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Revealing the Hidden Impacts: Insights into Biological Aging and Long-Term Effects in Pauci- and Asymptomatic COVID-19 Healthcare Workers

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Abstract: This study explores the role of inflammation and oxidative stress, hallmarks of COVID-19, in accelerating cellular biological aging. We investigated early molecular markers—DNA methylation age (DNAmAge) and telomere length (TL)—in blood leukocytes, nasal cells (NC), and induced sputum (IS) one-year post-infection in pauci- and asymptomatic healthcare workers (HCWs) infected during the first pandemic wave (February-May 2020), compared to COPD patients, model for "aged lung". Data by questionnaires, Work Ability Index (WAI), blood analyses, autonomic cardiac balance assessments, heart rate variability (HRV), and pulmonary function tests were collected. Elevated leukocyte DNAmAge significantly correlated with advancing age, male gender, daytime work, and an aged phenotype characterized by chronic diseases, elevated LDL and glycemia levels, medications affecting HRV, and declines in lung function, WAI, lymphocyte count, hemoglobin levels, and HRV ($p<0.05$). Increasing age, LDL levels, job positions involving intensive patient contact, and higher leukocyte counts collectively contributed to shortened leukocyte TL ($p<0.05$). Notably, HCWs exhibited accelerated biological aging in IS cells compared to both blood leukocytes ($p\leq0.05$) and NC ($p<0.001$), and were biologically older than COPD patients ($p<0.05$). These findings suggest the need for monitoring aging in pauci- and asymptomatic COVID-19 survivors, who represent the majority of the general population.

Keywords: biological aging; DNA methylation age; telomere length; post COVID-19; healthcare workers; pauci-symptomatic; heart rate variability; respiratory function; nasal cells; induced sputum

1. Introduction

Nearly four years after the World Health Organization (WHO) declared COVID-19 a pandemic, with over 775 million confirmed cases by May 2024 [1], the long-term consequences of the disease on the health of those infected remain largely unknown and are a significant area of research for global health [2]. The term "Long COVID" describes symptoms that persist or develop after the acute phase of COVID-19. This includes symptoms that last from 4 to 12 weeks after the acute phase, as well as post-COVID-19 syndrome, which refers to symptoms lasting more than 12 weeks and not explained by an alternative diagnosis [3].

While extensive research has focused on post-COVID-19 conditions in severely ill patients [4], there has been less attention on those with mild or asymptomatic infections, such as many healthcare workers (HCWs). Of 81 studies on post-COVID prevalence symptoms up to one year after infection

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[5–7], only 8 studies focused on pauci- and asymptomatic subjects after one year [8–15]. Furthermore, despite mild initial symptoms, 28% to 76% of these individuals developed post-COVID-19 syndrome. A one-year follow-up study on a mixed population (hospitalized and non-hospitalized patiens) conducted by Lombardo et al. [13] highlighted that more severe impairment in the acute phase did not appear to predict more serious complications. This gap is particularly important as these individuals represent a large portion of the population and underscores the importance of monitoring and supporting all COVID-19 patients, regardless of the initial severity of their symptoms, to adequately manage long-term complications.

Inflammation and oxidative stress, hallmarks of COVID-19 disease, play a key role in cell biological aging, supporting the hypothesis of its acceleration in COVID-19 [16]. At the cellular level, two interconnected "pillars of aging" are the earliest targets of cellular aging, i.e., telomere length (TL) and DNA methylation age (DNAmAge) [17,18]. One study reported evidence of biological age acceleration (i.e., epigenetic age acceleration and telomere shortening) in severe COVID-19 patients [16] as well as in COVID-19 survivors [19], whereas Franzen et al. [20] reported no epigenetic age acceleration in COVID-19 patients. Furthermore, in our recent work, we demonstrate that lung becomes older than blood, as measured by both TL and DNAmAge in induced sputum cells (IS) from the deep airways, compared to circulating blood leukocytes, in the same COPD patients, chronically exposed to inflammatory injury [21]. COVID-19 infection and the consequent pulmonary oxidative-inflammatory reaction lead to structural and functional pathological changes in the lung, also postulates an accelerated lung aging. To date, no one has investigated biological age indicators in pauci- and asymptomatic COVID-19 patients in different tissues other than blood, such as nasal cells (NC) and IS from the deep airways, which are tissues preferentially infected by SARS-CoV-2.

