental samples by establishing the relation between the mitochondrial (MT) DNA copy number and the single copy number of a gene (S) relative to the MT/S ratio of a reference assembled DNA sample (Pavanello et al., 2013). All samples were replicated threefold. The average of the three MT measurements was divided by the average of the three S measurements to calculate the MT/S ratio for each sample. The coefficient variation for the MT/S in samples examined on two distinct days was 6%. LmtDNAcn was treated as a continuous data variable in the statistical analysis. See complete description of LmtDNAcn analysis in the Supplementary Materials and Methods.

2.6 GSTM1 and GSTT1

A multi-PCR technique was applied to detect the presence or absence of the GSTM1 and GSTT1 genes, following the procedure as previously described (Pavanello et al., 2005). Briefly, the same amplification mix contained both GSTM1- and GSTT1-specific primer pairs and incorporateed a third primer pair for β-globin, the internal positive PCR control. The GSTT1 (480 bp), β-globin (285 bp), and GSTM1 (215 bp) amplified products were separated in a 2% agarose gel. The absence of the GSTM1- or GSTT1-specific fragment designated the corresponding null genotype (*0/*0), whereas the β-globin-specific fragment indicated the presence of amplified fragments in the reaction blend.

2.7 Statistical analysis

We used the Spearman's rank coefficient to calculate the pairwise correlation among the five variables of environmental exposure to PAHs (diet, indoor, home, traffic, outdoor), as well as age and sex. Through a mathematical model (see below: SEM), these variables were aggregated in the

latent variable “PAH” that represents an overall picture underlying physical reality, making it easier to understand and handle the data.

2.7.1 Analytic strategy

We used a conceptual framework describing the hierarchical relationships between risk factors, based on knowledge of the relevant literature and temporal considerations. As shown in Figure 3, the latent variable “exposure to PAH” was considered as the distal determinant, acting through the proximate determinant “anti-B[a]PDE–DNA” (intermediate variable or mechanism) to affect the final outcomes “tl50” (LTL median) or, alternatively, LmtDNAcn. Although it is uncommon, the notion of proximate and distal determinants is important because in an approach based entirely on statistical associations, distal factors are often improperly adjusted for proximate factors with a consequent reduction or elimination of the effects of the former (Victora et al., 1997).

All the above assumptions were converted into two models of structural equation modeling (SEM), one for each final outcome (either tl50 or LmtDNAcn). The STATA command syntax for each model was:

```
SEM (PAH -> diet indoor outdoor home traffic) (anti-B[a]PDE–DNA <- PAH sex smoking gstm1)
    (tl50 <- anti-B[a]PDE–DNA age sex smoking), stand vce(oim);
SEM (PAH -> diet indoor home traffic outdoor) (anti-B[a]PDE–DNA <- PAH sex smoking gstm1)
    (LmtDNAcn <- anti-B[a]PDE–DNA sex), stand vce(oim)
```

In the first, second and third set of parentheses we specified, respectively, the estimations of the latent variable “PAH”, the model for the mediator variable “anti-B[a]PDE–DNA” and the model for the final outcome (either tl50 or LmtDNAcn). Notice that “anti-B[a]PDE–DNA” was a dependent variable in the second set and an explanatory variable in the third set of parentheses. All the predictors shown in Figure 3 were used in preliminary analyses (data not shown) but only the statistically significant terms were included in SEM final models. In STATA commands, “stand” specifies that the effects are expressed as standardized (or beta) coefficients that make comparisons easily by ignoring the independent variable's scale of units, while “vce(oim)” specifies how the standard errors are calculated. “VCE” stands for variance–covariance matrix of the estimators, and “oim” stands for observed information matrix (OIM). The OIM estimator of the VCE is the default and is based on asymptotic maximum-likelihood theory. The VCE obtained in this way is valid if the errors are independent and identically distributed normal, although the estimated VCE is known to be reasonably robust to violations of the normality assumption.

We used three SEM goodness-of-fit statistics: (1) the chi square test for "model versus saturated" (the saturated model is the model that fits the covariances perfectly), (2) the standardized root mean squared residual (SRMR), and (3) the coefficient of determination (CD).

SEM results were both tabulated and presented graphically.

The sample size required for SEM is dependent on model complexity, the estimation method used, and the distributional characteristics of observed variables. The best option is to consider the model complexity (i.e., the number of exogenous variables) and the following rules of thumb: minimum ratio 5:1, with a recommended ratio of 10:1, or a recommended ratio of 15:1 for data with no normal distribution (Hair et al., 2006). With ten exogenous variables used in the SEM model, we should have a minimum of 150 (= 15 × 10) subjects; in total we reached 585 (537 with complete data), thus fulfilling these requirements. The analysis was conducted with the statistical package STATA 14.

3. RESULTS

In the present study, complete individual data are available for 537 (92%) out of 585 original subjects. Table 1 shows the main characteristics of subjects with number and row percent of categorical variables or mean and standard deviation of continuous variables. Age was relatively young given that the sample group targeted working-age individuals, sex was well balanced, and the majority of study subjects were nonsmokers and former smokers (79.7%). The intake of PAH-containing meals was on average equal to 46.8 times/year, which equates to less than once a week. Urban residence was predominant; outdoor exposure to traffic came mainly from occupations such as traffic police officers, gardeners and others. GSTM1 and T1 null frequencies were in line with what has been found in larger Caucasian population studies (Garte et al., 2001). Hardy-Weinberg Equilibrium (HWE) was tested for each polymorphism; the allele frequency was calculated, and the observed genotype frequency was compared with expected frequency using a X^2 test. The allele distributions for the polymorphisms were under HWE with p-value > 0.05 (data not shown). For the outcome variables, the main concern was the ascertainment of distribution. Anti-B[a]PDE–DNA did not follow a normal distribution, and while any transformations failed to reduce its skewness (data not shown) the variable was used as such. Considering this skewed distribution, telomere length was used as a 0/1 variable (below or above the median) since the median (0.9×10^8 nucleotides) and the mean (1.00×10^8 nucleotides) were rather close values. After square root transformation, the distribution of LmtDNAcn approached a normal distribution but still displayed a

significant departure from the normal distribution (data not shown). Therefore, LmtDNAcn was used as it was, given that the estimated VCE is reasonably robust in SEM, when considering violations of the normality assumption.

Spearman's rank coefficients and significance level for pairwise correlation of environmental exposure to PAHs as well as age and sex are reported in the Supplemental Information (Table 1S). The variable "outdoor" was highly related to sex – since only males were present in this category – but not correlated with other aspects of PAH environmental exposure. Perhaps further study can clarify this value for females as well. Diet and indoor exposure were significantly correlated with each other, and both were negatively correlated with "home" and "traffic" (Figure 4). It is worth noting that most rural households ranked 2 or 3 in the classification of indoor exposure to PAH, and were not affected by intense traffic in the neighborhood (data not shown). Interestingly, the times per year that subjects consumed PAH-containing meals (i.e. the "diet" category) significantly decreased with increasing age and was higher in males than in females (Figure 4).

Four groups of SEM results concerned with the analysis of tl50, which is the dichotomous variable indicating telomere length, are reported in the Supplemental Information (Table 2S). These are:

1. Structural equations. This includes the beta coefficients (with a "minus" sign indicating an inverse relationship), 95% confidence intervals and p-values for each of two structural equation models.
   - The first model shows that the endogenous variable anti-B[a]PDE–DNA significantly increased with increasing value of the latent variable PAH (beta = 0.178; p = 0.005) and with active smoking (0.149; p = 0.0001), whereas the presence of the detoxifying GSTM1 (beta = –0.098; p = 0.021) decreased adduct levels, as did sex (to a lesser extent).
   - The second model shows that the endogenous variable $LTL_{50\ percent}$ significantly decreased with anti-B[a]PDE–DNA (beta = –0.092; p = 0.028), age (beta = –0.135; p = 0.001), and sex (beta –0.117; p = 0.006), while active smoking (beta = 0.187; p = 0.0001) showed an opposite effect. Interestingly, PAH was not a significant predictor in the second structural equation.
2. Measurement. The standardized (beta) coefficients for this measurement model can be interpreted as correlation coefficients describing the direction (positive or negative) and degree (strength) of relationship between each indicator and the latent variable PAH. The most significant indicator was "diet" and "indoor", and the least significant was "outdoor". The positive coefficients (such as for "diet", "indoor" and "outdoor") indicate that the latent variable PAH tends to increase with increasing values of these exposure variables. The negative

coefficient of "home" and "traffic" means that these factors tend to go in the opposite direction, probably because the latter variables were false indicators of exposure.

Errors. The variability explained (1 – error) by the above fitting was about 7% for both anti-B[a]PDE–DNA (ε6 = 0.931) and telomere length (ε7 = 0.932).

Covariances. The findings demonstrated that age and sex were individually correlated with PAH.

The value of Chi square test for the discrepancy of the specified model versus the saturated model was 139.4 with p-value <0.0001. The size of residuals (SRMR) were equal to 0.055 and the coefficient of determination (CD) of 0.524, both of which demonstrated a good fit.

Using the graphical interface of SEM, the results shown in Table 2S were displayed as a path diagram in Figure 4. In this figure, an oval indicates the latent variable, square boxes indicate the observed variables, circles indicate errors, arrows specify the direction of causal flow, an arrowed route is a path, and the estimated beta coefficients appeared along the paths. The effect of one variable on another is called direct. There was also evidence of indirect effects (one variable affecting another variable which in turn affects a third), indicating that PAH exposure decreases LTL through formation of anti-B[a]PDE–DNA.

The analysis of LmtDNAcn was carried out with a STATA command similar to that used for tl50, except than the second structural equation with LmtDNAcn as outcome is reported in the Supplemental Information (Table 3S). The significant predictors of LmtDNAcn were anti-B[a]PDE–DNA (beta= –0.100, p = 0.018) and sex (beta = –0.146, p=0.0001). As for LTL, PAH exposure appeared to have no direct effect on LmtDNAcn but an indirect effect through the mediation of anti-B[a]PDE–DNA. Using the graphical interface of SEM, the results shown in Table 3S were displayed as a path diagram in Figure 5. Despite the fact that the variance explained by the above fitting was as low as 3.1% (1-0.969), the fit was good for the whole SEM model (chi square test = 116.2, p<0.0001; SRMR = 0.055; CD = 0.529).

3. DISCUSSION

The key finding of our study is that in a postulated chain of events, a hierarchical relationship was outlined between different PAH exposures (diet, indoor, outdoor, traffic, residential) as well as smoking that, acting through anti-B[a]PDE–DNA adduct formation, affected LTL and LmtDNAcn. This opens up the possibility that carcinogenic PAHs may also be gerontogenic for the general population (in particular males), by speeding up biological indicators of aging. In fact, LTL and

LmtDNAcn decreased with anti-B[a]PDE–DNA formation, particularly in males. In accordance with our previous findings, we found that anti-B[a]PDE–DNA was significantly increased with PAH exposure and active smoking, whereas the presence of the detoxifying GSTM1 decreased adduct levels. Lastly, male gender was also associated with decreased GSTM1 levels, to a lesser extent.

The direct negative relationship between LTL and DNA adducts is in line with our study in coke oven workers, who are extremely exposed to work-related PAH carcinogens (Pavanello et al., 2010), and thus would suggest that adduct formation might have a direct role in LTL erosion. In fact, anti-B[a]PDE–DNA adducts verified by means of HPLC-Fluorescence, are the consequences of the stereo-selective link between B[a]P and the exocyclic N2 of guanine, which is considered to be the crucial step in B[a]P carcinogenicity (Alexandrov et al., 2016). Furthermore, telomeres (as triple-G-holding chains) are a susceptible point for damage by such BPDE genotoxic metabolites. In fact, double-strand breakdowns and interference with the replication fork, which occur with extremely damaged telomeric bases, might directly generate telomere attrition (Oikawa et al., 2001). Furthermore, anti-B[a]PDE–DNA creation and subsequent telomere erosion may be regulated by a reduction in B[a]P detoxification, attributable to the presence of a specific GSTM1 detoxifying polymorphism. The formation of adducts within the proteins of the telomere-protecting complexes (including those regulated by GSTM1), can also be thought of as an extra outcome accounting for shorter LTL. In fact, altered mRNA expression levels of end-binding proteins associated with telomere injury were recently found in individuals with PAH exposure (Duan et al., 2019).

In this study, smoking was found to significantly increase LTL. Several studies, including large surveys such as those conducted by Bischoff et al. (2006) and Cassidy et al. (2010) were unable to substantiate the negative association between LTL and smoking discovered by others (Astuti et al., 2017). These inconsistent results seem to detect a moderate effect of smoking on LTL, if any, which may simply not be measurable. *In-vitro* experiments have shown that telomere length increases in younger inflammatory T cells (Weng et al., 1995) over the course of inflammation, which is a key process in mediating smoking-related health effects (Park et al., 2003). Therefore, the higher LTL in smokers that we discovered in the current study could be ascribed to the recall of younger inflammatory cells from the bone marrow to the blood circulation, in response to inflammatory signals (Weng et al., 1995). Likewise, short-term exposure to PM was associated with a quick rise in blood LTL (Dioni et al., 2011; Hou et al., 2012). Taken together, these observations indicate that short-term smoking exposure may generate a rapid increase in LTL, which may then contribute to sustaining the inflammatory mechanisms related to adverse health effects.

In the current study we also found that LmtDNAcn significantly decreased with anti-B[a]PDE–DNA. As for LTL, PAH exposure appeared to have no direct effect on decreased mtDNAcn but an indirect effect through the mediation of anti-B[a]PDE–DNA. Our results agree with those by Pieters et al. (2013) who found a reduction of LmtDNAcn associated with low chronic exposure to PAHs in house dust during wintertime. The ability of B[a]P to reduce mtDNA content was also validated *in vitro* in human TK6 cells (Pieters et al., 2013). In view of the genotoxic capability of B[a]P, this carcinogen may also have an impact on mtDNA dynamics. The lipophilic nature of B[a]P and its metabolite anti-B[a]PDE, combined with the very high ratio of lipid/DNA in mitochondria, expedites the rate at which these molecules enter mitochondria. Moreover, anti-B[a]PDE possesses 40 to 90-fold higher affinity for mtDNA than for nuclear DNA (Allen and Combs, 1980). Compared with nDNA, mtDNA has fewer protective histones and DNA repair ability, and is therefore extremely vulnerable to DNA injury. Consequently, the role of mtDNA-bound anti-BPDE could be a valuable fraction of the total cellular cargo of DNA adducts. Our results are consistent with a study that reported a significant reduction in LmtDNAcn in the lungs of long-term heavy cigarette smokers. Conversely, our previous study in non-smoking male workers with high exposure to PAHs (>3 μmol 1-pyrenol/mol creatinine) detected significantly higher LmtDNAcn in these subjects, compared with controls (Pavanello et al., 2013). It has been proposed that the oxidative stress, triggered by exposure to PAHs, has a dual impact on mitochondrial DNA content. Indeed, mild stress can promote mitochondrial DNA production and increased mitochondria number in order to sustain the higher respiratory needs of the cell and, as such, maintain cell viability (Lee et al., 2000). However, excessive oxidative stress spawned by tobacco smoke might instead lead to reduced (or no) synthesis of mtDNA, as tobacco smoke contains many toxic, carcinogenic and mutagenic compounds, as well as stable and unstable free radicals and reactive oxygen species (ROS), with the potential for oxidative DNA damage. Such damage may then eventually lead to cell senescence or cell death.

Next to active smoking, the most significant determinants of PAH exposure leading to increased anti-B[a]PDE–DNA, were “diet” and “indoor” exposure, while the least significant was “outdoor” exposure. Interestingly, much attention has been paid to the detrimental effects of outdoor pollution, even though the majority of individuals spend most of their time inside, with indoor air that could be even more contaminated than outdoor air. Indoor air pollution was actually listed as one of the ten leading risk factors for the worldwide burden of disease (Lim et al., 2012). Similarly, Delgado et al. (2005) clearly showed that exposure to wood smoke (without radon, which was the confounding factor in the Chinese studies) is a risk for lung cancer in non-smokers. Our findings show that indoor pollution (mainly due to wood burning) could be a considerable font of

B[a]P intake via inhalation that, by anti-B[a]PDE–DNA adduct formation, affects LTL and LmtDNAcn and thus should be thought of as a potential risk factor for lung cancer. Conversely, less attention has been given to risk analysis of gastrointestinal exposure to PAHs route. One study has shown that human exposure to environmental carcinogens such as PAHs occurs primarily from dietary sources (Bouvard et al., 2015), and epidemiological studies have revealed that a large proportion of human malignances is attributable, at least in part, to nutritional factors (Parkin et al., 2011). Furthermore, a clear relationship between aromatic-DNA adducts and food (e.g., grilled hamburgers) was found in subjects with low professional exposure to PAH, where chargrilled meat intake was verified by a questionnaire (Rothman et al., 1993). In two planned eating studies (involving grilled meat) with a small number of participants (n = 4 and 21), a significant enhancement of DNA adduct level was reported in a few subjects (van Maanen et al., 1994; Kang et al., 1995). These results, together with ours, would imply that PAH intake by means of diet is another crucial source of carcinogenetic exposure to consider, and is detectable by DNA adduct dosimetry in blood leukocytes.

Interestingly, both LTL and LmtDNAcn adduct-related erosion are evident in males, while females had much less. The majority of studies examining differences in LTL between women and men found that women have longer telomeres than men (Gardner et al., 2014). Several plausible biological arguments can be formulated to explain this. These include the action of oestrogens (Mayer et al., 2006), which can stimulate the production of telomerase (Nawrot et al., 2004) and protect against reactive oxygen species damage (Aviv, 2002). Furthermore, the rate of LTL shortening was slower in women than men (Gardner et al., 2014), as detected in cross-sectional and longitudinal studies (Chen e al., 2011). This same mechanism may also result in lower susceptibility to genotoxic damage in women, via anti-B[a]PDE-DNA adducts in the telomeric region. This is in agreement with our observation on LmtDNAcn in a study on 1,088 subjects of European descent, in which higher LmtDNAcn content was detected in women compared with men (Curran et al., 2007; Lee et al., 2014). Our findings are also consistent with previous studies that pointed to sex-specific differences in the effect of air pollution on cord blood LmtDNAcn, with boys being the most highly affected (Pieters et al., 2013; Rosa et al., 2017). On the other hand, some published reports have failed to demonstrate differences in LmtDNAcn content between men and women (Mengel-Form et al., 2014). Taken together, our data therefore supports the concept that males could be more vulnerable to the effect of PAH exposure on LTL and LmtDNAcn.

In our opinion, statistical analysis with SEM is a strength of the present research. In medicine and natural sciences, a given outcome is often affected or influenced by more than one factor simultaneously. Multivariate techniques try to statistically account for these differences,

adjusting an outcome measure Y to a 1-unit change in X, holding all other variables constant. However, it may be that other variables are not likely to remain constant; a change in X can produce a change in Z (direct effect) which in turn produces a change in Y (indirect effect). Both the direct and indirect effects of X on Y must be considered if we want to know what effect a change in X will have on Y. This can be done mathematically and statistically only using SEM. The procedure decomposes a correlation between two variables into their component parts: direct effects, indirect effects, common causes (X affects both Y and Z; this is a spurious association) and correlated causes (X is a cause of Z and X is correlated with Y). The user is required to state, often using a path diagram, the way that they believe the variables are inter-related. Via complex internal rules, SEM decides which model fits the data better. This method is more suitable for the analysis of complex interrelationships because it tests causal relationships rather than mere correlations.

4. Conclusions

This study supports evidence that LTL and LmtDNAcn erosion are critical events that capture everyday life exposure to PAHs within the general population. LTL and LmtDNAcn reduction, which are considered hallmarks of cellular aging, are associated with an increase in mortality rate and different kinds of tumors, including lung cancer (Liu et al., 2017; Raha and Robinson, 2000; Weischer et al., 2013). Therefore, our results suggest that male subjects with high exposure would be more likely to face premature aging and have an earlier onset of age-related diseases. The recent finding that LTL and LmtDNAcn erosion depends on some preventable everyday life exposure (which can in turn be affected by certain genetic factors) opens new perspectives in the prevention of age-related disorders including cancer.

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Conflicts of interest

There are no competing interests.

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Table 1. Main characteristics of subjects: demographic variables (age, sex); putative risk factors (smoking, diet, indoor, home, traffic, outdoor); genetic traits with modulating role (GSTM1, GSTT1), mediation variable (anti-B[a]PDE-DNA); outcomes (telomere length and LmtDNAcn).

FACTORS	STRATA	Number (row %)	Mean ± St Dev
Age (years)			41.90 ± 9.03
Sex	0 = Females	285 (52.6)	
	1 = Males	257 (47.4)	
Smoking [a]	0 = Non- Ex-smokers	432 (79.7)	
	1 = Current smokers	110 (20.3)	
Diet (times/year)			46.8 ± 43.1
Indoor [b]	0 = not exposed	325 (60.2)	0.63 ± 0.87
	1 = low	104 (19.4)	
	2 = medium	96 (17.9)	
	3 = high	14 (2.61)	
Home	0 = Rural	154 (28.4)	
	1 = Urban	388 (71.6)	
Traffic	0 = Scarce / moderate	277 (51.1)	
	1 = Intense	265 (48.9)	
Outdoor [c]	0 = < 4 hours/day	443 (81.7)	
	1 = ≥4 hours/day	99 (18.27)	
GSTM1[f]	0 = (*0/*0)	296 (55.1)	
	1 = *1/*1 and *0/*1	241 (44.9)	
GSTT1	0 = (*0/*0)	97 (18.06)	
	1 = *1/*1 and *0/*1	440 (81.9)	
Anti-B[a]PDE-DNA [d]			1.35 ± 2.87
Telomere length	0 = below 0.896	271 (49.9)	1.00 ± 0.45
	1 = equal /above 0.896	272 (50.1)	
LmtDNAcn			1.13 ± 0.31

[a]Smokers: current cigarette smokers were subjects smoking for at least 1 month before enrolment in the study.
[b]Subjects consuming charcoaled meat or pizza (times/year)
[c] Sum of several factors: presence of fireplace or coal- or wood-stoves at home; hobbies with introduction of PAHs; exposure to passive smoking.
[d] Exposure to outdoor pollution.
[e] A value of 0.125 adducts/108 nucleotides was assigned to subjects with non-detectable adducts. Samples with ≥ 0.5 adducts/108 nucleotides were positive.
[f] The absence of the specific GSTM1 fragment indicated the corresponding null genotype (*0/*0), and its presence corresponded to the *1/*1 and *0/*l genotypes.