The aim of this study is to assess the biological aging of blood leukocytes and target tissues of the infection (i.e., IS and NC) in SARS-CoV-2 positive HCWs of the first wave (February-May, 2020), recruited at the health surveillance visit approximately 12 months after infection, and to verify the long-term sequelae of the infection and the impact on work capacity.

Given the global impact of the COVID-19 pandemic, studying the potential influence of SARS-CoV-2 infection on accelerated biological aging is of significant public health, economic, and social relevance. For the first time, we are examining this effect not only in the blood but also in the target tissues primarily exposed to the virus. This research will enable the development of personalized strategies to facilitate a full return to work.

2. Results

2.1. Post-COVID Syndrome (PCS) and Symptom Prevalence

In Table 3 the prevalence of PCS symptoms in the HCWs cohort (n=76) at 12 weeks was higher in women than men (p=0.0043), but similar after 1 year (p=0.5238). However, after 1 year, symptoms decreased in women (p=0.0204), while persisting in men (p=0.9999). Table 4 shows the distribution and percentage of COVID-19 symptoms reported by the HCWs cohort (n=76) up to 4 weeks, from 4 to 12 weeks, beyond 12 weeks after diagnosis (NICE guidelines [22]), and at the 1-year follow-up. Dyspnea, palpitations, peripheral neuropathy, loss of concentration, memory problems, and anxiety, as well as rare symptoms like dermatological signs, persisted beyond 12 weeks and at the 1-year follow-up ($p>0.05$). However, brain fog, sleep disorders, depression, and less frequent symptoms, including ocular symptoms, persisted beyond 12 weeks but not after 1 year ($p>0.05$). Supplementary Figure 1 shows that in 30% of HCWs, symptoms persisted 1 year after SARS-CoV-2 infection.

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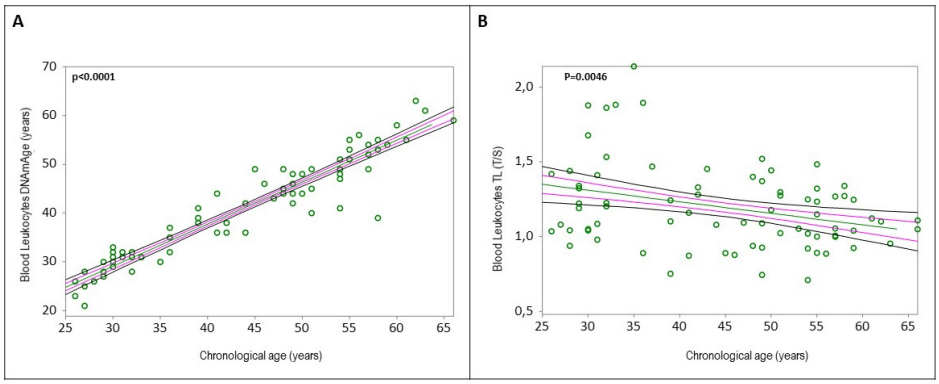

Figure 1. Correlation curves between blood leukocytes DNAmAge (A) or TL (B) and chronological age of $n = 76$ HCWs COVID19 survivors. In (A), a simple linear regression plot shows the correlation between blood leukocyte DNAmAge and chronological age [correlation coefficient (r) = 0.9433; two-sided $p < 0.0001$], while in (B), simple linear regression linear regression plot showing the correlation between blood leukocyte TL and chronological age [correlation coefficient (r) = -0.3217; two-sided $p = 0.0046$]. Mean, standard error (SE), and 95% coefficient intervals (CI) are represented as green, pink, and black lines, respectively.

Table 3. Prevalence of symptoms (Post Covid Syndrome - PCS), in the HCWs cohort (n=76) divided by gender and period since infection.

	PCS>12 weeks	PCS~1 year	P_Trend
HCWs	0.4605	0.3026	0.0663
Women	0.5741	0.3333	**0.0204**
Men	0.1818	0.2273	0.9999
P_Trend	**0.0043**	0.5238	

Table 4. Absolute and percentage distribution of COVID-19 symptoms reported by the HCWs up to 4 weeks, from 4 to 12 weeks, over 12 weeks after diagnosis, and at the time of the visit (1-year follow-up) (n=76 HCWs).