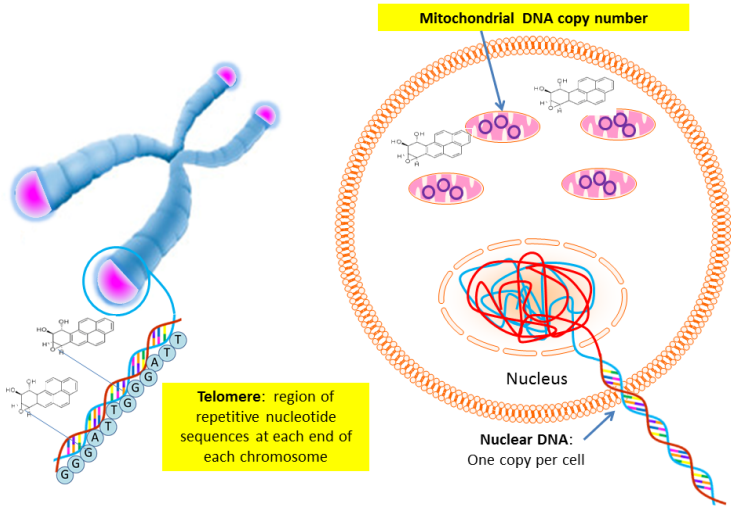


Figure 1. Polycyclic aromatic hydrocarbons decrease leukocytes telomere length and mitochondrial DNA copy number through anti-B[a]PDE-DNA adduct formation.

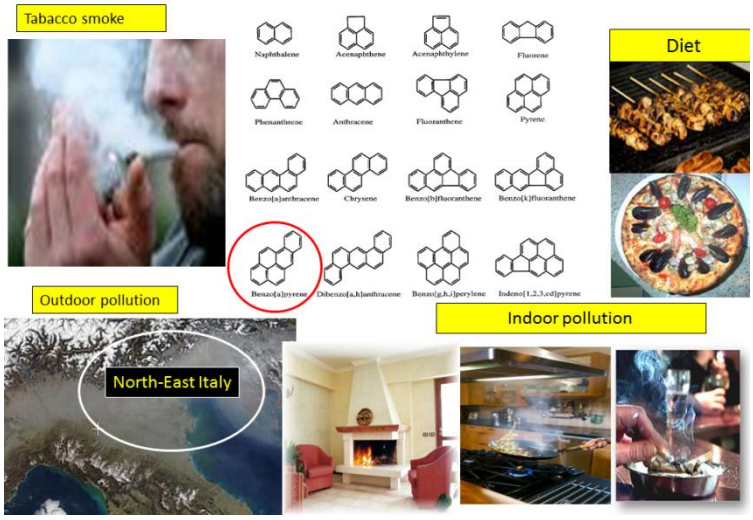
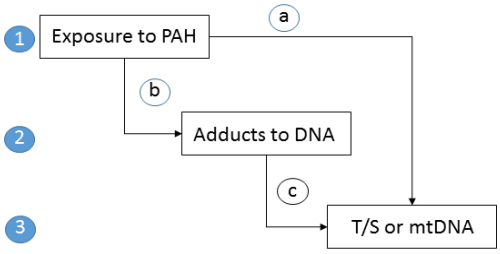


Figure 2. Sources of exposure to polycyclic aromatic hydrocarbons in the every day life exposures.



Model	Explanatory variables	Interpretation
1	Diet, home, traffic, indoor, outdoor exposure as well as age and sex	Overall estimate of exposure to PAH
2	PAH, age, sex, smoking, GSTM1, GSTT1	Effect of exposure on adducts adjusted for confounding
3	DNA adducts, PAH, smoking, age, sex, GSTM1, GSTT1	Effect of adducts on T/S (or mtDNA) adjusted for confounding

Figure 3. Conceptual hierarchical framework and summary of steps in the analysis.

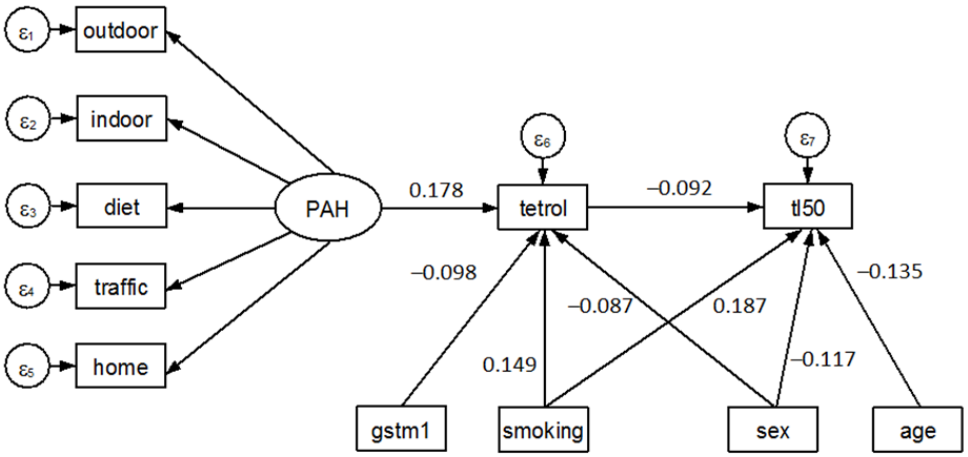


Figure 4. Path diagram of results shown in table 2S: latent variable (oval); observed variables (square boxes); variances (circles); causal flow (arrows); and paths (arrowed route). The estimated beta coefficients appeared along the paths.

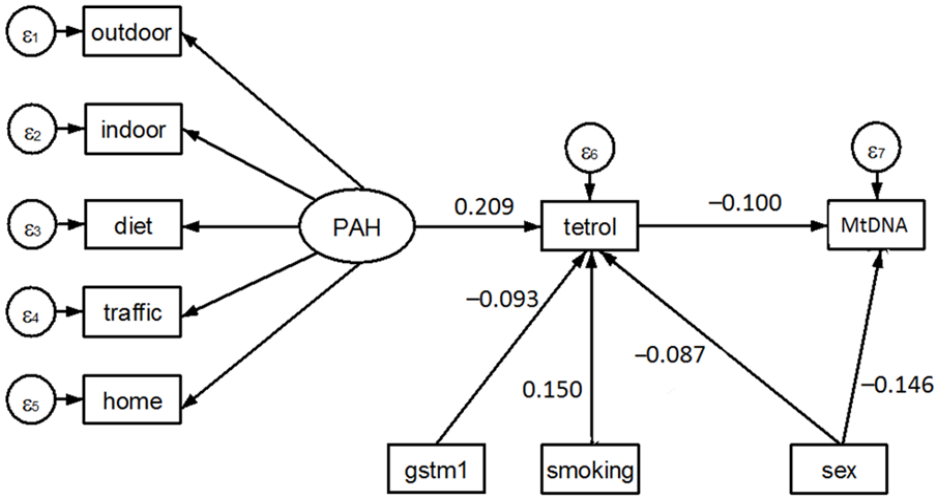


Figure 5. Path diagram of results shown in table 3S: latent variable (oval); observed variables (square boxes); variances (circles); causal flow (arrows); and paths (arrowed route). The estimated beta coefficients appeared along the paths.

Authors' Contributions

Conception and design: S. Pavanello

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Pavanello, M. Campisi, M. Hoxha

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Pavanello, M. Campisi, M. Hoxha, V. Bollati, G. Mastrangelo

Writing, review, and/or revision of the manuscript: S. Pavanello, M. Campisi, M. Hoxha, V. Bollati, G. Mastrangelo

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Pavanello, M. Campisi

Study supervision: S. Pavanello

SUPPLEMENTARY MATERIALS AND METHODS

HPLC/fluorescence analysis of the *anti*-B[*a*]PDE–DNA adduct

Anti-B[*a*]PDE–DNA adduct was detected by HPLC/fluorescence analysis of *anti*-B[*a*]P tetrol (tetrol I-1. See abbreviations) released after acid hydrolysis of DNA samples (Pavanello et al. 2006). Analysis was carried out as previously described (Pavanello et al. 2005) with some minor modifications, mainly regarding the automation of HPLC analysis, to minimize the batch effect, as well as the use of a Perkin Elmer L-7485P fluorimeter to improve sensitivity (see HPLC/fluorescence analysis of anti-B[a]PDE–DNA adduct in the Supplementary Materials). Briefly, about 100 μg of leukocyte DNA was dissolved in 0.1N HCl, and acid hydrolysis was carried out at 90 °C for 4 h. The equipment used included a Waters 717 Autosampler (Millipore Waters. Italy) coupled to a Waters Millipore automated gradient controller, plus a Waters 600 Multisolvent Delivery pump, and a model 510 solvent-delivery system. B[*a*]P-tetrol-I-1 was determined by comparison with a standard curve generated from the fluorescence areas of an authentic B[*a*]P-tetrol-I-1 standard (NCI Chemical Carcinogen Reference Standard Repository. Kansas City, MO, USA), analyzed in triplicate during the analysis of each set of samples. In total, eight batches each containing 75 samples were examined. The minimum correlation coefficient was 0.998 and the mean coefficient of variation (CV) for analyses repeated on different days was 10%. The highest CV value was 5.70% for those samples ($n = 8$) with more than 200 μg DNA repeated twice. In the present study, the detection threshold of B[*a*]P-tetrol-I-1 was 0.25 pg (signal/noise >3), i.e. this assay can measure 0.25 adducts/10^8 nucleotides in 100 μg DNA (1 fmol/μg DNA = 30 adducts/10^8 nucleotides).Samples with non-detectable DNA adducts were given a value of one-half the limit of detection of the method (LOD/2 = 0.125). Adduct levels were treated in the analyses both continuously and categorically (positive or non-detectable). Positive subjects were those with adduct levels of ≥0.5 adducts/10^8 nucleotides.

Leukocyte Telomere Length (LTL)

LTL was measured by using quantitative Real-Time PCR as previously described (Pavanello et al. 2018). This assay measures relative LTL 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 50 participants who were randomly selected from the study population

(500 ng for each sample). A fresh standard curve, from the pool of control samples, ranging from 30 to 0.23 ng/µl (serial dilutions 1:2), was included in every "T" and "S" PCR run, against a negative control (water). In total, 9 ng of DNA sample was added to each reaction, and each sample was run in triplicate. In brief, a high-precision MICROLAB STARlet Robot (Hamilton Life Science Robotics, Bonaduz AG, Switzerland) was used for transferring 7 µl of reaction mix and 3 µl of DNA (3 ng/µl) in a 384-well plate as previously described (Bollati et al. 2014; Pergoli et al. 2017). All PCR reactions were performed on a 7900HT Fast Real Time PCR System (Applied Biosystems). A primer pair for a beta-globin single copy gene (hbgu and hbgd) (Pavanello et al. 2010) as well as a telomere primer pair (telg and telc) as described in (Cawthon 2009) were used in the reaction mix. The thermal cycling profile for both amplicons began with 50°C for 2 min followed by incubation at 95°C for 2 min to activate the AmpliTaq DNA polymerase. For telomere PCR, activation was followed by 2 cycles of 15 sec at 95°C, 15 sec at 49°C, and 35 cycles of 15 sec at 95°C, which was then followed by 10 sec at 62°C, and 15 sec at 74°C. For hbg, activation was followed by 35 cycles of 15 sec at 95°C and 1 min at 58°C. At the end of each real-time PCR reaction, a melting curve was added for both T and S PCRs to verify the specificity of amplification. 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 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. LTL was treated in the analyses both as categorical tl50 (higher or lower than median: 0 = below 0.896; 1 = equal/above 0.896) or continuous variable.

Leukocyte mtDNAcn (LmtDNAcn)

LmtDNAcn was measured in the same DNA of LTL analysis using real-time quantitative PCR (qRT-PCR) as previously described (Pavanello et al. 2013). This assay measures relative mtDNAcn by determining the ratio of mitochondrial (MT) copy number to single copy gene (S) copy number in experimental samples relative to the MT/S ratio of a reference pooled sample. In brief, this method is based on quantification of MT and S quantities expressed as a cycle threshold (Ct; i.e. the number of cycles required for the fluorescent signal to cross the threshold) which is derived from a standard curve that is obtained from serial dilutions of reference DNA. The single-copy gene used in this study was human (beta) globin (hbg). The Mt PCR mix was composed of iQ SYBR Green Supermix (Bio-Rad) 1×, MtF3212 500 nmol/L, and MtR3319 500 nmol/L. The S (hbg) PCR mix was iQ SYBR Green Supermix (Bio-Rad) 1×, hbgF 500 nmol/L, and hbgR 500 nmol/L. In total 9 ng DNA

was loaded in a 10 μL PCR reaction. As a reference sample, we used the same randomly selected pooled DNA from 50 participants (from the current study) that was used for telomere analysis (500 ng from each sample). This was used to create a fresh standard curve, which ranged from 30 ng/μL to 0.23ng/μL, for every MT and S PCR run. The primers for qRT-PCR analysis of mtDNAcn and hbg were as previously described (Pavanello et al. 2013). All PCRs were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems). The thermal cycling conditions for mtDNAcn PCR were 3 min at 98°C to activate the hot-start iTaq DNA polymerase, followed by 35 cycles comprised of 15 sec denaturation at 95°C and 60 sec anneal/extend at 60°C. The thermal cycling conditions for the hbg PCR were the same as described above for LTL analysis: 3 min at 98°C to activate the hot-start iTaq DNA polymerase, followed by 35 cycles of 15 sec denaturation at 95°C and 60 sec anneal/extend at 58°C. Each run was completed by melting curve analysis to confirm the amplification specificity and absence of primer dimers. All samples were run in triplicate on a 384-well plate. The average of the three MT measurements was divided by the average of the three S measurements to calculate the MT/S ratio for each sample. The CV for the MT/S ratio in duplicate samples analyzed on two different days was 6%. LmtDNAcn was treated as a continuous data variable in the statistical analysis.

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Supplementary Tables

Table 1S. Spearman's rank coefficients and significance level for pairwise correlation of the different environmental exposures to PAHs (see Methods for definitions) as well as age and sex.

	Diet	Indoor	Traffic	Home	Outdoor	Age
Indoor	0.18					
	<0.001					
Traffic	–0.11	–0.09				
	0.008	**0.037**				
Home	–0.13	–0.28	0.25			
	0.003	**<0.001**	**<0.001**			
Outdoor	0.07	0.11	–0.01	0.03		
	0.088	**0.013**	0.824	0.498		
Age	–0.24	–0.05	0.13	0.14	0.03	
	<0.001	0.233	**0.003**	**0.002**	0.451	
Sex	0.09	0.08	–0.06	0.01	0.35	0.23
	0.035	0.060	0.202	0.864	**<0.001**	**<0.001**

Table 2S. SEM results of four groups (structural equations, measurement, variances and covariances) for the analysis of tl50. Standardized beta coefficients are shown (with the "minus" sign indicating an inverse relationship) with a lower and upper limit of 95% confidence intervals (95%CI) and p-values. The goodness-of-fit statistics of the SEM results are at the bottom of the table.

	Endogenous variables	Exogenous variables	Beta Coef.	95%CI		p-value
				Lower	Upper	
Structural Equations	anti-B[a]PDE–DNA	PAH	0.178	0.005	0.055	0.005
		smoking_gp	0.149	0.000	0.066	0.0001
		gstm1	–0.098	–0.021	–0.182	0.021
		sex	–0.087	0.057	–0.177	0.057
	$T/S_{50\ percent}$	anti-B[a]PDE–DNA	–0.092	–0.175	–0.010	0.028
		smoking_gp	0.187	0.105	0.268	0.0001
		age	–0.135	–0.218	–0.053	0.001
		sex	–0.117	–0.201	–0.033	0.006
Measurement	Diet ← PAH		0.439	0.317	0.561	0.0001
	Indoor ← PAH		0.436	0.311	0.561	0.0001
	Home ← PAH		–0.433	–0.573	–0.292	0.0001
	Traffic ← PAH		–0.309	–0.429	–0.189	0.0001
	Outdoor ← PAH		0.212	0.071	0.354	0.003
Errors	ε1 = var(e.diet)		0.807	0.707	0.921	
	ε2 = var(e.indoor)		0.810	0.708	0.927	
	ε3 = var(e.home)		0.813	0.700	0.944	
	ε4 = var(e.traffic)		0.904	0.833	0.982	
	ε5 = var(e.outdoor)		0.955	0.897	1.017	
	ε6=var(e.anti-B[a]PDE–DNA)		0.931	0.882	0.983	
	ε7 = var(e.T/S)		0.932	0.893	0.973	
	ε8 = var(PAH)		1	.	.	
Covariances	cov(sex. PAH)		0.230	0.082	0.378	0.002
	cov(age. PAH)		–0.318	–0.439	–0.197	0.0001

Goodness of fit statistics:

Likelihood ratio (LR) test of model vs. saturated: chi-square(28) = 139.400 ($p < 0.0001$)

Standardized root mean squared residual (SRMR) = 0.055

Coefficient of determination (CD) = 0.524

Table 3S. SEM results of four groups (structural equations, measurement, variances and covariances) for analysis of LmtDNAcn. Standardized beta coefficients are shown (with a "minus" sign indicating an inverse relationship) with a lower and upper limit of 95% confidence intervals (95%CI) and p-values. The goodness-of-fit statistics of the SEM results are shown at the bottom of the table.

	Endogenous variables	Exogenous variables	Beta Coef.	95%CI		p-value
				Lower	Upper	
Structural Equations	anti-B[a]PDE–DNA	sex	–0.087	–0.177	0.002	0.056
		smoking	0.150	0.067	0.233	0.0001
		gstm1	–0.093	–0.176	–0.009	0.030
		PAH	0.209	0.083	0.336	0.001
	Mt_DNA	anti-B[a]PDE–DNA	–0.100	–0.183	–0.017	0.018
		Sex	–0.146	–0.228	–0.064	0.0001
Measurement	Diet <- PAH		0.334	0.216	0.452	0.0001
	Indoor <- PAH		0.547	0.417	0.676	0.0001
	Home <- PAH		–0.469	–0.609	–0.329	0.0001
	Traffic <- PAH		–0.281	–0.409	–0.152	0.0001
	Outdoor <- PAH		0.187	0.045	0.329	0.010
Variances	ε1 = var(e.diet)		0.889	0.813	0.971	
	ε2 = var(e.indoor)		0.701	0.573	0.858	
	ε3 = var(e.home)		0.780	0.659	0.923	
	ε4 = var(e.traffic)		0.921	0.852	0.996	
	ε5 = var(e.outdoor)		0.965	0.914	1.019	
	ε6 = var(e. anti-B[a]PDE–DNA)		0.919	0.863	0.979	
	ε7 = var(e. LmtDNAcn)		0.969	0.941	0.998	
	ε8 = var(PAH)		1	.	.	
Covariances	cov(sex. PAH)		0.194	0.052	0.335	0.007

Goodness of fit statistics:

Likelihood ratio test of model vs. saturated: chi-squared (28) = 116.2, p <0.0001.

Standardized root mean squared residual (SRMR) = 0.055

Coefficient of determination (CD) = 0.529

TELOMERE LENGTH IN TRELONG STUDY POPULATION

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Introduction

The growing aging of the population, also called the "gray" revolution, is an emerging social and public health problem. The impact of the phenomenon is preannounced by alarming dimensions in Italy, amongst the oldest country of the world, especially the Northeast Veneto region, where the area of Treviso is characterized by the highest longevity, especially among women [National Institute of Statistics: www.istat.it; Caselli and Lipsi, 2002]. The reasons are poorly understood.

Aging is an individual and very complex process characterized by a progressive decline in the body's ability to respond to internal and/or external stressors, with consequent increase in the risk of illness and death [López-Otín et al., 2013]. This deterioration is the primary risk factor for major human pathologies [López-Otín et al., 2013]. However, there is great dissimilarity in aging trajectories and health outcomes. In fact, not all individuals grow older in the same way [Fulopet al., 2010], consequently chronological age may not be a reliable indicator of physiological decline [Levine 2013]. Quantifying the aging process, defining measurable estimates of 'biological aging' has become a major initiative in Geroscience Research [Kennedy et al., 2014].

Several key themes have been gleaned from the overlap observed in aging studies of model organisms, genetic epidemiology, and behavioral/pharmaceutical interventions. From these, two interconnected 'pillars of aging' have been proposed as the most promising early targets for aging and longevity research [Kennedy et al., 2014], i.e. telomeres shortening and epigenetic alterations in DNA. Telomere length and DNA methylation age (DNAmAge) - i.e. biological age-estimation based on the age-dependent methylation changes in certain CpG loci- are considered early hallmarks and the two most prominent markers of biological aging [Jylhava et al., 2017; Marioni et al., 2018]. Telomeres are hexameric nucleotide (TTAGGG) n-protein repeats that are critical in maintaining the genome stability of the cells [Blackburn et al., 2006]. Telomeric repeats in normal somatic tissue shorten by 30 to 200 bp after each mitotic division eroding chromosomal terminations. Hence, telomeres progressively shorten with each division of somatic cells, and their length, measured in peripheral blood leukocytes, is considered an indicator of biological aging

[Frenk et al., 1998; Mather et al., 2011]. However, telomeres, as triple-G- containing sequences, represent a sensitive target for damage by genotoxic compounds, cumulative oxidative and inflammatory stress that directly accelerate telomere shortening. Leukocyte telomere length (LTL) is a systemic index of aging [Pavanello et al., 2010; 2011] and age-related diseases [Sanders and Newman, 2013]. Interestingly, telomere length has also been proposed as a marker of prediction for health span and longevity [Bar and Blasco, 2016], in particular in elderly [Cawthon et al., 2003; Deelen et al., 2014; Lapham et al., 2015]. Longevity is a complex process resulting from genetic and environmental factors, as well as their interaction. The comparison among biomarkers of biological aging, environmental and occupational exposures, health status and other characteristics of the elderly people can help in understanding this phenomenon.

Aim of the Study

This study is part of a large research project with the purpose to appraise prospectively LTL and DNAmAge, in relation with the high longevity that characterizes this population taking into consideration demographic and health data, lifestyle, environmental and occupational exposures, clinical and chemistry biomarkers that could explicate the discrepancy between biological and chronological age.
In the present work we prospectively analyze LTL in TRELONG population at three different times: at baseline (T0), after 7-years (T1) and after 10-years (T2) follow up.

Materials and Methods

Study design

The Treviso Longeva (TRELONG) study is a prospective cohort study of women and men lived in the area of Treviso, which is characterized by the highest longevity in Nord-East Italy. The TRELONG Study started in 2003 with the recruitment of the resident population in the municipality of Treviso among the 13,861 (17%) adults older than 70 years. A total of 668 were selected (mean age 84 ± 8 years, range 70-105.5 years, 311 men and 357 women) as described elsewhere [Gallucci et al., 2007]. The TRELONG population was studied prospectively at three different times (each subject is self-control): at baseline (T0), after 7-years (T1) and after 10-years (T2). An interviewer-administered questionnaire clinical, lifestyle and demographic data and a blood sample were collected at participants' homes [Gallucci et al., 2007; Albani et al., 2014]. A list of the TRELONG variables is described in Table 1. The study was approved by the ethical committee of the National Institute on Research and Care of the Elderly (INRCA, Italy). The protocol included a written

informed consent for clinical and genetic studies. The population for which the extracted DNA was still available for the analyses of LTL consists of 576 subjects at T0, 300 and 200 subjects at T1 and T2, respectively. All samples of genomic DNA available were sent at our Laboratory of Environmental Mutagenesis and Genomic - Occupational Medicine, Department of Cardiac, Thoracic, and Vascular Sciences and Public Health, University Hospital of Padua- for the analyses of LTL, with a file reporting all information regarding qualitative (260/280 nm) and quantitative (ng/µl) description and volume of each sample (µl).

LTL analysis

LTL was determined in genomic DNA previously extracted from the whole blood samples at early stage during recruiting (T0) and after a 7-years (T1) and 10-years (T2) follow up. TL was appraised by the real-time quantitative polymerase chain reaction (PCR) method established by Cawthon [2009] as previously developed [Pavanello et al. 2017; 2018]. In brief, this method measures the relative LTL by estimating in genomic DNA the proportion of telomere repeat copy number (T) in relation to a single copy gene (S) (T:S fraction) as previously described [Pavanello et al., 2017; 2018]. The single copy gene employed in this investigation was the human β-globin (hbg) [Pavanello et al., 2010]. A "six- point" reference curve was built from a serially diluted DNA pool, varying from 15 to 0.47 ng in each plate, in order to calculate the relative quantities of T and S in ng of samples to be examined. The DNA pool was realized by taking an aliquot of genomic DNA from samples examined in the present study at random designated and derived from baseline collection (T0), and consecutive collection after 7-years (T1) and 10-years (T2) follow up. All samples and standards were analysed in triplicate and the average of the 3 T/S ratio measurements was considered in the statistical analyses. 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. All PCR reactions were performed on a SteponePlus Real-Time PCR System (Applied Biosystems). A primer pair for a beta-globin single copy gene (hbgu and hbgd) [Pavanello et al., 2010] as well as a telomere primer pair (telg and telc) as described in Cawthon [2009] were used in the reaction mix. The thermal cycling profile for both amplicons began with 50°C for 2 min followed by incubation at 95°C for 2 min to activate the AmpliTaq DNA polymerase. For telomere PCR, activation was followed by 2 cycles of 15 sec at 95°C, 15 sec at 49°C, and 35 cycles of 15 sec at 95°C, which was then followed by 10 sec at 62°C, and 15 sec at 74°C. For hbg, activation was followed by 35 cycles of 15 sec at 95°C and 1 min at 58°C. LTL was analysed in triplicate for all samples of genomic DNA available (n=576 subjects at T0, n=301 and n=202 subjects at T1 and T2, respectively) and replicated in different days. 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 among triplicate measures was <0.25. The coefficient of variation for the average T:S ratio of samples analysed over three consecutive days was 10%, which was similar to the reproducibility originally reported for this method.

Statistical analysis

Statistical analyses were performed on data collected for each subject enrolled in the study. Univariate and multivariate methods were used selecting the appropriate models. The analyses were performed using the statistical software StastDirects.

Results and Discussion

We prospectively determined LTL in the TRELONG population. LTL was analyzed in n=576 subjects at T0, n=301 and n=202 subjects at T1 and T2, respectively. Our results show a decline in LTL from baseline [mean 2.35±1.14 (T/S), T0] to follow up [mean 1.60±0.95 (T/S), T1; mean 0.83±0.32 (T/S), T2; $p<0.0001$] in all subjects, as reported in Figure 1, and also in only n=161 subjects who have LTL measurements at all three time points ($p<0.0001$, Figure 2). Furthermore, Figure 3 displays the average trajectories of LTL stratified by age over the three different time-points, with younger subjects who have longer telomeres than older. These results are in accordance with previous longitudinal studies on telomere dynamics that reported a decrease in LTL with increasing age [Barrett et al., 2013; Müezzinler et al., 2013].

Future Directions

Statistical analyses are currently underway to investigate how LTL correlates with information regarding demographic data, prognosis, lifestyle and therapy collected (Table 1), in order to:

1) establish the determinants of LTL at T0 in the TRELONG population characterized by the high longevity
2) evaluate whether LTL determined at T0 (2003) is predictive of longevity/mortality in the TRELONG population.

Authors' Contributions

Conceived and designed the study: MC, SP and MG. Performed the samples' analysis: MC and SP. Provided samples: MG. Analyzed the data: SP, MC and MG. Wrote the paper: SP and MC.

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Conflict of interest

The authors declare no conflict of interest.

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Table 1. TRELONG variables.

TRELONG VARIABLES	
1) Personal information and cognitive impairment (MMSE)	a) date of birth b) gender c) first menstruation at ..... years old d) menopause at ..... years old e) Does he/she live in Nursing Home? f) literacy g) Mini Mental State Examination (MMSE)
2) Marital status, education and occupation	a) marital status b) qualification c) education in years d) what job have you done mainly during the life?
3) Family and social network	a) family structure and social relations
4) Lifestyle	reading, sports, walking, gardening, friends, volunteer activities, etc
5) Risk factors	a) family history of chronic diseases b) smoking c) alcohol intake

6) Chronic diseases (also DCI and CCI)	a) medical history with a detailed list of chronic diseases that arose before or after the date of the previous interview with particular reference to those included in the construction of the two indices Disease Count Index (CCI) and Charlson Comorbidity Index (CCI)
7) General physical examination, sight, hearing	a) pulsations per minute b) blood pressure c) sight d) hearing e) right or left handed
8) Physical performance tests also for the calculation of SPPB:	a) Balance b) Walking speed test c) Chair stand up test d) SPPB e) Lifting weight Kg. 2 f) Strength (hand-grip) g) Arms mobility (1. hands behind neck and 2. behind shoulders)
9) Nutrition	a) medical history with a detailed list of chronic diseases that arose before or after the date of the previous interview with particular reference to those included in the construction of the two indices Disease Count Index (CCI) and Charlson Comorbidity Index (CCI)
10) Disability	a) Activities of daily living (ADL) b) Instrumental activities of daily living (IADL)
11) Depression	a) Geriatric Depression Scale (GDS)
12) Multidimensional Prognostic Index (MPI) with its eight domains:	a) Activities of Daily Living (ADL) b) Instrumental ADL (IADL) c) Short Portable Mental Status Questionnaire (SPMSQ) d) Comorbidity Index (CIRS-CI) e) Mini Nutritional Assessment (MNA) f) Exton Smith Scale (ESS) (risk of bed sores) g) Number of medications (drugs) h) Social support network
13) Economic aspects	a) economic situation over the past five years b) activities carried out after retirement
14) Home care	
15) Exposure to environmental pollution (collected only in 2013 follow up)	a) Past occupation. Have you had the following occupations in the past? If yes, how long? b) Diet of high-PAH content food: How often do you eat the following type of foods? If you eat those foods, please note the type and amount of the food of intake c) Time-activity in microenvironments: How much time (in minutes) per day (on a typical day) you spend in each location? d) Residence characteristics
16) Blood chemistry variables	a) Blood glucose (mg/dl) b) Serum albumin (g/dl) c) Total cholesterol (mg/dl) d) LDL cholesterol (mg/dl) e) HDL Cholesterol (mg/dl) f) Triglycerides (mg/dl) g) Fibrinogen (mg/dl) h) Creatinine (mg/dl) i) Hemoglobin (g/dl) j) Platelets (n x 103/mm3) k) White cells (n x 103/mm3) l) Uric acid (mg/dl) m) Erythrocyte sedimentation rate (ESR) (mm/hr) n) C-reactive protein (CRP) (mg/dl)
17) Blood samples for serum and genetic analysis	
18) Survival data	

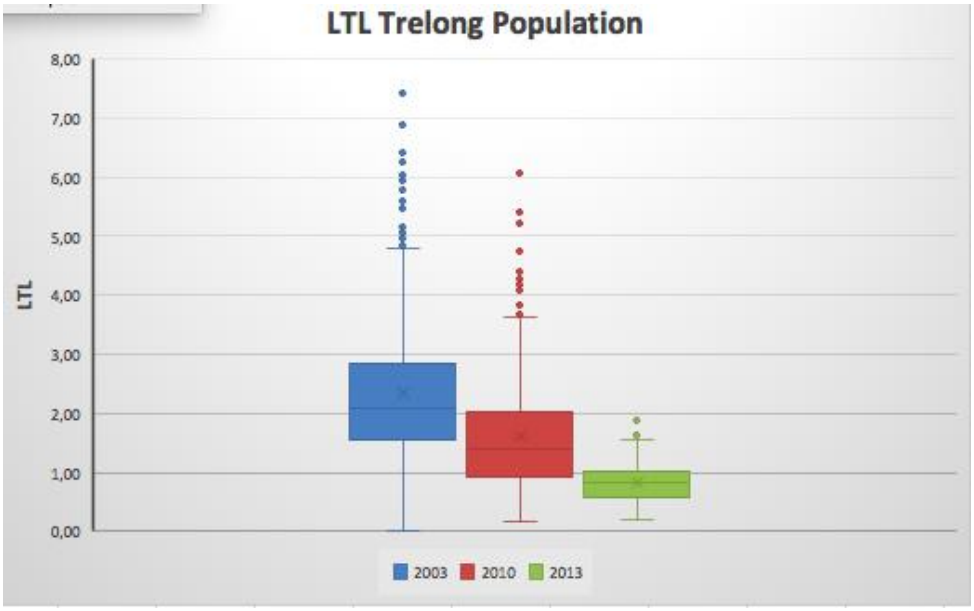

Figure 1. LTL of TRELONG population at baseline (T0), ater a 7-years (T1) and a 10-years (T2) follow up.

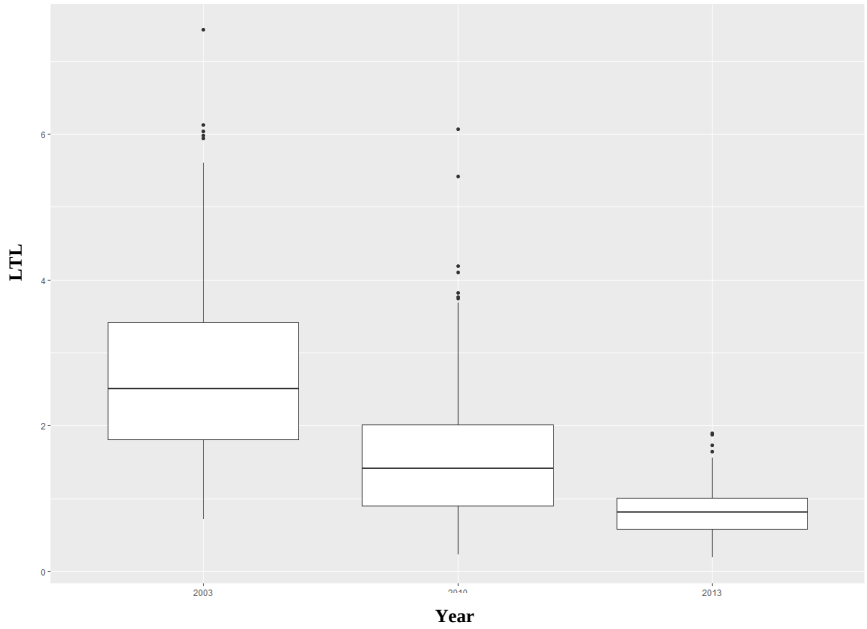

Figure 2. LTL of n=161 subjects whit measurements at baseline (*T0*), after a 7-years (*T1*) and a 10-years (*T2*) follow up.

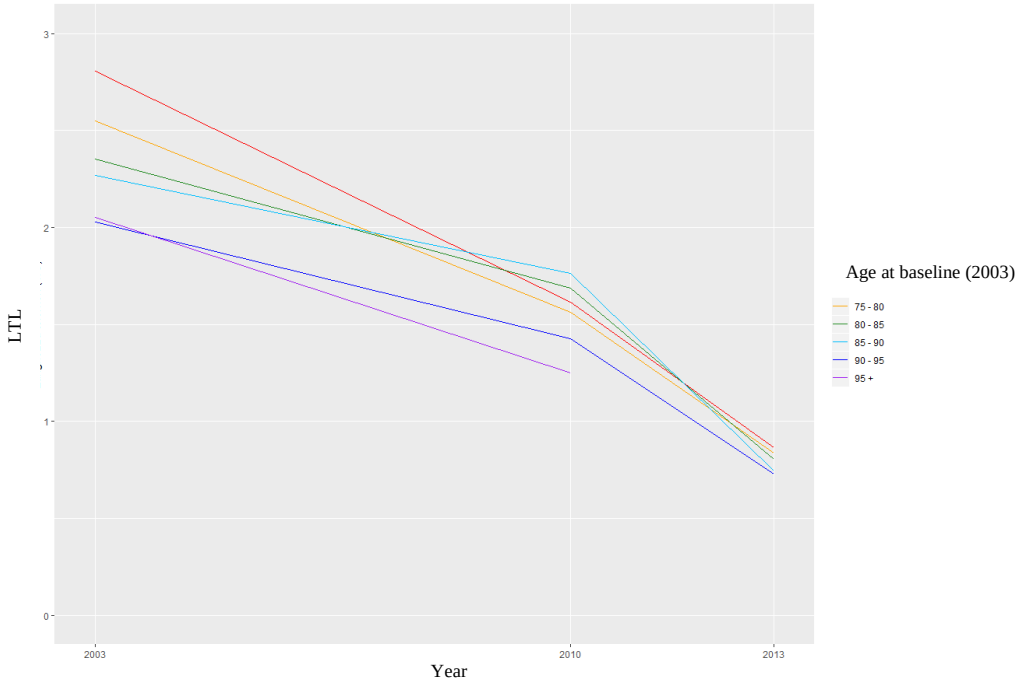

Figure 3. Average trajectories of LTL measurements stratified for age over the years.

Chapter 3. BIOLOGICAL AGING IN SUBJECTS AFFECTED BY AGE-RELATE DISEASES

RELATIONSHIP BETWEEN TELOMERE LENGTH, GENETIC TRAITS AND ENVIRONMENTAL/OCCUPATIONAL EXPOSURES IN BLADDER CANCER RISK BY STRUCTURAL EQUATION MODELLING.

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Article

Relationship between Telomere Length, Genetic Traits and Environmental/Occupational Exposures in Bladder Cancer Risk by Structural Equation Modelling

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Abstract: *Background*: Telomere length (TL) maintenance plays an important role in bladder cancer (BC) and prognosis. However the manifold influence of everyday life exposures and genetic traits on leucocyte TL (LTL), is not fully elucidated. *Methods*: Within the framework of a hospital-based case (n = 96)/control (n = 94) study (all Caucasian males), we investigated the extent to which LTL and BC risk were modulated by genetic polymorphisms and environmental and occupational exposures. Data on lifetime smoking, alcohol and coffee drinking, dietary habits and occupational exposures, pointing to aromatic amines (AAs) and polycyclic aromatic hydrocarbons (PAHs) were collected. Structural equation modelling (SEM) analysis appraised this complex relationships. *Results*: The SEM analysis indicates negative direct links ($p < 0.05$) between LTL with age, DNA adducts, alcohol and NAT2, and positive ones with coffee, MPO and XRCC3; and between BC risk ($p < 0.01$) with cigarettes, cumulative exposure to AAs and coffee, while are negative with LTL and age. There was evidence of indirect effects ($p < 0.05$) on BC risk, probably via LTL reduction, by age and NAT2 (positive link), MPO and XRCC3 (negative link). Conclusions: Our study supports evidence that LTL attrition is a critical event in BC. The new finding that LTL erosion depends on some preventable everyday life exposures genetically modulated, opens new perspectives in BC prevention.

Keywords: telomere length; environmental exposures; occupational exposures; DNA adduct; bladder carcinogenesis; cancer prevention; genetic polymorphisms; structural equation modelling; case-control study

1. Introduction

Dysfunction in telomere maintenance plays an important role in human carcinogenesis; however, the complex relationship between telomere protection and the risk of developing bladder cancer (BC) has not been thoroughly studied. Telomeres are hexameric nucleotide (TTAGGG) n-protein repeats on the distal ends of eukaryotic chromosomes that are critical in maintaining the genome

stability of the cells [1]. Telomeric repeats in normal somatic tissue shorten by 30 to 200 bp after each mitotic division eroding chromosomal terminations [2]. A specific enzyme, telomerase is involved in telomere synthesis after mitosis, but is active only in progenitor cells and in certain diseases [3]. Telomeres, therefore, progressively shorten with each division of somatic cells, and their length, measured in peripheral blood leukocytes (LTL), is considered an indicator of biological age [4]. Continuing attrition of telomeres causes genetic instability that can favour, over time, the typical diseases associated with aging, including cancer [3]. Telomere shortening is a function of oxidative stress and antioxidant defences [4]. Environmental (e.g., cigarette smoking) and occupational exposure to bladder carcinogens, directly, by damaging DNA, and indirectly, by favouring the cumulative effect of oxidative damages and the onset of chronic inflammation, might accelerate the physiological process of telomere erosion and, consequently, facilitate the onset of chronic degenerative pathologies, cancer included [5]. Furthermore, it has been documented an association between leukocyte telomere length (LTL) reduction and some known risk factors for BC, such as exposure to nitrosamines [6,7] and polycyclic aromatic hydrocarbons (PAHs) [8,9]. Some retrospective case-control studies associated shorter LTL in patients with BC [7,10,11]. Long LTL was instead strongly associated with an increased BC risk in Egyptians [12]. These conflicting results are hard to be explained from the biological viewpoint. Moreover, no study apparently estimated the complex interactions between LTL, multiple genetic polymorphisms, occupational and everyday life exposure to aromatic amines (AAs) and PAHs, even evaluated by DNA adduct estimation and BC risk.

We previously assessed the interaction between occupational and environmental exposures with metabolic and DNA-repair polymorphisms on the risk of BC in a retrospective hospital based case-control study [13–16]. BC that is the fourth most common cancer in men and the 11th one in women, is a typical example of multifactorial disease, whose etiology is characterized by the interaction between environmental/occupational and genetic risk factors [17,18]. Tobacco smoking and occupational exposure are major risk factors attributable (about 50% and 4–20%, respectively) to BC via exposure to AAs and PAHs, which lead to increase oxidative stress and DNA damage [13]. In particular, occupational exposure to AAs (e.g., dyestuffs industry and rubber textile and printing) and to PAHs (e.g., metallurgy and metalworking sectors, drivers and exposed to diesel emissions) has long time been associated with an increased incidence and mortality for BC [18,19]. On the other hand, increasing evidence suggests a significant influence of genetic predisposition, including that related to metabolism and DNA damaging repair, on BC incidence [13–15] accounting for almost 20% of the BC risk. In particular, the application of genome-wide analyses (GWAS), beside confirming the involvement of genetic variants linked to detoxification pathways of bladder carcinogens (NAT2, UGT1A, GSTM1), has led to the identification of a number of variants, among which the most accredited are those involved in the telomere's biology, conferring an increased BC risk [20].

The aim of the present study is twofold: to investigate the extent to which LTL and BC risk were modulated by genetic polymorphisms, and environmental/occupational exposures, where exposure was also evaluated by leucocytes DNA adducts analysis; to explore whether LTL involved an additional increase in BC risk. These complex interrelationships have been appraised using the analysis of structural equation modelling (SEM).

2. Materials and Methods

2.1. Subjects

The present study includes study population stemming from an earlier hospital-based case-control study fully described in previous publications [13–15]. Inclusion criteria were being male, aged 20–80 and Italian. In all the cases involved 199 newly diagnosed, histologically confirmed BC patients, admitted to the urology departments of two large hospitals from 1997 to 2000. Controls were a total of 214 non-neoplastic urological patients matched to cases by age (±5 years), period and hospital of admission. Exposure to occupational and environmental carcinogens was evaluated by a standardized

questionnaire (cumulative exposure to AA and PAHs) and by level of DNA adducts in leucocytes (dose to the target). A written informed consent was obtained from each subject; the local Ethical Committee approved the study (protocol number 2859/9185, 4 September 1996). In the present study, we had considered 96 cases and 94 controls for which was still available a sufficient DNA aliquot for the analysis of telomeres.

2.1.1. Data Collection

A trained interviewer collected information on demographic variables, lifetime smoking history, coffee and other liquid consumption, dietary habits, lifetime occupation history by questionnaire. Occupational exposures to PAHs and AAs were estimated according to methodology described in previous publication [13–15]. An index of cumulative exposure to AAs and PAHs, separately, was calculated as product ($i \times f \times l$) of length (l), intensity (i) and frequency (f) of exposure in each job, summing up as many products as were necessary to take into account all jobs done. Life-long consumption of cigarettes was calculated as pack-years. The lifelong time-weighted average of cups/day of coffee was recoded as 0 (never drinkers), $\leq$3, 4, $\geq$5 cups/day. PAHs containing food, fruit, large leaf vegetables and other vegetables consumption was divided into four categories (less than once/month; less than once/week; 1–3 times/week; more than 3 times/week). Job titles and individual activities, as well as occupational exposures to AAs, were blindly coded by an occupational physician according to methodology described in previous publication [13–15]. Occupations involving exposure to AAs were attributed to 11 International Standard Classification of Occupations codes for job tasks (1-61.30: Painter, Artist; 3-70.20: Mail Sorting Clerk; 5-70.30: Barber-Hairdresser; 7-41.40: Mixing- and Blending-Machine Operator, Chemical and Related Processes; 8-01.10: Shoemaker, General; 8-11.20: Cabinetmaker; 8-73.70: Vehicle Sheet-Metal Worker; 9-01.35: Rubber Moulding-Press Operator; 9-31.20: Building Painter; 9-39.20: Brush-Painter, except Construction; 9-39.30: Spray-Painter, except Construction) and 11 International Standard Industrial Classification of all Economic Activities codes for industrial activities (3240: Manufacture of footwear, except vulcanized or moulded rubber or plastic footwear; 3320: Manufacture of furniture and fixtures, except primarily of metal; 3521: Manufacture of paints, varnishes and lacquers; 3559: Manufacture of rubber products not elsewhere classified; 3819: Manufacture of fabricated metal products except machinery and equipment not elsewhere classified; 3824: Manufacture of special industrial machinery and equipment except metal- and wood-working machinery; 3843: Manufacture of motor vehicles; 5000: Construction; 9415: Authors, music composers and other independent artists not elsewhere classified; 9513: Repair of motor vehicles and motorcycles; 9591: Barber and beauty shops).

2.1.2. Analysis on DNA from Peripheral Blood Leucocytes

Blood samples were collected from all the subjects during hospital admission and on the same day processed by centrifugation for obtaining peripheral blood leucocytes. The protocol for automated DNA extraction was performed according to Extragen kit (Extragen, by ELITech Group S.p.A., Torino, Italy) following the manufacturer's instructions as previously described [13–16]. In particular 2.5 mL of buffy coats prepared from up to 10 mL of whole blood were processed for DNA extractions. A typical yield ranged from 150 to 400 μg DNA/extraction from a normal donor.

2.1.3. 32P-Post-Labeling Analysis of DNA Adducts

Aliquots of 5 μg DNA were assayed for the presence of aromatic-DNA adducts by 32P-postlabeling after enrichment with Nuclease P1 as previously described [21]. Resolution of DNA adducts was performed by multidirectional thin-layer chromatography (TLC), using polyethyleneimine (PEI)-cellulose plates [21]. DNA were enzymatically digested to 3′-mononucleotides with 0.14 U/μg DNA of micrococcal nuclease and 1 mU/μg DNA of spleen phosphodiesterase for 3–4 h at 37 °C. After the enrichment procedure by Nuclease P1 digestion, DNA bases were labelled with 50 μCi of [gamma-32P] ATP with a specific activity of 5000 Ci per mmol

by using 2.5 units of T4 polynucleotide kinase. 20 µL of postlabeled sample were spotted on the origin of a premarked PEI cellulose sheet and run for the multidirectional TLC chromatography. DNA adducts levels were measured as relative adduct level per 10^8 nucleotides.

2.1.4. Genotyping

Genotyping of glutathione S-transferase M1 (GSTM1) null, GSTT1 null, GSTP1 I105V, *N*-acetyltransferase 1 (NAT1) fast, NAT2 slow, cytochrome P450 1B1 (CYP1B1) V432L, sulfotransferase 1A1 (SULT1A1) R213H, myeloperoxidase (MPO) G-463A, catechol-*O*-methyltransferase (COMT) V108M, manganese superoxide dismutase (MnSOD) A-9V, NAD(P)H:quinone oxidoreductase (NQO1) P187S, X-ray repair cross-complementing group 1 (XRCC1) R399Q, XRCC3 T241M, and xeroderma pigmentosum complementation group (XPD) K751Q polymorphisms was assessed using Amplification Refractory Mutation System assay [13–15].

2.1.5. Leucocytes Telomere Length Analysis

Leucocytes telomere length (LTL) measured by the real-time method (RT-PCR) following the Cawthon procedure [22] as we previously used [9]. Briefly, after DNA extraction from leukocytes, LTL was determined by the ratio between the number of repeated copies of the telomere (T), compared to a single-copy gene known (S), (T/S ratio) [9]. The single copy gene used in this study was human b-globin (HBG). The analysis was conducted using the RT-PCR system StepOne plus equipment (Life Technologies, Applied Biosystems, Milan, Italy). In each series of analysis it has been inserted a standard calibration curve made up of six points and generated by a pool of DNA diluted in series, the concentration of which varies between 40 ng and 1.25 ng. All samples were examined in triplicate and the average of the three T/S ratios was used in the statistical analysis. To evaluate the reproducibility of the ratio T/S, the test was repeated for the 10% of the samples on different days. The coefficient of variation in the analysis was 3.0% [16].

2.2. Statistical Analysis

The test of Wilcoxon-Mann-Whitney was used to compare the key characteristics of cases and control, that include age, years of school, tobacco smoke, coffee and/or alcohol consumption, body mass index (BMI), vegetables consumption, cumulative exposure to AA and PAHs, levels of DNA adducts and LTL.

Structural equation modeling (SEM) analysis was used to appraise the complex relationships among the variables. In fitting SEM, age at diagnosis, lifelong consumption of cigarettes (pack-years), coffee consumption and alcohol intake (cumulative consumption), myeloperoxidase (MPO), cumulative exposure to aromatic amines—variables associated with BC risk according to previous publications—plus DNA adducts (transformed in logarithm), X-ray repair cross-complementing protein 1 and 3 (XRCC1 and XRCC3), *N*-Acetyl Transferase 2 (NAT2), manganese superoxide dismutase (MnSOD) and cytochrome P450 1B1 (CYP1B1) were used as exogenous variables (corresponding to predictors in regression based techniques). BC risk and LTL (expressed as square root to normalize the distribution) were the endogenous variables (corresponding to outcome variables). There were two alternative hypotheses which stated: each endogenous variable could be affected by one or more exogenous variables (hypothesis 1); BC risk could also be influenced indirectly through the variable "LTL" (hypothesis 2). The two competing hypotheses were converted in two models of structural equations, to find which model fitted best the observed data. SEM structural equations were fitted with "asymptotic distribution free" method because it did not make assumption on joint normality of all the variables. The effect of each exogenous variable was expressed as standardized (or beta) coefficients that make comparisons easily by ignoring the independent variable's scale of units. Both direct and indirect effects were estimated by SEM. SEM results were both tabulated and presented graphically. We used two SEM's goodness-of-fit statistics: (1) the chi square test for "model versus saturated" (the saturated model is the model that fits the covariances perfectly); and (2) the stability index obtained

from the analysis of simultaneous equation systems. The analysis was carried out with the statistical package STATA 13 (StataCorp., College Station, TX, USA).

The sample size required for SEM is dependent on model complexity, the estimation method used, and the distributional characteristics of observed variables [23]. The best option is to consider the model complexity (i.e., the number of exogenous variables) and the following rules of thumb: minimum ratio 5:1 [23,24]; recommended ratio 10:1 [25,26]; recommended ratio 15:1 for data with no normal distribution [25]. With 12 exogenous variables used in the SEM model, we should have 180 (= 15 × 12) subjects but they were actually 190, fulfilling the above requirements. The power should therefore be >0.80.

3. Results

Table 1 shows that the main characteristics of 96 cases and 94 controls were similar, except for pack-years (significantly higher in cases) and LTL (significantly higher in controls).

Table 2 shows the beta coefficients ("minus" sign indicating an inverse relationship) with 95% confidence intervals and p-values for two structural equation models estimated by SEM. LTL and BC risk were the endogenous variables for the first and second model, while the exogenous variables were the same for both models, respectively. Both direct and indirect effects are shown.

1. Direct effects. The first model shows that LTL is negatively associated with age ($p = 0.000$), DNA adducts ($p = 0.017$), alcohol intake ($p = 0.017$) and NAT2 ($p = 0.018$), and positively association with coffee ($p = 0.016$), MPO ($p = 0.009$) and XRCC3 ($p = 0.004$). The second model shows that BC risk significantly increased with consumption of cigarettes ($p = 0.000$), cumulative exposure to AAs ($p = 0.003$) and coffee ($p = 0.006$), while it decreased with LTL ($p = 001$) and age ($p = 0.019$).
2. Indirect effects. The first model shows no indirect effects. The second model shows that, via LTL reduction, BC risk increased with age ($p = 0.007$) and NAT2 ($p = 0.011$), while it decreased with MPO ($p = 0.029$) and XRCC3 ($p = 0.003$).

These findings supported the hypothesis that BC risk in our population was increased directly by LTL reduction, and was further affected through shortened LTL by several other variables, such as age and polymorphisms in NAT2, XRCC3, and MPO, that did not have a direct effect on BC risk.

Concerning the goodness-of-fit statistic, the chi square test was 0.00 ($p = 1.00$) indicating no difference against a saturated model, and the stability index was 0.0, signifying that SEM model satisfied stability condition.

Using the graphical interface of SEM, the same results (only direct effects) shown in Table 2 were displayed as path diagram in Figure 1. In this figure, square boxes stand for variables, arrows specify the direction of causal flow, an arrowed route is a path, and the estimated beta coefficients appeared along the paths. The "error term" for each equation is represented by a circle, and the correlation between errors is displayed as a curved path. It can be seen that error1 and error2 have a contemporaneous cross-equation correlation ($p = 0.007$). Therefore, the two equations were related through the correlation in their errors.

4. Discussion

In this paper we report direct negative links between LTL with age, DNA adducts, alcohol and NAT2, and positive (protection) ones with coffee, MPO and XRCC3; and between BC risk with cigarettes, cumulative exposure to AAs and coffee, while are negative with LTL and age. There was evidence of indirect effects on BC risk, via LTL reduction, by age and NAT2, MPO and XRCC3 (negative link).

The negative relationship between LTL and DNA adducts, is in line with our previous findings in coke over workers highly exposed to occupational PAHs carcinogens [9], and would suggest that adduct formation may perhaps have a direct role in shortening LTL. DNA adducts, in fact, such as those we determined by P32 post-labelling are the results of the stereoselective binding

of polyromantic compounds, AA included, to the exocyclic N2 of guanine nucleotides, that are considered the primary essential damaging event in bladder carcinogenicity [27]. In particular telomeres, as triple-G-containing sequences, may represent a sensitive target for damage by such AA genotoxic compounds. Double-strand breaks and interference with replication fork generated by the bulky-damaged telomeric bases may directly induce telomere shortening [28]. Then AA-adduct formation and the consequent telomere attrition may be modulated by a decrease in AA detoxification due to the specific NAT2 slow polymorphism. Furthermore, the formation of adduct in the proteins of the telomere-sheltering complexes, NAT2-modulated too, could be also considered as an alternative event accounting for shorter LTL, as an additional mechanism.

LTL is found also to be modulated by some other genetic polymorphisms such as MPOA and XRCC1399Arg. The genetic polymorphism of enzymes involved in individual response to oxidative stress (MPO) and repair (XRCC3) is likely involved in modulating the individual response to environmental exposures such as tobacco smoking, coffee drinks, AAs exposure and DNA adducts formation too [13,21,29]. In particular, on one hand MPOA allele is associated with a reduced mRNA expression that in turn may shrunk its action on procarcinogen activation of tobacco smoke carcinogens [30], while XRCC1399Arg polymorphism, that presents higher DNA repair activity [15,29], was previously associated with lower levels of bulky DNA adducts [31,32]. In the present study LTL are longer in carriers MPOA and XRCC1399Arg, confirming the protective role of MPOA and XRCC3 even on telomere stability.

The significance of the studied polymorphisms is related to the fact that the studied variants are those of enzymes that participate to the metabolic and DNA repair pathways of bladder carcinogens such as AAs and PAHs, and these are relevant functional variants.

The literature findings regarding age and LTL is also supported giving a qualitative meaning to our results. The relationship of LTL with alcohol drinking is in line with our previous findings on shorter LTL in abusers with higher alcohol intake [33] confirming alcohol drinking as an important biological aging factor.

We found a positive (protective) effect of coffee consumption on LTL. Although the associations between coffee intake, disease, and mortality have been investigated multiple times, research evaluating the relationship between caffeine intake and telomere length relationship is extremely rare. Our results is in line with a previous study by Liu et al. [34] that showed a significant association between longer telomeres and caffeinated coffee consumption in 4780 women. While previous studies on coffee consumption and telomere length have provided inconsistent findings [35,36] because did not exclude decaffeinated coffee consumption. Present findings and those by Liu et al. [34] would suggest that some specific compounds contained in coffee may protect DNA integrity. In fact intervention studies have shown that coffee consumption reduces spontaneous DNA strand breaks [37] and protects against chemical-induced DNA damage [38,39] and oxidative DNA damage [40]. These intervention studies had study periods ranging from 3 days [39] to 4 weeks [37] and coffee consumption amount ranging from 600 mL/day [38] to 1 L/day [39]. In addition animal and cell line studies have shown that specific compounds in coffee such as chlorogenic acid [40,41] and diterpenes [42,43] may protect against DNA damage.

Table 1. Characteristics of the study population.

Variables	Cases (N = 96)		Controls (N = 94)		Mann-Withney
	Average ± SD	N (%)	Average ± SD	N (%)	p
Age (years)	61.5 ± 10.9	96 (100)	60.3 ± 11.7	94 (100)	0.6224
Years of school	7.41 ± 3.35	95 (99)	8.38 ± 3.67	93 (99)	0.0530
Tobacco smoke (pack-years)	32.8 ± 20.6	96 (100)	23.2 ± 20.6	94 (100)	0.0008
coffee consumption (coffee-years)	2.76 ± 2.67	96 (100)	2.05 ± 1.49	94 (100)	0.1158
Alcohol consumption (alcohol-years)	93.4 ± 104	96 (100)	107 ± 117	94 (100)	0.4154
BMI (Kg/m^2)	26.0 ± 3.61	85 (86)	25.9 ± 3.20	88 (94)	0.8329
Vegetables consumption (vegetables/week)	2.43 ± 0.80	96 (100)	2.52 ± 0.60	94 (100)	0.8142
Cumulative exposure to AA	79.7 ± 78.0	11 (11)	18.6 ± 17.5	6 (6)	0.1880
Cumulative exposure to PAHs	46.5 ± 39.1	37 (38)	31.5 ± 31.6	35 (37)	0.4922
DNA adducts (ln)	1.11 ± 1.31	96 (100)	0.84 ± 1.11	94 (100)	0.1831
LTL (T/S)	1.55 ± 1.14	96 (100)	2.03 ± 1.42	94 (100)	0.0123

SD: standard deviation; BMI: body mass index; AA: aromatic amines; PAH: polycyclic aromatic hydrocarbons; LTL: leucocytes telomere length.

Table 2. SEM results (beta coefficients, 95% confidence intervals and *p*-values) for endogenous variables of structural equations: direct and indirect effects.

Endogenous Variables	Exogenous Variables	Direct Effects			Indirect Effects		
		Beta Coefficient	95% Confidence Interval	*p*-Value	Beta Coefficient	95% Confidence Interval	*p*-Value
LTL (RQ)	**Age**	**−0.0090**	**−0.0132; −0.0049**	**0.000**	0		
	DNA Adducts (ln)	**−0.0431**	**−0.0787; −0.0075**	**0.017**	0		
	Alc	**−0.0702**	**−0.1278; −0.0127**	**0.017**	0		
	PY	0.0385	−0.0107; 0.0879	0.125	0		
	Cof	**0.0729**	**0.0138; 0.1320**	**0.016**	0		
	AA	−0.0240	−0.0865; 0.0383	0.450	0		
	Nat2	**−0.0869**	**−0.1591; −0.0147**	**0.018**	0		
	MPO	**0.0942**	**0.0238; 0.1647**	**0.009**	0		
	XRCC3	**0.1055**	**0.0333; 0.1777**	**0.004**	0		
	XRCC1	0.0335	−0.0360; 0.1031	0.345	0		
	MnSOD	−0.0014	−0.1202; 0.1174	0.981	0		
	CYP1B1	0.0098	−0.0673; 0.0870	0.803	0		
BC risk	**LTL (RQ)**	**−1.3605**	**−2.1939; −0.5271**	**0.001**	0		
	Age	**−0.0107**	**−0.0198; −0.0017**	**0.019**	**0.0123**	**0.0034; 0.0213**	**0.007**
	DNA Adducts (ln)	−0.0439	−0.1127; 0.0249	0.212	0.0587	−0.0033; 0.1207	0.064
	Alc	−0.0663	−0.1761; 0.0434	0.236	0.0956	−0.0081; 0.1992	0.071
	PY	**0.1478**	**0.0726; 0.2231**	**0.000**	−0.0525	−0.1283; 0.0233	0.174
	Cof	**0.1747**	**0.0505; 0.2989**	**0.006**	−0.0992	−0.2058; 0.0073	0.068
	AA	**0.0944**	**0.0314; 0.1575**	**0.003**	0.0328	−0.0517; 0.1172	0.447
	Nat2	0			**0.1183**	**0.0275; 0.2090**	**0.011**
	MPO	0			**−0.1283**	**−0.2438; −0.0128**	**0.029**
	XRCC3	0			**−0.1436**	**−0.2385; −0.0487**	**0.003**
	XRCC1	0			−0.0456	−0.1379; 0.0466	0.332
	MnSOD	0.0904	−0.0757; 0.2566	0.286	0.0019	−0.1600; 0.1638	0.982
	CYP1B1	0			−0.0134	−0.1186; 0.0918	0.803

Legend: AA = aromatic amines (cumulative); Age = age at diagnosis; Alc = alcohol (cumulative); BC risk = bladder cancer risk; Cof = coffee (cumulative); CYP1B1 = cytochrome P450 1B1; DNA Adducts (ln); XRCC1 = X-ray repair cross-complementing protein 1; XRCC3 = X-ray repair cross-complementing protein 3; LTL (RQ) = leucocytes telomere length (expressed as square root); MnSOD = manganese superoxide dismutase; MPO = mieloperoxidase; Nat2 = *N*-acetyl transferase 2; PY = pack-years. Bold = statistically significant results.

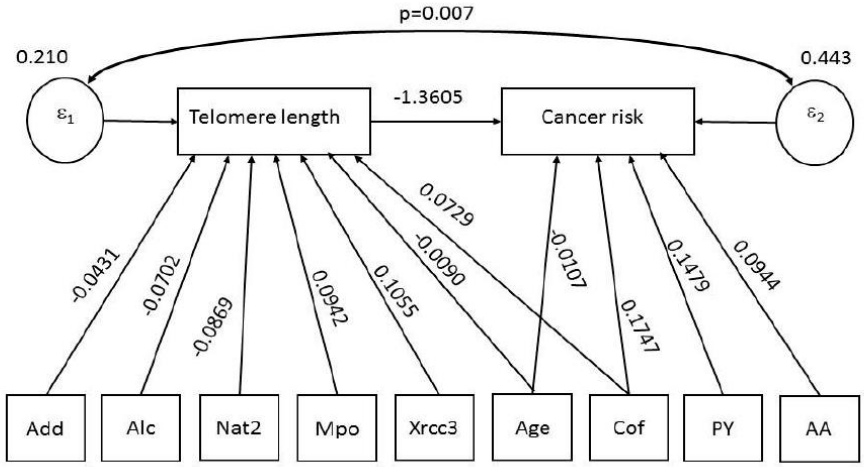

Figure 1. Path diagram of results shown in Table 2: variables (square boxes); causal flow (arrows); and paths (arrowed route); error terms for each equation (circles) and correlation between errors (curved path) with the corresponding p-value. The estimated beta coefficients appeared along the paths. Figure legend: Age = Age at diagnosis; Mpo = Mieloperoxidase; PY = Pack-years; Add = DNA adducts (ln); Cof = Coffee, (cumulative); AA = Aromatic amines (cumulative); Alc = Alcohol (cumulative); XRCC3 = X-ray repair cross-complementing protein 3; Nat2 = *N*-acetyl transferase 2.

With regard to BC risk, the literature is scanty on the relationship with occupational exposures to AAs and PAHs, and DNA adducts; those few published studies have only found an exposure-independent association between adducts and BC risk [44]. Our study precisely evaluated the occupational exposure history to AAs and PAHs, and noted a significant correlation between occupational exposure to AAs and BC (direct effect). Therefore occupational exposure to AAs is confirmed as central risk factor for BC development. In addition, the correlation we found between AAs exposure and BC risk is biologically plausible, because AAs are activated in liver and transported by blood proteins to the bladder where, under acidic conditions [45] or, enzymatically by O-acetylation of *N*-hydroxyarylamine (predominantly by the *N*-acetyltransferase 1 (NAT1) isozyme), are further activated to the ultimate carcinogen [46]. Unlike AAs, cumulative exposure to PAHs was not associated with BC risk in our study population. Experimental evidence suggests that PAHs are slowly absorbed through most tissues. For instance, in the case of dermal exposure, considered the main route in the industry [47,48], absorption accounts for a small fraction of applied dose, and PAHs are enzymatically activated and degraded at this site of entry [49–51]. The concentration and persistence of PAHs in the lung is largely related with inhalation of PAHs containing dust [51,52]. The high propensity of PAHs to act as carcinogens at the sites of entry is supported by several experimental studies [53].

There was evidence of indirect effects on BC risk, probably mediated by LTL reduction, of age and NAT2 (positive link), MPO and XRCC3 (negative link). The latter associations have been previously reported [13–16]; this study adds that these relations are indirect effects likely mediated by LTL reduction.

As described by Cawthon and colleagues in a study on an elderly population [54], LTL shortening, considered a hallmark of cellular aging, is associated with an increase in mortality rate. Recently, some studies have coherently shown lower survival and lower LTL, in patients with different kinds of tumours, including BC [11,54–56]. Therefore, our results seem to be suggestive that patients with BC who are older and with shorter LTL could be at poorer prognosis. This aspect is warranted to be prospectively scrutinized. Further studies in larger populations should investigate the complex

interrelationships among LTL, environmental and occupational variables as well as genetic endpoints, especially taking into account the relevant clinical and prognostic parameters along the course of BC.

5. Conclusions

In conclusion, the new relevant findings are that LTL erosion associates directly and directly with BC risk, strengthening the evidence of a central role of LTL in bladder carcinogenesis. Moreover, at the moment of BC diagnosis, LTL was found to decrease with some genetic polymorphisms and higher levels of DNA adduct and alcohol intake and to increase with coffee consumption.

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Conflicts of Interest: The authors declare no conflict of interest.

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BIOLOGICAL AGE IN COPD PATIENTS REVEALS AN ACCELERATED LUNG AGING

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Introduction

Chronic Obstructive Pulmonary Disease (COPD) is one of the major causes of chronic morbidity and mortality worldwide [Lozano et al., 2012], and it represents an important public health challenge, being both a preventable and treatable disease [GOLD, 2019]. Globally, the COPD burden is projected to increase in coming decades because of continued exposure to risk factors and aging of the population [Mathers and Loncar, 2006].

COPD is characterized by a progressive airflow obstruction, not fully reversible, as the result of the interaction of environmental agents and inherited genetic factors [Vestbo et al., 2013]. Cigarette smoking is considered the major risk factor for COPD worldwilde, although occupational exposures [Melville et al., 2010] as well as exposure to air pollution [Andersen et al., 2011; Hansel et al. ,2013; Gordon et al., 2014], respiratory infections [Sethi and Murphy, 2008] and factors linked to low socio economic status [Burney et al., 2014] are associated with the disease development.

Among the generally accepted pathophysiological mechanisms is that chronically inhaled tobacco smoke penetrates into the lung, deposits in the alveolar area, and locally triggers oxidative stress and inflammatory response [van Eeden and Sin, 2013]. This pulmonary local oxidative-inflammatory reaction damages lung tissue and a consequent narrowing of the airways makes breathing difficult [https://www.europeanlung.org/en/lung-disease-and-information/lung-diseases/copd]. Furthermore, there is growing evidence suggesting that the pathogenesis of COPD is linked to an accelerated aging process of the lung [Ito and Barnes, 2009; Maciewicz et al., 2009; Mercado et al.,2015] as the result of the chronic inflammation and oxidative stress responses [van Eeden and Sin, 2013; Barnes, 2016]. The exposure to gerontogenic cigarette smoke and inflammation leads to a significant decline in forced expiratory volume in one second (FEV1) that mirrors the increased lung obstruction, postulating that in COPD, lung aging is accelerated.

Our cells have a biological clock in telomere length (TL) [Blackburn et al., 2006] and DNA methylation (DNAm) that is an emerging biomarker of biological aging, defined as DNAmAge [Horvath, 2013]. The discrepancy between DNAmAge and chronological age, defined as age

acceleration (AgeAcc), informs about the speed of biological clock, mirroring the real biological state [Horvath and Raj, 2018].

Several studies report that COPD patients show shorter telomeres in circulating leucocytes compared to age matched smokers and nonsmokers control subjects [Savale et al., 2009; Mui et al., 2009; Houben et al., 2009; Cordoba-Lanus et al., 2017] providing evidences of an accelerated aging that may be related to lung inflammation [Mui et al., 2009] and a disturbed oxidant/antioxidant balance relationship [Houben et al., 2009]. A significant correlation between TL in peripheral leucocytes and in lung tissue suggests that telomeres in blood may be considered a biomarker of senescence of the lung on COPD patients [Saferali et al., 2014]. Telomere shortening has been also related to increased risk of poor clinical outcomes and mortality [Lee et al. 2012; Jin et al. 2018]. Furthermore, some studies showed an association between smoking-related altered methylation signals in peripheral blood and the risk for development of COPD and lung function decrease [Sood et al., 2010; Qiu et al., 2011; Wielscher et al., 2015; Lee et al., 2017; Machin et al., 2017]. Altered promoter methylation of the p16 or GATA4 genes in sputum was correlated with low lung function and increased risk of COPD among smokers [Sood et al., 2010; Meek et al., 2015]. However, no one study determined the DNAmAge in blood and induced sputum of COPD patients.

Aim of the study

The aim of this study is to investigate in COPD patients whether biological aging, determined by TL and DNAmAge, proceeds at the same rate in the target organ, i.e. lung, using induced sputum sample derived from lung, and in the systemic level i.e., blood, and relate it with demographic data (age, gender), life style and occupational exposure, pulmonary function, clinical and blood parameters.

Materials and methods

Study design

The present study includes n=18 moderate COPD patients (GOLD 2019; mean age 72±8 years; mean 63.1±16.6 FEV1%pred; 50% males) enrolled at the ambulatory of Respiratory Physiopathology Ward – Occupational Medicine, Department of Cardiac, Thoracic and Vascular Sciences 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 inclusion criteria were post-bronchodilator FEV1/VC ratio < 0.70 and no acute exacerbation for at least 6 weeks. 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. All patients gave their written informed consent and 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, clinical determinants (e.g., therapy, lymphocytes, C-Reactive protein (CRP)), diseases medical history and therapy. All patients underwent to a physical examination and pulmonary function was assessed by spirometry recording forced expiratory volume in one second (FEV1), forced vital capacity (FVC), vital capacity (VC), total lung capacity (TLC), residual volume (RV) and FEV1/VC ratio. For each patient blood samples were collected in vacutainers K3EDTA 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 recorded using a spirometer (Master Screen PFT, PRO, Viasys Sanità) according to the guidelines /recommendations of the American Thoracic Society/European Respiratory Society (ATS/ERS) [Miller et al., 2005]. FEV1, FVC and the FEV1/VC ratio were used as the primary variables of lung function. Lung function was measured both before and 10 minutes after the use of post-bronchodilator. FEV1 and FVC were expressed as liters and as a percentage of the predicted normal value (FEV1% and FVC%) 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 [Sterk et al., 1993]. These values obtained are in turn used as reference assessments for consecutive spirometries performed during induced sputum standard procedure [Paggiaro et al., 2002]. 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 FEV1 was measured for the detection and monitoring of lung function during the process, stopping the procedure when FEV1 decreased over 20 percent compared with 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 [Paggiaro et al., 2002]. 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 ~300,000 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 [Pavord et al., 1997] 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 DNAeasy Blood&Tissue kit (QIAGEN, Milano, Italy) on a QIAcube System (QIAGEN, Milano, Italy) for automated high-throughput DNA purification, following the manufacturer's instructions according to a customized protocol. 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 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 180 μL of PBS. DNA extraction was performed on automated QIAcube System (QIAGEN, Milano, Italy) using QIAmp®

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 [Pavanello et al., 2017]. 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 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 (5 µl 5 ng/µl) of DNA sample was added to each reaction, and each sample was run in triplicate. All PCR reactions were performed on 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 [Cawthon, 2002].

DNAmAge analysis and AgeAcc evaluation

DNAmAge was determined by analysis the methylation levels from selected markers using bisulfite conversion and Pyrosequencing® methodology as previously reported [Pavanello et al., 2019]. 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. [2015] with some modifications based on the fact that the method was almost completely automated using the PyroMark Q48 Autoprep (QIAGEN, Milano, Italy). Briefly, 2 µg DNA was submitted to bisulfite

conversion: unmethylated cytosines in extracted DNA were converted to uracil using Epitect Fast® DNA Bisulfite (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 AgePlexMono 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 (54–56 °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 1. 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 [Zbieć-Piekarska et al. 2015]. 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%.

Statistical analysis

Statistical analyses were performed with StastDirects software. Data are expressed as mean ± SD or number and percentage. AgeAcc was also evaluated as the difference between the observed DNAmAge and chronological age. The diversity among the two groups of patients split per therapy were appraised with Mann-Whitney U Test and Chi-square test, respectively. Levels of TL, DNAmAge, and AgeAcc in lung as derived from pulmonary 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. Clinical determinants (e.g., therapy, lymphocytes, C-Reactive

protein (CRP), FEV1, FVC, FEV1%pred) of AgeAcc in blood were appraised by multiple regression analysis. Results were considered significant when a p value of ≤0.05 was obtained.

Results

Characteristics of the study subjects are reported in Table 2 and 3. Table 2 shows interval variables (mean ± SD) in all COPD patients (n=18) and in COPD patients (n=9) with long-acting β2 agonist /long-acting muscarinic antagonist (LABA/LAMA) administration and patients (n=9) with combined inhaled corticosteroids (ICS)/ LABA/ LAMA administration, also defined as dual and triple therapy. The Mann Whitney U test comparing the two groups indicates that patients treated with LABA/LAMA have a better lung function: they present higher values of FEV1 (p=0.0003), FEV1% (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 patients treated with ICS/LABA/LAMA therapy. Table 3 shows the number and percentage of categorical variables in the same groups. The Chi-square test doesn't show any significant difference between patients with dual and triple therapy. All characteristics are therefore equally distributed among the two groups.

Table 4 reports the results of TL analysis in COPD patient' lung and blood. 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 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).

As shown in Table 5, 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). Also lung AgeAcc (Table 6) is extremely older than blood AgeAcc 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 blood AgeAcc of all patients (n=16) (Mann Whitney U test: mean -4.5±5.02 years vs mean -10.3±3.63 years; p=0.0156). Blood AgeAcc, but not TL, highly correlates with those of lung (Figure 1: r = 0.916, p=0.0037). Amongst clinical determinants considered by multiple regression analysis (table 7) we found that CRP (p=0.0378) and FEV1 (p=0.0422) significantly correlate with blood AgeAcc.

Discussion

Our study explores the hypothesis that biological aging in COPD patients, determined by TL and DNAmAge, proceeds at different rate in the disease target organ, i.e. lung, and at systemic level, i.e. blood, and relates with demographic data (age, gender), life style and occupational exposure, pulmonary function, clinical and blood parameters. The main findings stemming from this work reveal that in these patients:

a) lung, i.e., pulmonary cells obtained from induced sputum, is biologically older than blood, as determined by TL and DNAmAge.
b) blood AgeAcc, but not TL, highly correlates with lung AgeAcc.
c) blood AgeAcc significantly correlates with the main clinical features (CRP and FEV1) of COPD.

Increasing evidences suggest that an accelerated aging process of the lung is linked to the pathogenesis of COPD [Ito and Barnes, 2009; Maciewicz et al., 2009; Mercado et al., 2015]. Our findings support this hypothesis revealing that lung presents shorter TL and higher DNAmAge in respect to blood. We disagree with the only study that reported shorter TL in blood compared to lung tissue [Saferali et al., 2014]. The telomere shortening, we observed in lung, could be due to an elevated oxidative stress and increased release of pro-inflammatory cytokines derived from smoking pollutants, leading to telomere damage. In accordance with results on TL, we found that lung DNAmAge is extremely older than blood. To the best of our knowledge, this is the first time that DNAmAge was determined in COPD patients' lung and blood. Several studies showed an association between smoking-related altered methylation pattern in peripheral blood and sputum, and the risk for developing COPD and decrease in lung function [Lee et al., 2017; Machin et al., 2017; Qiu et al., 2011; Sood et al., 2010; Wielscher et al., 2015; Meek et al., 2015; Sood et al., 2010]. These associations suggest that methylation process could be the target mechanism of cigarette smoke damage. The greater lung AgeAcc confirms an accelerated biological aging of the lung, probably due to the reason that it is the target organ of the disease. Moreover, we found a close nexus between biological aging in the target organ and at systemic level: blood AgeAcc mirrors that of lung, proposing that it could be a replacement indicator of the lung aging. Furthemore, we found that blood AgeAcc is related with the main clinical features of COPD, i.e., inflammation and lung function decline (CRP and FEV1). The link between AgeAcc and lung function (FEV1), we found, is in line with a recent study on longitudinal data from SAPALDIA and ECRHS cohorts that reports an association between AgeAcc (estimated as residuals from regression between epigenetic and chronological ages using Horvath method) and lung function decline, particularly in women above age of 50 years [Rezwan et al., 2020]. Similarly, the correlation between CRP and

AgeAcc reinforces the possibility that AgeAcc may be associated with increased inflammatory response in COPD patients. This is in accordance with previous studies that reported an association between increased DNAmAge and elevated levels of CRP [Quach et al., 2017; Irvin et al., 2018; Morrison et al., 2019], beside to others inflammatory markers [Irvin et al., 2018].
Moreover, we hypothesize that the remarkably younger AgeAcc of blood could be explained through the effect of the therapy required by COPD and/or the several comorbidities of these patients. Our assumption is supported by a recent study showed a significant decrease in epigenetic age as a result of drug therapy with recombinant human growth hormone (rhGH), dehydroepiandrosterone (DHEA) and metformin [Fahy et al., 2019], the latter proposed as a candidate for slowing aging in humans [Barzilai et al., 2016].

In conclusion, we demonstrate that lung of COPD patients is remarkably biologically older than blood. The correlation of AgeAcc with the main clinical features (CRP and FEV1) of COPD supports the hypothesis that lung aging is accelerated. This finding opens new research challenge on novel therapeutic approaches to counteract the advanced biological aging in the lung. 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 validate and further characterize these findings.

Authors' Contributions

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

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Conflict of interest

The authors declare no conflict of interest.

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Table 1. 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	

Table 2. Interval variables in COPD patients with LABA/ LAMA and inhaled corticosteroid (ICS)/long-acting β2 agonist (LABA) /long-acting muscarinic antagonist (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	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	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 3. Distribution of categorical variables in COPD patients 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
Smoking	Non smokers	1(5)	0(0)	1(11)	0.500
	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
Charlson index	≤1	1(5)	1(11)	0(0)	0.500
	≥ 2 ≤4	10(55)	4(44)	6(67)	0.395
	≥5	7(39)	4(44)	3(33)	0.667

Table 4. 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±0.35§	1.48±0.21§†	1.47±0.26§†

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

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

Table 5. DNAmAge of COPD patients' lung and blood.

	Chronological age (years)	DNAmAge(years)		
		Lung	Blood	Blood
N	**16**	**7**	**7**	**16**
Mean±SD	74±7	67.4±5.80§	61.6±5.40§	63.3±5.60

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

Table 6. AgeAcc of COPD patients' lung and blood.

	Chronological age (years)	AgeAcc (DNAmAge-chronological age)		
		Lung	Blood	Blood
N	16	7	7	16
Mean±SD	74±7	-4.5±5.02§†	-10.8±3.50§	-10.3±3.63†

§ 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 7. Multiple regression analysis: the influence of therapy, Lymphocytes (%), CRP (mg/ml) and lung functional parameters (FEV_1, VC, CPT, VR) on AgeAcc in blood.

Variables	b	r	t	p
Therapy	b1 = -1.482	r = -0.233	t = -0.588	P = 0.578
Lymphocytes %	b2 = -0.023	r = -0.063	t = -0.154	P = 0.882
C-reactive protein (mg/ml)	b3 = -0.638	r = -0.735	t = -2.655	**P = 0.0378**
FEV_1	b4 = 11.213	r = 0.724	t = 2.572	**P = 0.0422**
VC	b5 = -152.444	r = -0.185	t = -0.462	P = 0.660
CPT	b6 = 143.624	r = 0.175	t = 0.435	P = 0.679
VR	b7 = -142.982	r = -0.174	t = -0.432	P = 0.681

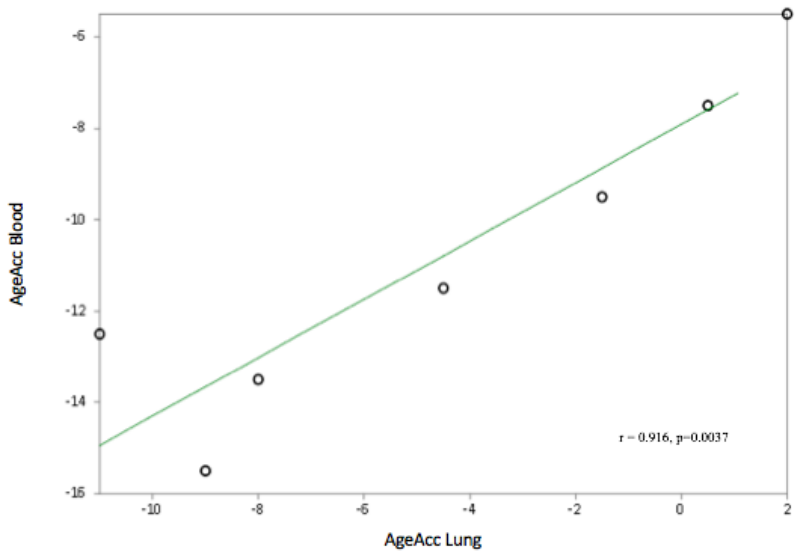

Figure 1. Non-parametric linear regression plots showing correlation between AgeAcc in blood and in lung

TELOMERE LENGTH IN IDIOPATHIC PULMONARY FIBROSIS PATIENTS

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Introduction

Idiopathic pulmonary fibrosis (IPF) is an irreversible, progressive and fatal lung disease of elusive etiology [Lederer and Martinez, 2018], characterized by interstitial remodeling, leading to compromised lung function. Mortality rate in IPF is high with a median survival after diagnosis of 2–3 years [Raghuet al., 2014; Strongmanet al., 2018].

IPF is a rare disease, although it is the commonest idiopathic interstitial pneumonia [Sauleda et al., 2018]. Historical estimates of incidence in industrialized countries have ranged from 2.8-19 per 100,000 persons/year [Olson et al., 2018; Wakwaya et al., 2019]. Epidemiological study suggests that incidence of IPF is increasing, particularly in older men [Sgalla et al., 2016]. Older age, male sex, and cigarette smoking are considered risk factors for IPF [Baumgartner et al., 2000], as well as occupational exposures with the 26% of attributable fraction to the burden of IPF [Blanc et al., 2019]. Also genetic predisposition, in particular telomere-related gene (TRG) mutations are noteworthy, linking risk to disease development [Armanios et al., 2007; Allen et al., 2017].

Although our understanding of disease pathogenesis remains incomplete, increasing evidence involves accelerated mechanisms of aging, including cellular senescence, in IPF pathogenesis [Faner et al. 2012; Schafer et al, 2017]. Recent investigations have implicated telomere shortening in the pathogenesis of the disease [Stanley and Armanios, 2014]. Compared with the other disorders of telomere dysfunction caused by a single gene defect (such as dyskeratosis congenita, aplastic anaemia, and liver cirrhosis), IPF is the most common manifestation of telomere-mediated disease [Armanios, 2012].

Telomeres are repetitive functional complexes of DNA/protein located at the ends of the chromosomes. Telomeres preserve the integrity of DNA, which, in their absence, would be gradually lost with each cell division [Blackburn et al., 2015]. A specific enzyme, telomerase, is critically important for telomere maintenance by replenishing the lost DNA after mitosis, but is active only in progenitor cells and in certain diseases, such as cancer [Calado et al., 2009]. Telomeres therefore shorten progressively with each division of somatic cells and their length measured in peripheral blood leucocytes (leucocyte

telomere length - LTL) is considered an indicator of biological age and physiological stress alike [Epel et al., 2004].

Telomere length in both peripheral blood leucocytes [Stuart et al. 2014] and lung tissue [Alder et al., 2008; Snetselaar et al., 2017], has been reported to be shorter in IPF patients than in controls, also in patients without mutation in the telomerase genes, suggesting that it might be a marker of increased disease susceptibility [Alder et al., 2008].

IPF has a poor prognosis and there isn't cure. At present two antifibrotic therapies are available, nintedanib and pirfenidone, which has been shown to reduce lung function decline, albeit their mechanism of action is unclear [Noble et al., 2011; King et al., 2014; Richeldi et al. 2014]. However, some evidence suggests that these drugs may reduce mortality [Richeldi et al. 2016; Nathan et al., 2017].

Aim of the Study

The aims of this study were: 1) to analyze LTL in well phenotypically characterized IPF patients (T0); 2) to prospectively investigate a possible change in the rate of telomere shortening by combining collected clinical, functional, biological and antifibrotic therapy data.

Materials and Methods

Study design

Study population consists of n=101 IPF patients identified according to ATS/ERS/JRS/ALAT Committee on Idiopathic Pulmonary Fibrosis guidelines [Raghu et al., 2018]. The recruitment was conducted at Pneumology Ward - Department of Cardiac, Thoracic and Vascular Sciences and Public Health, University of Padova. The local Ethics Committee - University of Padova, in accordance with principles of the Helsinki Declaration, approved the study (practice number 3843/AO/16). Patients' enrollment started on July 2014. All patients recruited, before starting treatment with antifibrotic drugs (nintedanib and pirfenidone), gave their written informed consent to participate to this study. All patients underwent to a physical examination and pulmonary function was measured by spirometry, recording forced expiratory volume in one second (FEV1) and forced vital capacity (FVC). All measurements were obtained and interpreted in accordance with the recommendations of the American Thoracic Society/European Respiratory Society [Raghu et al., 2018]. Disease progression was defined

as FVC decline >5%/year. At enrolment (T0), characteristics of the patients including demographic data (age, gender), lifetime history of smoking (pack-years) and body mass index (BMI), age at diagnosis and antifibrotic therapy were acquired through a questionnaire specifically structured. Blood samples were collected in vacutainers K3EDTA tubes and Paxgene tubes, for laboratory tests (total white blood cell counts) and LTL determination.

A subgroup of n=24 patients underwent to a follow up medical examination (T1) with the same operating method described at enrollment. Data on patients at follow up, have been integrated with occupational history.

DNA extraction

DNA extraction was performed on all samples of whole blood using an automated QIAcube System (QIAGEN, Milano, Italy) according to the DNAeasy Blood&Tissue kit (QIAGEN, Milano, Italy) procedure for high-throughput purification from human blood, following the manufacturer's instructions and a customized protocol. 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).

LTL analysis

LTL was measured by the real-time quantitative PCR method developed by Cawthon [2009] and previously described [Pavanello et al., 2017]. This method measures the relative LTL in genomic DNA by determining the ratio of telomere repeat copy number (T) to single-copy gene (S) (T:S ratio) in experimental samples relative to the T/S ratio of a reference pooled sample [Pavanello et al., 2017]. The single copy gene employed in this investigation was the human β-globin (hbg). A "seven-points" standard curve was generated from a serially diluted DNA pool (obtained from DNA samples randomly selected) varying from 40 to 0.625 ng in each plate, in order to determine relative quantities of T and S (in nanograms). All samples and standards were analyzed in triplicate and the average of the 3 T/S ratio measurements was considered in the statistical analyses. 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. 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., relative telomere length. A measure of T/S ratio was considered acceptable if the SD among triplicate measures was <0.25. To test

the reproducibility of measurements, PCR runs of these samples were replicated in different days and the coefficient of variation (CV) for the average T/S ratio should be at least 10% [Pavanello et al., 2017].

Statistical analysis

Statistical analyses were performed on data based on information collected for each patient enrolled in the study. Continuous data will be expressed as mean and standard deviation (SD), while categorical variables were given as percentages. Univariate and multivariate regressions were used selecting the appropriate models. The analyses were performed using the statistical software Stata and StastDirects.
The influence of the independent variables at T0 such as BMI (Kg/m^2), gender, pack-years ((number of cigarettes/20)×years), age, FVC, FVC percent of the predicted value (FVC%pred), leucocytes (x10^3/ml), neutrophils (x10^3/ml), lymphocytes (x10^3/ml) and monocytes (x10^3/ml), on the dependent variable LTL was appraised by multiple regression analysis. Multiple linear regression analysis was also performed to evaluate the influence of BMI (Kg/m^2), gender, LTL at beginning of follow-up (T0), age at diagnosis (years), months from diagnosis to therapy, pack-years ((cigarettes/20)×years), occupational risk factor, lung function decline (FVC%pred T1-T0), and follow-up (days of treatment) on LTL at follow up (T1).

Results

Characteristics of the IPF patients at enrollment (T0), such as demographic data and information acquired through questionnaire (age, gender, pack-years, BMI), lung function measurements (FVC and FVC%pred), total white blood cell count and differential counts, therapy (nintedanib and pirfenidone), and LTL are reported in Table 1. The majority of enrolled patients were male (83.2%) with a mean age of 69±9 years and LTL at T0 1.27±0.34 (T/S). Nonparametric linear regression didn't show any correlation between LTL and age ($p = 0.780$) of patients at T0. Amongst determinants of LTL considered in the multiple linear regressions (Table 2) none was significantly correlated.
Among IPF patients, a subgroup of 24 patients underwent to a follow up medical examination (T1).
In Table 3 are shown the main characteristics of these patients as mean±SD or number (%), including LTL at the beginning of follow up (T0) and at the end of follow up (T1), follow up (days of treatment), and the decline in FVC%pred, in addition to age, gender, BMI kg/m^2, total white blood cell count and differential counts, age at diagnosis, months from diagnosis to therapy, history of smoking (pack-years)

and occupational risk factors. Our results show a slight increase in average of LTL at end of follow-up (T1) if compared to LTL at beginning of follow up (T0) (mean LTL (T/S) at T1, 1.29 ± 0.26 vs mean LTL (T/S) at T0, 1.21 ± 0.27; p=0.09). Multiple linear regression analysis (Table 4) reveals that LTL at T1 is significantly related to the follow up considered as time of treatment (p=0.002), LTL at the beginning (T0) (p<0.0001) and decline in lung function (p=0.057). The other variables are not significantly related.

Discussion

The main finding stemming from our study is that in the subgroup of IPF patients with follow up we observe an increase in LTL positively related to the duration of antifibrotic treatment, both with pirfenidone and nintedanib, to the misure of LTL at the beginning (T0) and with the reduction of lung function decline. These results would suggest that telomere shortening in IPF patients treated with antifibrotic drugs may be reversed leading to an increase in LTL.

Our results are in line with a recent study from the CAPACITY (Clinical Studies Assessing Pirfenidone in IPF: Research of Efficacy and Safety Outcomes) and ASCEND (A Study of Cardiovascular Events in Diabetes) trials conducted by Dressen and co-workers [2018] that reported a beneficial effect of the antifibrotic drug, pirfenidone, on LTL. Currently, there is no specific therapy targeting telomere shortening that has demonstrated a benefit in patients with IPF [Planas-Cerezales et al., 2019]. Our results would suggest that the antifibrotic drugs currently available and recommended for use in patients with IPF, not only pirefenidone but also nintedanib, may interfere with telomere shortening in leucocytes. Furthermore, we observe a correlation between an increase in LTL at the end of follow up and reduction in lung function decline probably mediated by antifibrotic treatment. Indeed, previous studies reported a reduction in lung function decline as result of antifibrotic therapy [Noble et al., 2011; King et al., 2014; Richeldi et al., 2014; Dressen et al., 2018].

Another finding is that, LTL didn't correlate with age in IPF patients. This result could be due to the small range of age in patients enrolled and probably to their unhealthy status, taking into account that IPF is considered the most common manifestation of telomere-mediated disease [Armanios, 2012]. Moreover, the large number of males in our study population confirms the higher susceptibility of the male for IPF development [Baumgartner et al., 2000; Sgalla et al., 2016].

The longitudinal design represents a strong point of our study, although we recognize some weak points. Main limitations are the small sample size of patients in the longitudinal subgroup, the lack of

an age-matched control group, and the lack of additional genetic investigations in particular regarding telomere-related gene (TRG) mutations linking to risk of disease development, including TERT, TERC, DKC1, TINF2, RTEL1 and PARN [Allen et al., 2017; Barratt et al. 2018].

In conclusion, taking into account our findings, we are in accordance with other authors [Planas-Cerezales et al., 2019; Molina-Molina, 2019] to further explore the implication of telomere length in pathogenesis, progression and treatment of disease.

Future Developments

Thanks to a financial support received by Boehringer Ingelheim International GmbH for the study titled "Exploring Biological Aging Mechanisms in The Pathogenesis and Progression Of Idiopathic Pulmonary Fibrosis" (BI study number 1199.0356) the research on IPF disease will continue.

The project will aim to explore other biomarkers of biological aging, in the peripheral blood (leucocytes) that include mitochondrial dysfunctions (measured by number of copies of mitochondrial DNA (mtDNAcn)) and epigenetic alterations (DNA methylation age (DNAmAge)).

Authors' Contributions

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

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Conflict of interest

The authors declare no conflict of interest.

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Table 1. Characteristics of IPF patients (n=101) at T0.

VARIABLES	Mean ± SD	Number (%)
Gender (male %)		84 (83)
Age (years)	69.0±9.00	
BMI (kg/m^2)	26.0±4.00	
Pack years ((cigarettes/20)×years)	21.0±23.0	
Leucocytes (10^3/ml)	7.94±3.55	
Neutrophils (10^3/ml)	5.00±2.00	
% Neutrophils	58.0±8.00	
Lymphocytes (10^3/ml)	2.30±0.75	
% Lymphocytes	30.0±8.00	
Monocytes (10^3/ml)	0.67±0.20	
% Monocytes	8.52±2.21	
FVC (L)	2.61±0.73	
FVC (% pred)	76.0±20.0	
Therapy		
No therapy		2 (2)
No information		2 (2)
Nintedanib		39 (39)
Pirfenidone		58 (57)
LTL	1.27±0.34	

Table 2. Multiple regression analysis: the influence of independent variables at T0 as BMI (Kg/m^2), gender, pack-years ((cigarettes/20)×years), age, FVC, FVC percent of the predicted value (FVC%pred), leucocytes (x10^3/ml), neutrophils (x10^3/ml), lymphocytes (x10^3/ml) and monocytes (x10^3/ml), on the dependent variable LTL.

Variables	**B**	**R**	**t**	**p**
BMI	b1 = -0.001	r = -0.016	t = -0.140	P = 0.889
Gender	b2 = -0.063	r = -0.040	t = -0.355	P = 0.723
Pack Years	b3 = 0.001	r = 0.067	t = 0.597	P = 0.552
Age	b4 = -0.005	r = -0.106	t = -0.945	P = 0.347
FVC	b5 = 0.066	r = 0.063	t = 0.559	P = 0.577
FVC%pred	b6 = 0.001	r = 0.021	t = 0.185	P = 0.854
Leucocytes (x10^3/ml)	b7 = 0.0003	r = 0.099	t = 0.881	P = 0.381
Neutrophils (x10^3/ml)	b8 = 0.034	r = 0.132	t = 1.181	P = 0.241
Lymphocytes (x10^3/ml)	b9 = -0.040	r = -0.081	t = -0.721	P = 0.473
Monocytes (x10^3/ml)	b10 = -0.077	r = -0.040	t = -0.357	P = 0.722

Table 3. Main characteristics of n=24 IPF patients at T1.

Variables	Mean ± SD.	Number (%)
Gender (Males %)		20 (83)
Age (years)	70.2± 7.67	
BMI (Kg/m^2)	27.1 ± 3.67	
Pack-years ((cigarettes/20)×years)	21.7 ± 19.6	
Leucocytes (10^3/ml)	12.0±15.3	
Neutrophils (10^3/ml)	4.89±1.19	
% Neutrophils	58.9±7.32	
Lymphocytes (10^3/ml)	2.13±0.68	
% Lymphocytes	29.2±6.73	
Monocytes (10^3/ml)	0.61±0.17	
% Monocytes	8.10±1.90	
Occupational risk factor		6 (25)
Months from diagnosis to therapy	2.81 ± 19.6	
Follow-up (days of treatment)	297.2 ± 123.5	
Decline (FVC %pred T1- T0)	-3.63 ± 7.03	
LTL at beginning of follow-up (T0)	1.21 ± 0.27	
LTL at end of follow-up (T1)	1.29 ± 0.26	

Table 4. Multiple regression analysis: the influence of BMI (Kg/m^2), gender, LTL at beginning of follow-up (T0), age at diagnosis (years), months from diagnosis to therapy, pack-years ((cigarettes/20)×years), occupational risk factor, lung function decline (FVC%pred T1-T0), and follow-up (days of treatment) on LTL at follow up (T1).

Variables	B	R	t	p
BMI	b1 = -0.020	r = -0.474	t = -2.01	P = 0.063
Gender	b8 = -0.101	r = -0.251	t = -0.97	P = 0.349
LTL T0	b2 = 0.748	r = 0.834	t = 5.67	**P < 0.0001**
Age diagnosis	b3 = -0.002	r = -0.083	t = -0.31	P = 0.759
Months diagnosis	b4 = -0.003	r = -0.377	t = -1.52	P = 0.149
Pack-years	b5 = 0.0003	r = 0.050	t = 0.19	P = 0.854
Occupation	b6 = 0.072	r = 0.219	t = 0.84	P = 0.415
Decline	b7 = -0.011	r = -0.484	t = -2.07	P = 0.057
Follow-up (days of treatment)	b9 = 0.001	r = 0.707	t = 3.74	**P = 0.002**

Chapter 4. HEALTHY SUBJECTS AND PATIENTS EXPOSED TO REJUVENATING FACTORS

EXPLORING EPIGENETIC AGE IN RESPONSE TO INTENSIVE RELAXING TRAINING: A PILOT STUDY TO SLOW DOWN BIOLOGICAL AGE.

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Article

Exploring Epigenetic Age in Response to Intensive Relaxing Training: A Pilot Study to Slow Down Biological Age

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Abstract: DNA methylation (DNAm) is an emerging estimator of biological aging, i.e., the often-defined "epigenetic clock", with a unique accuracy for chronological age estimation (DNAmAge). In this pilot longitudinal study, we examine the hypothesis that intensive relaxing training of 60 days in patients after myocardial infarction and in healthy subjects may influence leucocyte DNAmAge by turning back the epigenetic clock. Moreover, we compare DNAmAge with another mechanism of biological age, leucocyte telomere length (LTL) and telomerase. DNAmAge is reduced after training in healthy subjects ($p = 0.053$), but not in patients. LTL is preserved after intervention in healthy subjects, while it continues to decrease in patients ($p = 0.051$). The conventional negative correlation between LTL and chronological age becomes positive after training in both patients ($p < 0.01$) and healthy subjects ($p < 0.05$). In our subjects, DNAmAge is not associated with LTL. Our findings would suggest that intensive relaxing practices influence different aging molecular mechanisms, i.e., DNAmAge and LTL, with a rejuvenating effect. Our study reveals that DNAmAge may represent an accurate tool to measure the effectiveness of lifestyle-based interventions in the prevention of age-related diseases.

Keywords: epigenetic age; DNA methylation age; relaxing training; telomere length; myocardial infarction patient; telomerase

1. Background

DNA methylation is an emerging robust biomarker of biological aging. The advent of epigenome-wide high-throughput sequencing analyses has led to a successful identification of a large number of genomic sites strongly associated with age [1]. These discoveries have allowed the creation of an "epigenetic clock" with unprecedented accuracy for age estimation with an average error of only 3.6 years; this is defined as DNA methylation age (DNAmAge) [1–3]. The methylation-based biological age was mostly developed based on DNA extracted from blood as an easy available source [1–4]. Researchers have developed several multiple age-prediction models with various statistical methods to determine the age of a person based on the age-dependent methylation changes in certain CpG loci [1,2,5,6]. The number of CpG sites employed in developing these age prediction models fluctuates from a small number to the 100 s. An attempt has been made to increase the feasibility of age predictors with the utilization of as few loci as possible. Amongst the most robust DNAmAge predictors is the model proposed by Zbieć-Piekarska et al. [3] that was developed using data from 5 CpG sites. This method shows that DNAmAge highly correlates (r = 0.94) with chronological age

with a mean deviation from the calendar age (4.5 years) analogous to that from Horvath et al. [1] and Hannum et al. [2] (r = 0.96 and r = 0.91), with 3.6 and 4.9 years considered as the reference methods. Zbieć-Piekarska et al. [3] developed the algorithm from a larger sample (n = 420) and then they validated it using a smaller one (n = 300), covering the entire adult life span.

The discrepancy between DNAmAge and chronological age (ΔDNAmAge) [7], defined as age acceleration, that provides information regarding the speed of epigenetic clock [7], is closely associated with age-related disorders, including cardiovascular disease [8,9] and mortality risk [10,11]. Environmental factors have been found to impact age acceleration and include economic hardship [12], dietary factors [13], pollution [14,15] and education [16]. Cumulative lifetime stress hormones are one of the factors described to accelerate epigenetic aging [17,18]. On the other hand, one study examines the beneficial impact of physical exercise [19] and long-term meditation on DNAmAge [20]; however, no longitudinal study has been performed.

Relaxing practices, including meditation and music listening, are novel and inexpensive interventions that have become a focus of scientific interest. The American Heart Association (AHA) recommends the potential benefits of relaxing techniques for primary and secondary prevention of cardiovascular disease [21]. Relaxing practices are able to counteract stress and stress-induced disorders, through the activation of specific brain areas, evoking the relaxation response (RR) [22]. The RR is the complement of the stress response. Millennia-old rituals arousing the RR comprise meditation, yoga and repetitive prayer. The RR reduces levels of stress hormones, inflammation and oxidative stress [23–25] that are molecular pathways involved in cellular aging processes [26]. Studies show that some forms of meditation that help to mitigate psychological stress have a favorable effect on telomeres by activating telomerase activity [27,28]. The potential effect of intensive meditation training on telomere length elongation is suggested by two studies on healthy subjects [29,30], while no effect has been observed in breast cancer survivors [31,32] and depressed patients [33]. However, it is unexplored whether relaxing experiences modulate the rate of the epigenetic aging.

In our previous work, the beneficial impact of relaxing training has been demonstrated in patients after myocardial infarction (MI) with respect to inflammatory genes, across a cascade of neuro-endocrine-immune (NEI) messengers, and on clinical recovery of endothelial function and the initial regression of carotid atherosclerosis [34].

In this work, within the framework of a pilot longitudinal study, with a before-and-after design, we examine the hypothesis that an intensive relaxing training (twice a day) for 60 days may influence leucocyte DNAmAge by turning back the epigenetic clock in patients after MI and in healthy subjects. To explore this hypothesis we applied the prediction model proposed by Zbieć-Piekarska et al. [3] that presents high prediction accuracy and makes easier the application of age predictors' analysis. Moreover, we compare DNAmAge with another mechanism of biological age, leucocyte telomere length (LTL) and telomerase. The longitudinal assessment of age-related biomarkers is the appropriate tool to determine accelerated aging, since confounding factors can be controlled.

2. Materials and Methods

2.1. Study Subjects

Within the framework of a pilot longitudinal study (before and after), in which each subject is a control of him/herself, we enrolled consecutively from October to December 2015, n = 20 patients who were admitted to our Cardiology Wards for ST elevated (STEMI) or non-ST elevated myocardial infarction (NSTEMI) and also with carotid atherosclerosis, and n = 10 healthy control individuals age- and gender-matched as previously described [34]. Patients and healthy controls were all Caucasians. Subjects were asked to participate in relaxing training. Each subject signed a consent form giving his or her agreement to take part in the study. All subjects were instructed in relaxing practices as previously described [34]. In brief, the initial four days of training took place in our hospital and the rest of the relaxing sessions was carried out independently by the subjects at home for 20 min, 2 times

a day. Subjects, from whom a sufficient quantity of DNA and RNA was available, were analysed to evaluate DNAmAge, LTL and telomerase. In the group of patients and healthy subjects, individuals were 14 and 6 with 3 (21%) and 2 (33%) females per group, respectively. In these groups, age was quite differently distributed. However, no significant difference was found among age, sex and body mass index (BMI), except for smoking habits. Patients, however, soon after MI and during relaxing training, stopped active smoking. Our institutional ethic committee (protocol number 3487/AO/15) authorized the study.

In Figure 1, we describe the research plan. Briefly, at enrolment (T0) and after 60 days (T1) of relaxing practice, for each subject, blood samples were collected in order to analyse biological age DNAmAge and LTL from extracted DNA and telomerase from RNA. For each subject at T0 and T1, vital indicators were also measured and blood samples for biochemical parameters and stress hormones, inflammatory cytokines, oxidative stress markers, endothelial progenitor cells (EPCs) and the expression of inflammatory genes (p53, Nuclear Factor kappa-light-chain-enhancer of activated B cells, Toll-like receptor 4) were also taken [34]. An electrocardiogram and transthoracic echocardiography were completed in order to evaluate the arterial pressure as previously reported [34]. The environmental conditions at the time of data collection were the same for all the subjects. In particular, the coaching, the relaxing sessions and blood withdrawals were performed in the same laboratory of our clinic located near our echocardiography test centre. A treatment diary was kept by participants. Patients and controls were also evaluated by the Clinical Psychology Unit of our hospital to attest the individual personality characteristics, neuro-cognitive reserve and the grade of perceived stress. Briefly, all patients were free of cognitive deficit and had no other comorbidities. All patients received the best medical treatment in agreement with AHA and European Society of Cardiology recommendations for the therapy of ischemic heart disease and adhered to the same cardiac rehabilitation plan (physical exercise and nutrition training).

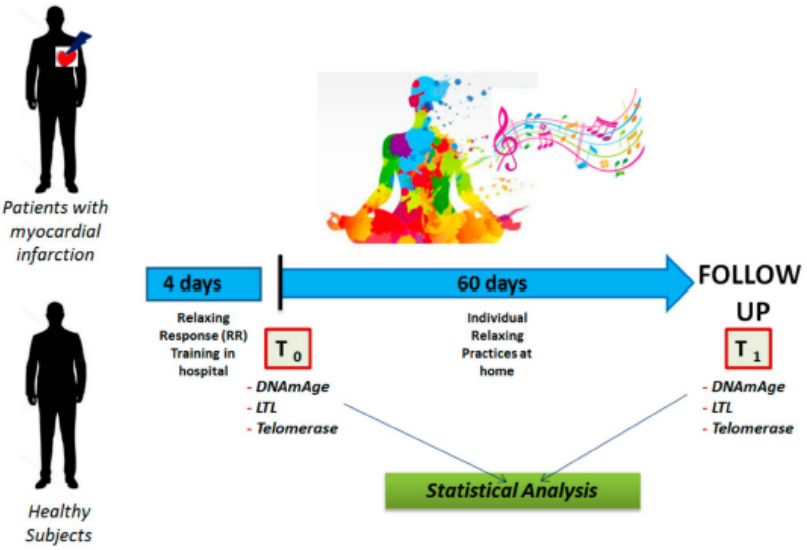

Figure 1. Longitudinal Plan of the Study.

2.2. DNA Extraction

DNA was extracted from whole blood by the DNAeasy Blood&Tissue kit (QIAGEN, Milano, Italy) on a QIAcube System (QIAGEN, Milano, Italy) for automated DNA purification, following the manufacturer's instructions according to a customized protocol. DNA was quantified and checked for quality using both Quantus™ Fluorometer (Promega, Milano, Italy) and QIAexpert Quantification System (QIAGEN, Milano, Italy). We obtained genetic material suitable for subsequent analytical

procedures both from a qualitative (260/280 nm 1.8 for DNA) and quantitative (mean DNA recovery 98.6 ng/μL) point of view.

2.3. DNAmAge

DNAmAge was determined by analysis the methylation levels from selected markers using bisulfite conversion and Pyrosequencing® methodology. 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 [3] with some modifications based on the fact that the method was completely automated using the PyroMark Q48 Autoprep (QIAGEN, Milano, Italy). Briefly, 2 μg DNA was submitted to bisulfite conversion: unmethylated cytosines in extracted DNA were converted to uracil using Epitect Fast® DNA Bisulfite (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 AgePlexMono kit (Biovectis, Warszawa, Poland). Details of sites and sequences for analysis are reported in Supplementary Materials Table S1. 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 (54–56 °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 containing in AgePlex Mono kit (Biovectis) and loaded into a 48 well-plate (Pyromark Q48 Discs, QIAGEN, Milano, Italy). Pyrosequencing was performed on a Pyromark Q48 Autoprep instrument using Pyromark Q48 Advanced Reagents (QIAGEN) 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 [3]. All samples were analyzed 3 times for each marker to verify the reproducibility of our results, and their average was 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%.

2.4. LTL and Telomerase Expression

LTL was measured by the real-time quantitative PCR method (qPCR) as previously described [35,36]. This analysis determines the TL in genomic DNA by establishing the ratio of telomere repeat copy number (T) to single-copy gene (S), T/S [35,36]. As a single-copy gene in this study, we used human β-globin (hbg). A "seven-points" standard curve was produced from a consecutively diluted DNA pool (obtained from DNA samples randomly chosen) in order to calculate the relative quantities of T and S (in nanograms). All samples and standards were examined in triplicate, and the mean of the 3 T/S ratio measures was employed in the statistical evaluations. The PCR runs were conducted in triplicate on a SteponePlus Real-Time PCR System (Life Sciences Solutions, Thermo Fisher Scientific, Monza, Italy). To test the reproducibility of measurements, samples were replicated on different days, and the CV for the average T/S ratio was 8.5%, which was similar to the CV previously reported [35,36]. The expression of hTERT was analyzed by real-time PCR using TaqMan Gene Expression assay (Life Sciences Solutions, Thermo Fisher Scientific, Monza, Italy).. hTERT expression was standardized on GAPDH expression. All the reactions were performed in 96-well plates by the Steponeplus Real Time PCR System. The profile of thermal cycles and the PCR reaction components for both cDNAs were those recommended by the Protocol TaqMan ® Gene Expression Assays (Life Sciences Solutions, Thermo Fisher Scientific, Monza, Italy). The relative gene expression levels were determined on the basis of the ΔΔCt method of Livak and Schmittgen [37]. The comparative cycle

threshold method (ΔΔCt), which compares the difference in cycle threshold values between groups as previously described [37], was used to obtain the relative fold change in gene expression.

2.5. Sample Size Estimation

Sample size estimation for before-and-after study (Paired T-test) was applied to calculate the sample size for all subjects, patients and healthy subjects. For each group, the mean of the change Δ_{T1-T0} DNAmAge and the relative standard deviation (SD) were calculated (see Table 1). The effect size, obtained using the T statistic and non-centrality parameter, was 2.33, 8.29 and 2.88 for patients, healthy subjects and all subjects, respectively. It was calculated by considering α (two-tailed) = 0.05, β = 0.20 and SD = 2.88, 5.78 and 4.36 for patients, healthy subjects and all subjects, respectively. The calculation of sample size was computed through a STATA command by specifying the sample size (14 patients, 6 healthy subjects and 20 all subjects), the SD and the effect size. The group size to obtain statistical significance with α (two-tailed) = 0.05 and β = 0.20 was estimated to be n = 18, 14 and 4, respectively, for all subjects, patients and healthy subjects.

Table 1. DNAmAge and Δ_{T1-T0} DNAmAge at enrolment T0 and after 60 days of relaxing practices T1.

	T0	T1		p §
	DNAmAge		Δ_{T1-T0} DNAmAge	
	Mean (SD)			
All subjects	51.4 (9.37)	49.9 (10.0)	1.50 (4.36)	0.143
Patients	55.7 (5.66)	55.6 (4.29)	−0.14 (2.88)	0.428
Healthy subjects	41.3 (8.73)	36.7 (5.85)	−4.67 (5.78)	**0.053**

§ Paired two sided t tests.

2.6. Statistical Analysis

In the text, continuous variables are expressed as the mean and standard deviation, while dichotomous variables are expressed as percentages. DNAmAge and the difference between DNAmAge after and before the relaxing practices (Δ_{T1-T0} DNAmAge) are expressed as the mean and standard deviation, such as LTL and telomerase expression. Statistical analysis was applied to compare DNAmAge and LTL before and after 60 days of relaxing practices and to analyse their relationship. Comparison between two groups was made using a (two-tailed) paired *t* test, and correlation between means was evaluated by non-parametric linear regression models (Spearman's and Kendall's ranks). Multiple linear regression analysis was performed to examine the influence of disease, gender, treatment, and chronological age (independent variables) on DNAmAge and LTL (dependent variable) of all study subjects. We used Chow's test, a test to appraise whether the coefficients, of two linear regressions on different data sets, presented a similar trend. All statistical tests and *p*-values were two-sided and were conducted with Statsdirect Statistical software (Ashwell). Results were considered significant when a *p*-value of ≤0.05 was obtained.

3. Results

3.1. DNAmAge Correlation with Chronological Age

Linear regression analysis showed that in all subjects (n = 20) DNAmAge is high positively correlated with chronological age both before (T0) and after 60 days (T1) as displayed in Figure 2a/Figure 2b (Kendall's rank correlation, a) tau = 0.78, $p < 0.01$; b) tau = 0.89, $p < 0.0001$), with a mean deviations from calendar age of 4.36 and 1.3 years. This result is a quality validation of our analysis, confirming the extreme accuracy for epigenetic age estimation of this model. The multiple linear regression results (data not shown) confirmed that DNAmAge is highly dependent both before and after intervention (analysis of variance F = 21.01 $p < 0.0001$ and F = 54.06 $p < 0.0001$) on chronological

age (r = 0.758, $p < 0.001$ and r = 0.719, $p < 0.001$) but not from gender (r = 0.045, $p = 0.859$ and r = −0.409, $p = 0.092$) and from to be patients (r = −0.446, $p = 0.063$ and r = 0,137, $p = 0.587$).

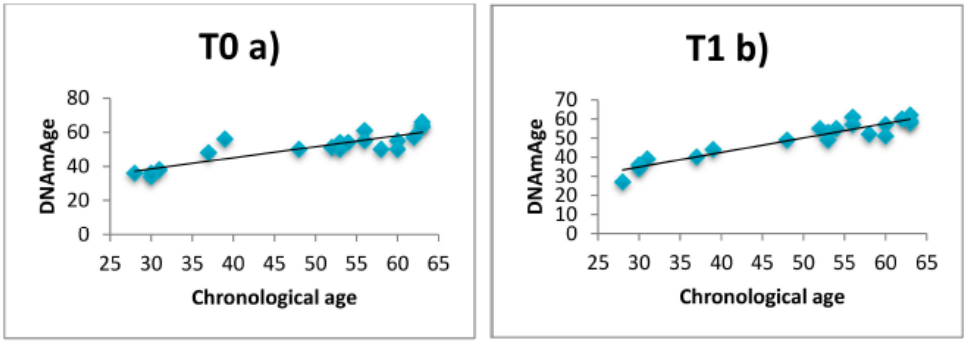

Figure 2. Correlation curves between DNAmAge and chronological age at enrolment T0 (**a**) versus after 60 days of relaxing practices T1 (**b**).

3.2. DNAmAge after 60 Days of Relaxing Practices

DNAmAge and Δ_{T1-T0} DNAmAge of all subjects, patients and healthy subjects, before and after intervention, are shown in Table 1. DNAmAge of healthy subjects after 60 days (T1) of relaxing practices is significantly younger (Δ_{T1-T0} DNAmAge = −4.66 years, $p = 0.053$), but not that of patients (Δ_{T1-T0} DNAmAge = −0.14 years; $p = 0.428$). This indicates a decrease in DNAmAge after relaxing practices in healthy subjects but not in patients. Multiple linear regression results (Table 2) confirm that Δ_{T1-T0} DNAmAge decrease/ rejuvenation is highly dependent on healthy subjects (r = 0.631, $p = 0.005$) and declines with chronological age (r = −0.507, $p = 0.032$), but not gender (r = −0.443, $p = 0.075$).

Table 2. Multiple linear regression of the influence of being healthy subject, age and gender on Δ_{T1-T0} DNAmAge for all subjects (n = 20).

	b	r	t	*p*
Healthy subjects	14.836	0.631	3.260	**0.005**
Chronological age	−0.400	−0.507	2.350	**0.032**
Gender	−3.497	−0.443	1.977	0.075

Analysis of variance from regression: F = 5.297 $p = 0.01$.

Table 3 shows DNA methylation status at the CpG sites of each of the five genes analyzed for DNAmAge determination (ELOVL2, C1orf132, KLF14, TRIM59 and FHL2). We find a significant decrease in DNA methylation pattern of KLF14 in all subjects after intervention (T1 versus T0 mean KLF14 % methylation (met) = 11.5 versus 13.3; Paired *t* test = 2.23; $p = 0.037$) suggesting that this gene is more susceptible to epigenetic changing.

3.3. LTL, Telomerase and Relaxing Practices

LTL and telomerase were evaluated in 14 patients and 6 healthy subjects before (T0) and after intervention (T1). Results of LTL and telomerase expression are reported in Supplementary Materials Table S2. After 60 days of intervention LTL is preserved in healthy subjects, while is continues to decrease in patients (T1 versus T0 mean LTL = 1.25 versus 1.49, $p = 0.05$). Telomerase expression did not differ in all groups. Moreover, we observed that the conventional negative correlation between LTL and chronological age is negative at enrolment (T0) but becomes significantly positive at T1, i.e., after 60 days of relaxing practices, in both patients (Figure 3) and healthy subjects (Figure 4). Comparison of the correlation curves by Chow's test at T0 versus T1 shows a significant difference in patients and healthy subjects (Chow Test F (2, 24 df) = 7.40 ($p < 0.01$); Chow Test F (2, 8 df) = 1.20 ($p < 0.05$)].

Correlation between DNAmAge and LTL was not significant in patients (T0 tau b = 0.012, $p > 0.999$; T1 tau b = 0.268, $p = 0.206$) and healthy subjects (T0 tau b = −0.276, $p = 0.566$; T1 tau $b = 0.467$; $p = 0.259$) both before and after intervention (data not shown).

Table 3. Methylation levels (% met) of five selected markers at enrolment T0 and after 60 days of relaxing practices T1.

	T0	T1	$p^{\S}$
ELOVL2 % Met Mean (SD)			
All subjects	61.2 (4.46)	61.9 (6.02)	0.253
Patients	63.0 (2.66)	64.6 (3.86)	0.071
Healthy subjects	56.8 (5.04)	55.7 (5.61)	0.135
C1orf132 % Met mean (SD)			
All subjects	49.7 (10.9)	40.9 (8.52)	0.554
Patients	45.4 (9.35)	46.9 (6.55)	0.517
Healthy subjects	59.8 (6.88)	60.2 (3.97)	0.935
TRIM59 % Met mean (SD)			
All subjects	50.6 (6.57)	51.9 (7.67)	0.204
Patients	53.9 (4.26)	56.1 (3.61)	0.131
Healthy subjects	43.2 (4.62)	42.2 (5.04)	0.110
KLF14 % Met mean (SD)			
All subjects	13.3 (3.21)	11.5 (1.99)	**0.037**
Patients	13.4 (2.95)	12.5 (1.45)	0.260
Healthy subjects	12.8 (4.02)	9.2 (0.41)	0.087
FHL2 % Met mean (SD)			
All subjects	45.0 (8.09)	45.7 (6.88)	0.609
Patients	47.7 (8.06)	48.6 (6.06)	0.671
Healthy subjects	38.5 (2.95)	39.0 (2.76)	0.774

§ Paired *t* tests.

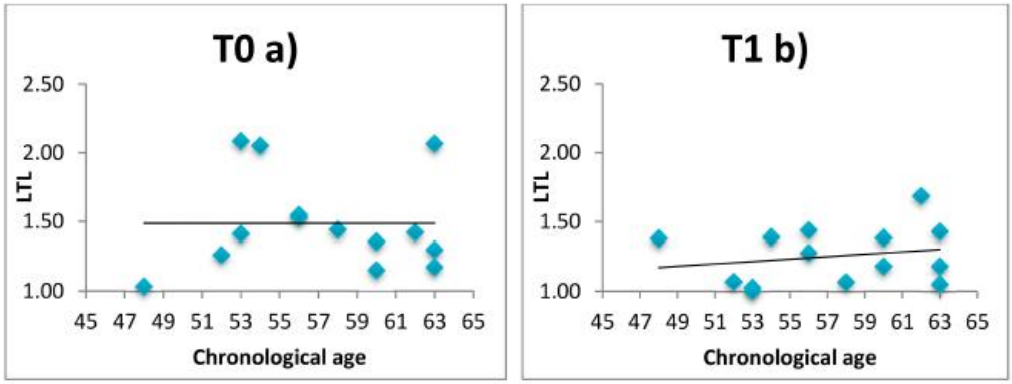

Figure 3. Comparison of the correlation curves between LTL and chronological age at enrolment T0 (**a**) versus after 60 days T1 (**b**) based on Chow's test for patients. Chow Test F (2, 24 df) = 73,975 ($p < 0.01$).

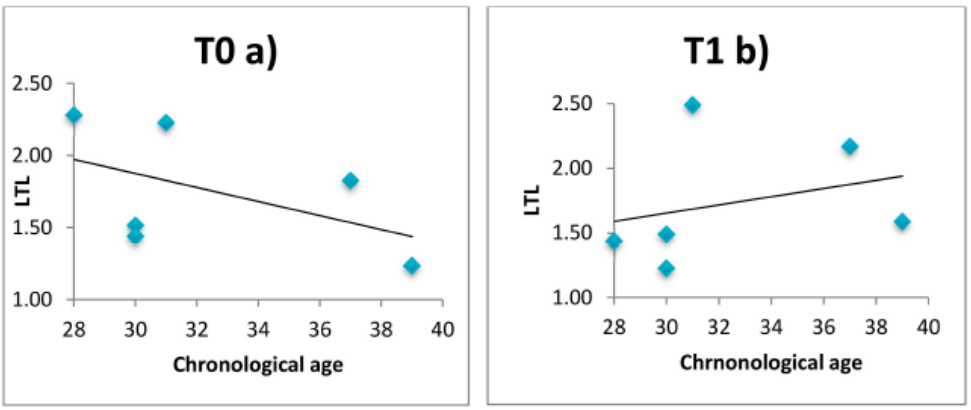

Figure 4. Comparison of the correlation curves between LTL and chronological age at enrolment T0 (**a**) versus after 60 days T1 (**b**) based on Chow's test for healthy subjects. Chow Test F (2, 8 df) = 11,889 ($p < 0.05$).

4. Discussion

In this longitudinal study, we explored the hypothesis that intensive (twice a day) relaxing training may influence leucocyte DNAmAge, turning back the epigenetic clock, in patients after myocardial infarction, and in healthy subjects, using the model proposed by Zbieć-Piekarska et al. [3]. This method has high prediction accuracy and make easier the application of age predictor analysis. Moreover, we compared DNAmAge with LTL and telomerase, other indicators of biological aging.

We found a reduction in DNAmAge in healthy subjects after 60 days of intensive relaxing training, but not in patients. The multiple linear regression results confirm that DNAmAge rejuvenation is highly dependent on healthy subjects but decreases with chronological age. Our results are in line with those obtained by Chaix and co-workers [20], who investigated in a cross-sectional study the effects of regular meditation practices on DNAmAge in healthy long-term meditators. They found a beneficial effect on DNAmAge in relation with several years of meditation. The results of our longitudinal study add that meditation practices have a potential rejuvenating effect on DNAmAge even after 60 days of relaxing practices in healthy subjects but are not sufficient in patients after myocardial infarction and in elderly subjects. One plausible mechanism that may support the positive effect of relaxing practices is the epigenetic switching through stress hormones. Stressors can drive a stable alteration in DNA methylation [17,18,38]; this change is mediated by the genomic action of glucocorticoids, primary molecular effectors to stress response [17,18]. Glucocorticoids act in every bodily organ, by stimulation of a transcription factor, the glucocorticoid receptor (GR) that regulates gene expression with the binding of its homodimer to glucocorticoid response elements (GREs) in regulatory regions [39]. Beyond regulating gene transcription, GRE binding can locally produce demethylation, a form of molecular signature that influences ensuing responses to glucocorticoids [40]. In both groups (patients and healthy subjects), we observed a significant reduction of stress hormones (cortisol, ACTH, copeptin, epinephrine and norepinephrine) after relaxing training [34]. Therefore, it is plausible that stress hormone reduction in our healthy subjects throughout the relaxing practices could influence cellular aging through DNA demethylation. The null findings for DNAmAge in the intervention group of patients could be explained by the fact that stress hormone disruption is higher in patients than in healthy subjects. In our patients, for example, at time T0 we observed that epinephrine and norepinephrine were higher than in healthy subjects [34]. This would suggest that more time and therefore a longer period of relaxing therapy is necessary to reduce hormone levels in patients than in healthy subjects. The fact that the rejuvenation effect decreases with age is in agreement with the fact that elderly people are more sensitive to stress and epigenetic changing mediated by glucocorticoids, due to the decline in DNA repair and maintenance mechanisms [41].

Moreover, we found a significant decrease in the DNA methylation pattern of KLF14 after intervention. KLF14 is part of the Krüppel-like factor family, which is a zinc-finger family of transcription factors capable of linking to GC-rich sequences, and has appeared as a key controller of important functions in various organs [42]. It especially regulates transcription cytokines and markers of inflammation [43] and appears to be associated with coronary artery disease [44]. Among the ageing-associated CpG sites, the KLF14 promoter region has been related to chronological age in several populations with distinctive ethnic backgrounds [45,46]. Therefore, the reduction detected in the methylation pattern of KLF14 after relaxing practices could provide a new indicator of the favorable effect, in combination with the decrease in cytokines and markers of inflammation (ESR, fibrinogen, HS-CRP, IL-6 and TGFβ-1) we found [34].

The methylation-based biological age is an attractive biomarker of aging because it can be measured in the DNA of most human tissues with high accuracy [1,4]. We chose to use the model proposed by Zbieć-Piekarska et al. [3] to increase the practicability of the test by detecting methylation levels of only 5 genes: ELOVL2, FHL2, KLF14, TRIM59 and C1orf132. Specifically in the first four genes, methylation is fostered with age, while in the latter one, methylation declines with age [3]. In this work, DNAmAge was highly correlated with chronological age in all subjects, confirming the prediction power of this model with a mean deviation from calendar age very comparable to those of Horvath [1] and Hannum et al. [2] and the quality validation of our analysis.

We found that after relaxing practices, LTL is preserved in healthy subjects, but continues to decrease in patients. Furthermore, we found that the conventional negative correlation between LTL and chronological age becomes positive after training both in patients and in healthy controls. This result suggests that relaxing training may have a positive impact on LTL regulation, both in healthy subjects and patients. Our results agree with those reporting in prospective studies a beneficial influence of meditation practices on LTL in healthy subjects, specifically after 3 weeks [30] and 12 weeks intervention [29], and in breast cancer survivors in whom LTL was instead maintained after 8 weeks of mindfulness practices [31].

Moreover, we did not find any differences in telomerase expression after relaxing practices. Our results are in accordance with those observed in non-regular meditators after 3 weeks [30] and after 6 days [47] of a meditation retreat or relaxing practices. However, other studies found an increase in telomerase activity in healthy subjects [27,48] and in breast cancer survivors [32]. The heterogeneity of telomerase activity findings makes it tricky to discriminate a clear relationship between meditation practice and telomerase across studies. As observed by Conklin and co-workers [30] the increase in LTL may result from both telomerase-mediated and telomerase-independent pathways.

No correlation between DNAmAge and LTL was found in all subjects, both before and after intervention. This result is in line with recent studies [49–51] that did not show LTL to be associated with epigenetic age. These biological age indicators respond to molecular mechanisms completely differently.

qPCR is the most frequently applied method for LTL testing even if crucial phases of analysis may contribute to the assay variability [52]. These include preanalytical factors, such as DNA extraction methods which are the most important sources of experiment-induced variation in qPCR LTL analyses, and analytical factors, i.e., telomere primer sequences and concentration, single copy gene, master mix, PCR program conditions, inclusion of quality control samples, PCR instruments and data analysis method. In this longitudinal study, the DNA was extracted from whole blood samples with the same DNA extraction kit. Moreover, analytical factors did not differ among analyses, and the same pool DNA was used as a reference standard in each qPCR reaction. All together, these procedures make possible to decrease the assay variability, reducing the measurement error.

The weakness of our study is mainly related to the low number of subjects, in particular in the healthy group; however, the sample size estimation we calculated is sufficient to obtain statistical significant results. Another weakness of the study could be the imbalanced ratio of males to females (i.e., 3 in patients and 2 in healthy subjects group), smokers to nonsmokers and the different distribution of age. This is a longitudinal study in which each subject was studied twice, before-and-after 60 days

of relaxing practices; hence, each subject is the control of him/herself with the second observation reinforcing the first. Moreover, the multiple regression analyses do not show any influence of gender on DNAmAge and LTL, neither in patients nor in healthy subjects, both before and after relaxing practices. Concerning smoking, a previous study failed to detect an effect of smoking on DNAmAge in blood [13]; several studies, including large investigations such as that conducted by Bischoff et al. [53] and Cassidy et al. [54], were unable to confirm the negative correlation between LTL and smoking found by others [55]. Furthermore, patients soon after MI and during relaxing training stopped active smoking. The inconsistency between several studies likely seems to reflect a complex effect of smoking, if any, on LTL that might not be easily detectable. Conversely, the association of an overall impact of smoking on DNAmAge is still unexplored and deserves further study.

The longitudinal characteristic is one strength of the present research, indeed all subjects (patients and healthy subjects) were analyzed before and after 60 days of relaxing practices and each subject is the control of him/herself in a short-term period. The before-and-after design offers better evidence about intervention effectiveness than a cross-sectional study and is most useful in demonstrating the impacts of a short-term program. To minimize the threats of validity, several precautions were taken for our short-term study. These include the scrupulous timing of blood sampling, the environmental conditions at the time of data collection; in particular, the coaching, the relaxing sessions and blood withdrawals were performed for all subjects in the same laboratory of our clinic located near our echocardiography test center; a treatment diary was kept by each participant; patients and healthy subjects were also evaluated by the Clinical Psychology Unit to attest to any individual personality characteristics; assessment of parameters specifically connected to RR (including stress hormones, inflammatory genes, across a cascade of neuro-endocrine-immune (NEI) messengers, and on clinically regaining of endothelial function and the initial regression of carotid atherosclerosis), whose changes can be ascribed to relaxing practices according to the methods used, as reported in our previous work [34]. All these measures were taken in order to preclude any other influences. Moreover, our data are in line with the biological assumption that relaxing practices by eliciting RR may influence leucocyte DNAmAge by turning back the epigenetic clock. Taking into account the above and the longitudinal nature of the study, we can assume that sample size, individual and technical conditions did not affect our findings, and the differences we observed before and after relaxing practices are related to treatment.

The innovation introduced by this study consists not only of the simultaneous evaluation of two indicators of biological aging but also of the perspective nature of the study design in which each subject was a self-control. In this way, it is possible to overlook the influence of previous events that might have contributed to modify indicators of biological aging and the other functional, vital, genetic and biochemical parameters we detected.

5. Conclusions

The new relevant finding stemming from our study is a potential decrease of DNAmAGE after 60 days of intensive relaxing practices especially in healthy subjects, suggesting a rejuvenating effect of these practices. We also observed a reduction in the methylation pattern of KLF14 after relaxing practices that may provide a new indicator of the favorable effect on prevention coronary artery disease. The analysis of the epigenetic clock therefore may represent an accurate tool to measure the effectiveness of lifestyle-based interventions for the prevention of age-related diseases. Moreover, the conventional negative correlation between LTL and chronological age that became positive after relaxing training suggests that these techniques may have a positive influence on telomere regulation. Our findings, on epigenetic age, corroborated by those on LTL, support our hypothesis of the benefits of intensive relaxing practices, which influence two key molecular mechanisms involved in cellular aging, and could represent a novel and inexpensive preventive strategy for stress- and age-related chronic diseases. However, we recognize the fact that the low number of people analyzed in this study

raises the question of chance findings. Longitudinal studies in larger cohorts are therefore required to validate and further characterize these findings.

Supplementary Materials: The following are available online at http://www.mdpi.com/1660-4601/16/17/3074/s1, Table S1: Details of markers, AgePlex sequences to analyze, CpG sites and location of the prediction model. Table S2: LTL and Telomerase expression at enrolment T0 and after 60 days of relaxing practices T1.

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DEFINING BIOLOGICAL AGE OF THE HEART

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The number of patients suffering from end-stage organ failure that are waiting for a transplant is steadily increasing, but the current organ shortage does not allow all patients to benefit from this optimal therapeutic option. Additionally, compared to previous eras, a current donor is likely to be older than 65y/o and to have died from the consequences of chronic comorbidities, such as cerebrovascular disease. Current strategies to face organ shortages demand a critical re-examination of donor eligibility, so as to include elders[1]. Defining the biological age of organ tissue may contribute to supporting this process. Aging trajectories are in fact greatly heterogeneous among coeval individuals; therefore, chronological age may not be a reliable indicator of the rate of physiological breakdown of the body or it's organs. Consequently, in donors, the correlation between chronological and biological ages may also not be reliable, and the clinical implications of such a discrepancy may hold a significant impact on heart transplants. DNA methylation is an emerging robust biomarker of biological aging[2]. This discovery has allowed the creation of an "epigenetic clock" with unprecedented accuracy for biological age determination, which is defined as DNA methylation age (DNAmAge)[2]. In our previous work, we demonstrated that biological age analysis, using DNAmAge technology, may represent an accurate tool to measure a rejuvenating effect of such a practice[3]. Furthermore, biological age acceleration (AgeAcc), i.e. the discrepancy between DNAmAge and chronological age, is predictive of age-related disorders. These include cardiovascular disease and mortality risk[4], thus mirroring the real biological state.

Over a timespan of 12 months (February 2018-February 2019), 31 heart transplants were performed at our institution. Amongst these, tissue sampling was performed in 17 consecutive cases. During surgery, donor atrial walls were trimmed and adapted to recipient atrial cuff for anastomosis, and samples of excess tissue from right (RA) and left atrium (LA) were collected. Biological heart age, in terms of DNAmAge and AgeAcc, were determined in donors' RA and LA, as well as in the blood, and as control in recipients' RA, LA, and blood (Table S1 and Table S2).

The median age of enrolled donors was 54 years (16-65), with 13 (76%) male, and all were brain dead donors (n=4 post anoxic damage, n=5 politrauma, n=7 cerebral hemorrhage, n=1 drowning hypoxia) (donors' characteristics are described in Table 1). Biological donors' heart age was found to be younger than chronological age (Table 2, median AgeAcc RA and LA =-14 and -16 years, $p<0.0001$), suggesting that donors' cardiac tissues are biologically younger than chronologically measured. Biological RA and LA age had comparable values in donors (Table 2), suggesting that although anatomically diverse and exposed to different physiological conditions, they express the same biological age. Differently the biological recipients' RA and LA age are different: on average LA is 3 years older than RA (Table S2). However, the biological donors' LA age is 5 years younger than recipients' LA age (median LA AgeAcc -16 versus -11, paired t-test, $p<0.05$). This would

indicate that patients' LA who underwent heart transplantation have received a heart with a younger LA. Additionally, we found that biological donors' AgeAcc of RA, but not of LA, is positively related to females ($p=0.038$), suggesting that AgeAcc of the RA tissue in females is greater than in males (data not shown). This result is consistent with a female-based vulnerability in cardiac tissue. As expected, biological heart age of both RA ($p=0.0002$) and LA ($p<0.0001$) correlate with chronological age (Figure S1,A-B). Furthermore, biological heart AgeAcc is inversely correlated with chronological age (Figure S1,C-D), indicating that older donors' hearts are even biologically younger and safer than previously anticipated. Biological age analysis on blood is confirmed to be correlated with the donor's chronological age (Figure S1, E $p=0.0008$), but it showed to be much older than both RA and LA tissues ($p<0.0001$, Table 2). Biological blood AgeAcc does not show correlation with those of RA and LA tissues ($p=0.721$ and $p=0.653$, respectively).

Differently from the previous experiences of Hovarth et al.[2], which reported comparable results on dilated cardiac ventricles, we used healthy cardiac tissues from donors with the purpose of determining whether their biological age reflects the chronological age, in comparison with unhealthy cardiac tissues from recipients. According to our findings, biological heart age is younger than chronological age and donors LA age is much younger than recipients one. Furthermore, biological age of the blood does not correlate with that of the heart. The possibility that transfusions given to trauma victim donors could have a part in this difference could be excluded since also recipients show the same difference (Table S2). This strengthens the idea that biological blood age cannot be considered a reliable indicator of the cardiac biological age, although other studies have already described it as predictor of morbidity and mortality in patients with cardiovascular disease[4]. The adoption of ex-vivo perfusion devices[5] will be of paramount importance in order to transfer the methodology herein described into the clinical realm because will allow the biological age determination during donor assessment.

In conclusion, accepting donors older than 50y/o has already contributed to widening the pool of available organs, but to date, there was no evidence that the chronological age of donors corresponds to the biological status of their organs. Our study indicates that the chronological age limit of 65y/o for donors might be able to be extended, as it does not reflect the real biological age with regards to cardiac tissues, as these are consistently younger than chronologically measured. Our preliminary findings represent a milestone in the process of donor organ evaluation, and unquestionably demands a critical review of the currently accepted clinical criteria, thus opening up new research platforms in the field of all solid organ transplantation.

Author Contributions

Conceived and designed the study: SP, MC and GG. Performed the samples' analysis: SP and MC. Provided samples: GT, VT and GC. Analyzed the data: SP, MC and AF. Wrote the paper: SP, MC, AF and GG.

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Disclosure

None relevant disclosures for all the authors.

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Table 1. Donors' Characteristics (n=17) and Myocardial Protection Techniques.

Age (y)	54 (16-65)
Male (n, %)	13, 76%
Organ Care System (n, %)	7, 41%
Cold Ischemic Time (min)[§]	29 (20-38)
Time in OCS (min)[§§]	265 (160-360)
Cold Cardioplegia (n,%)	10, 59%
Cold Ischemic Time (min) *	108 (-270)
Blood parameters	
Leucocytes (n *10^3/µl)	10.5 (7.29-24.02)
Red blood cells(n *10^3/µl)	3.10 (2.58-5.04)
Mean corpuscular volume (fL)	90.7(65.7-97.7)
Creatinine (mg/dL)	1.00 (0.42-87.0)
Glucose mg/dl	129.0 (6.40-239.0)

Data are expressed in median (range) or number (percentage).

[§] For the OCS, the time of cold ischemia is obtained by calculating the time from aortic cross clamping at the donor site to the aortic de-clamping at the recipient site minus the time the organ was inserted in the OCS device.

[§§] While in OCS, the heart is beating and the coronary flow is guaranteed by an extracorporeal blood perfusion.

*Cold Ischemic Time: time (in min) from aortic cross clamping at the donor site to the time (in min) of recipient aorta declamping.

Table 2. Chronological Age, DNAmAge and AgeAcc for RA*, LA^ and blood in donors.

Donors	Chronological age (years)	DNAmAge (years)				AgeAcc (years)		
		RA	LA	Blood	RA - LA	RA	LA	Blood
N	17	16	15	11	14	16	15	11
Median	54	41	35	55	1.00	-14[§§]	-16[§§]	-4
Min/Max	16/65	4/49	9/53	12.5/65	-7/10	-22/-5	-25/-5	-9/4

[§§] Paired t tests: right atrium versus blood AgeAcc and left atrium versus blood AgeAcc P<0.0001
*Right atrium, RA; ^Left Atrium, LA
RA – LA= difference between DNAmAge of Right atrium - Left Atrium

Supplementary Material S1: Study Protocol, Methods and Materials

Our Local Ethical Committee approved the study (protocol number 2246P), allowing a waiver from consent.

At the donor site, hearts were explanted following standard surgical practice. After an initial dose of cardioplegic arrest, myocardial protection and safe organ transportation to the recipient site were obtained or i) using hypothermia and cold cardioplegia to maintain low levels of cellular metabolism (n= 10 pts) or ii) guaranteeing adequate coronary blood flow in a beating and normothermic heart, using machine perfusion devices (n=7), such as OCS (Organ Care System, Transmedics Inc.).[1] For the purpose of this study, a blood sample (3-4 ml) from the donors, was collected in PAXGENE tubes (BD biosciences, USA).

Tissue Sampling and Storage

Once reached the recipient site, donor's cardiac tissues were collected. Surgery was performed using a "bicaval technique" for all patients as standard. During surgery, donor atrial walls were trimmed and adapted to recipient atrial cuff for anastomosis, as usual.

Two samples of exceeding tissue from right (RA) – by means of venous caval tissue - and left atrium (LA), at least approximately 3 mm^3, were collected and placed in all protect tissue reagent-RNA Later (Qiagen) for DNA/RNA stabilization. Blood and cardiac tissue samples (RA and LA) were also collected from recipients. All collected samples were, then, transferred to the laboratory of Genomic and Environmental Mutagenesis of the University of Padova for genetic analyses and stored at -20°C, until the molecular analysis was performed.

DNA extraction from blood and tissue samples

DNA extraction was performed on all samples of whole blood using an automated QIAcube System (QIAGEN, Milano, Italy) according to the DNAeasy Blood&Tissue kit (QIAGEN, Milano, Italy) procedure for high-throughput purification from human blood, following the manufacturer's instructions and a customized protocol. In particular, 400 µL of whole blood from each sample were processed for DNA extraction. DNA extraction from cardiac tissue samples was also performed on QIAcube System (QIAGEN, Milano, Italy) with some procedural differences compared to blood. In brief, a maximum of 25 mg of tissue sample was disrupted using TissueLyser II system (QIAGEN, Milano, Italy) through high-speed shaking with beads, which beat and grind samples. The TissueLyser II system delivers thorough and rapid disruption of samples to fully release biomolecules, and also simultaneously homogenizes samples to facilitate subsequent purification

procedures on QIAcube System (QIAGEN, Milano, Italy) by using DNAeasy Blood&Tissue kit (QIAGEN, Milano, Italy) following the manufacturer's instructions. After extraction, DNA was quantified and checked for quality using QIAexpert Quantification System (QIAGEN, Milano, Italy).

DNAmAge analysis

After DNA extraction, methylation-based biological age was determined by analysis the methylation levels from selected markers using bisulfite conversion and Pyrosequencing® methodology. This method is based on determination of methylation level of a set of five markers (ELOVL2, C1orf132, KLF14, TRIM59 and FHL2) in genomic DNA, as previously described[2] with some modifications relatively to the fact that the method was completely automated using the PyroMark Q48 Autoprep (QIAGEN, Milano, Italy).

AgeAcc assessment

AgeAcc was also evaluated. It is defined as the difference between the observed DNAmAge (tissue) and the chronological age of the donors. The predicted DNAmAge^ was calculated by regressing DNAmAge on chronologic age and AgeAcc^ by calculating the difference between the predicted DNAmAge^ and chronological age. No difference was detected with results obtained with data from AgeAcc and AgeAcc^, therefore we used both DNAmAge and AgeAcc data.

Sample size

Estimating that a significant correlation would be in the order of r = 0.80, we calculated that the sample to obtain statistical significance (α 0.01) should be n = 15 (power 0.9).

Statistical analysis

Statistical analyses were performed with StastDirects software. Data are expressed as median, minimum and maximum values unless otherwise specified. Comparison between two groups was made using (two-tailed) Paired t test and correlation between means was evaluated by simple linear regression models (Spearman's and Kendall's Ranks). Results were considered significant when a p value of $\leq$0.05 was obtained.

References

1. Ardehali A, Esmailian F, Deng M, et al. Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective, open-label, multicentre, randomised non-inferiority trial. *Lancet* 2015; **385**:2577–84.

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Table S1. Recipients' Characteristics (n=17).

Age (y)	54 (23-74)
Male (n, %)	15, 88%
Diagnosis (n, %)	
Ischemic cardiomyopathy post acute myocardial infarction	7, 41%
Hypertrophic cardiomyopathy	7, 41%
Other (Neonatal critical aortic stenosis, Hypoplastic left heart syndrome, Valvular cardiomiopathy)	3, 18%
Blood parameters	
Leucocytes (n *10^3/µl)	7.72 (4.43-16.83)
Red blood cells(n *10^3/µl)	4.43 (3.36-5.83)
Mean corpuscular volume (fL)	87.4 (73.4-102.3)
Creatinine (mg/dL)	84.0 (0.82-232.0)
Glucose mg/dl	94.0 (5.10-137.0)

Data are expressed in median (range) or number (percentage).

Table S2. Chronological Age, DNAmAge and AgeAcc for RA*, LA^ and blood in recipients.

Recipients	Chronological age (years)	DNAmAge (years)				AgeAcc (years)		
		RA	LA	Blood	RA - LA	RA	LA	Blood
N	15	15	15	14	15	15	15	14
Median	54	39	40	55	-3	-14[§§]	-11[§§]	-4
Min/Max	23/74	12/58	16/55	18/66	-16/7	-32/5	-25/3	-16/7

[§§]Paired t tests: right atrium versus blood AgeAcc and left atrium versus blood AgeAcc P<0.0001
*Right atrium, RA; ^Left Atrium, LA
RA – LA= difference between DNAmAge of Right atrium - Left Atrium

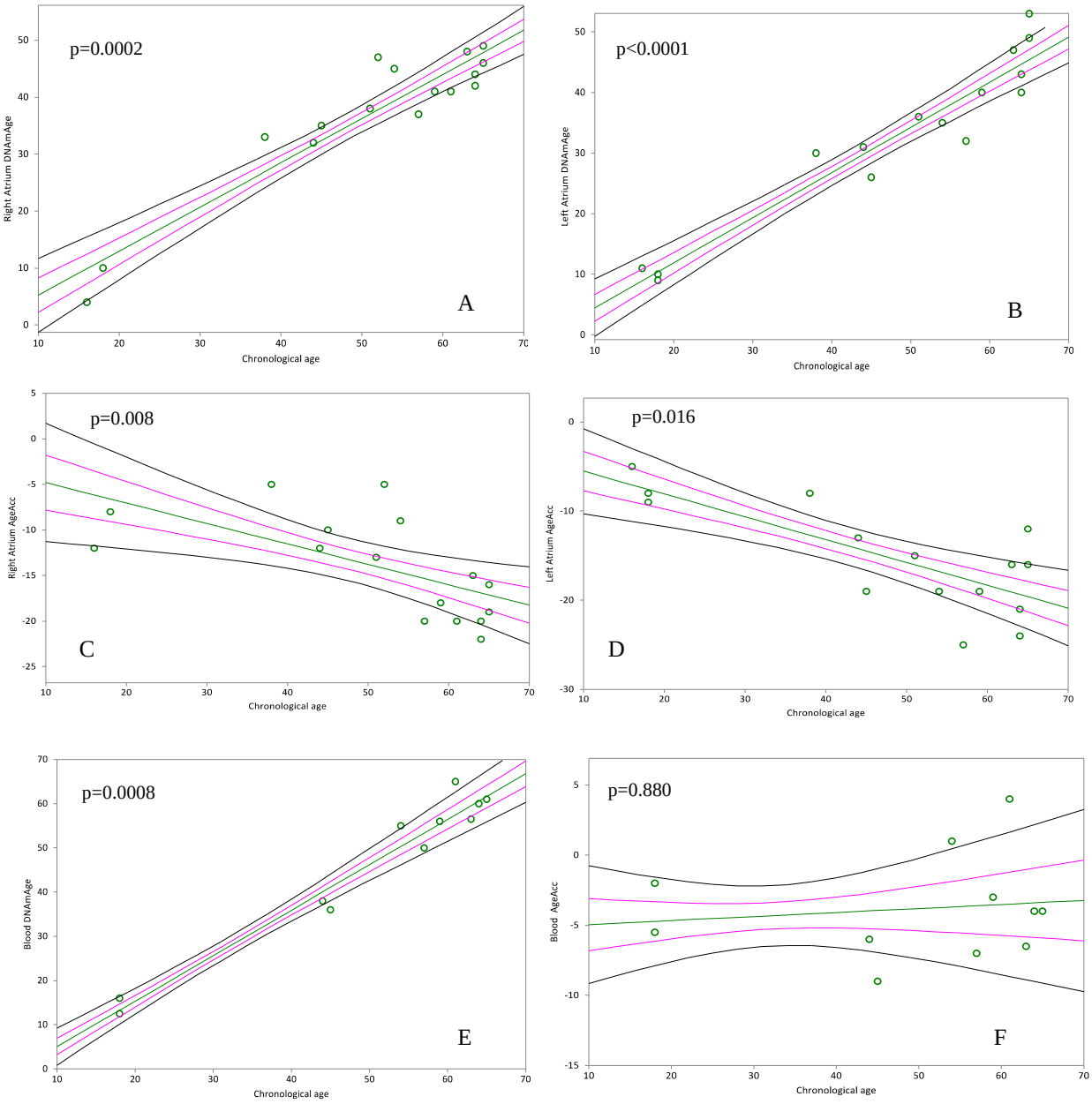

Figure S1. In A and B, non-parametric linear regression plots showing correlation between donors' chronological age and DNAmAge of the right atrium (RA) and the left atrium (LA) (Kendall's rank correlation coefficient tau b for RA= 0.700, for LA =0.806). In C and D, non- parametric linear regression plots showing the correlation between AgeAcc and the chronological age, of RA in A and LA donors in B (Kendall's rank correlation coefficient tau b= -0.506 and tau b= -0.485). In E and F, non- parametric linear regression plots showing the correlation between DNAmAge of the circulating blood leucocytes (indicated as "blood age") and the chronological age of the donor in A (Kendall's rank correlation coefficient tau b= 0.807), whereas in B no correlation is shown between chronological age and blood AgeAcc (Kendall's rank correlation coefficient tau b= 0.056).

Mean, Standard Error (SE) and 95% coefficient intervals (CI): green, pink and black lines, respectively.