Symptoms		Up to 4 weeks after diagnosis n, (%)	From 4 to 12 weeks after diagnosis n , (%)	Over 12 weeks after diagnosis n, (%)	P Chi2	1-year follow-up	P Chi2
General symptoms							
	Asthenia	46 (60.53)	34 (44.74)	19 (25.00)	<0.0001	12 (15.79)	<0.0001
	Fever	46 (60.53)	1 (1.32)	0 (0.00)	<0.0001	0 (0.00)	Na
	Pain	20 (26.32)	8 (10.53)	6 (7.89)	0.0026	6 (7.89)	0.0013
Respiratory symptoms							
	Dyspnoea	11 (14.47)	7 (9.21)	7 (9.21)	**0.4873**	5 (6.58)	**0.4217**
	Cough	36 (47.37)	7 (9.21)	4 (5.26)	<0.0001	2 (2.63)	<0.0001
	Rhinitis	2 (2.63)	1 (1.32)	0 (0.00)	Na	0 (0.00)	Na

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Cardiovascular symptoms						
Chest pain	8 (10.53)	1 (1.32)	1 (1.32)	0.0059	0 (0.00)	Na
Thoracic oppression	14 (18.12)	5 (6.58)	2 (2.63)	0.0022	0 (0.00)	Na
Palpitations	11 (14.47)	7 (9.21)	6 (7.89)	**0.3761**	3 (3.95)	**0.1495**
Neurological symptoms						
Brain fog	10 (13.16)	7 (9.21)	3 (3.95)	**0.1316**	2 (2.63)	0.0453
Headache	37 (48.78)	10 (13.16)	5 (6.58)	<0.0001	3 (3.95)	<0.0001
Delirium	2 (2.63)	0 (0.00)	0 (0.00)	Na	0 (0.00)	Na
Sleep disorders	20 (26.32)	11 (14.47)	10 (13.16)	**0.0668**	8 (10.53)	0.0412
Peripheral neuropathies	4 (5.26)	4 (5.26)	3 (3.95)	**0.9089**	3 (3.95)	**0.9601**
Loss of concentration	14 (18.42)	13 (17.11)	11 (14.47)	**0.8017**	9 (11.84)	**0.6858**
Memory problems	11 (14.47)	14 (18.42)	13 (17.11)	**0.8017**	11 (14.47)	**0.8833**
Dizziness	5 (6.58)	0 (0.00)	0 (0.00)	0.0056	0 (0.00)	Na
Hypersomnia	2 (2.63)	0 (0.00)	0 (0.00)	Na	0 (0.00)	Na
Gastrointestinal symptoms						
Diarrhoea	18 (23.68)	1 (1.32)	1 (1.32)	0.0059	1 (1.32)	<0.0001
Abdominal pain	5 (6.58)	0 (0.00)	0 (0.00)	0.006	0 (0.00)	Na
Nausea	9 (11.84)	0 (0.00)	0 (0.00)	<0.0001	0 (0.00)	Na
Anorexia	16 (21.05)	0 (0.00)	0 (0.00)	<0.0001	0 (0.00)	Na
Vomiting	2 (2.63)	0 (0.00)	0 (0.00)	Na	0 (0.00)	Na
Other	2 (2.63)	0 (0.00)	0 (0.00)	Na	0 (0.00)	Na
Musculoskeletal symptoms						
Joint pain	39 (51.32)	15 (19.74)	13 (17.11)	<0.0001	8 (10.53)	<0.0001
Muscle pain	37 (48.68)	17 (22.37)	8 (10.53)	<0.0001	6 (7.89)	<0.0001
Psychological or psychiatric symptoms						
Anxiety	11 (14.47)	9 (11.84)	7 (9.21)	**0.6040**	5 (6.58)	**0.4245**
Depression	7 (9.21)	5 (6.58)	4 (5.26)	**0.6247**	0 (0.00)	Na
Other	1 (1.32)	0 (0.00)	0 (0.00)	Na	0 (0.00)	Na
Otorhinolaryngological symptoms						
Ageusia	40 (52.63)	10 (13.16)	3 (3.95)	<0.0001	2 (2.63)	<0.0001
Anosmia	41 (53.95)	12 (15.79)	4 (5.26)	<0.0001	2 (2.63)	<0.0001
Sore throat	8 (10.53)	1 (1.32)	1 (1.32)	0.0059	0 (0.00)	Na
Otalgia	1 (1.32)	0 (0.00)	0 (0.00)	**0.3663**	0 (0.00)	Na
Dermatological signs	3 (3.95)	2 (2.63)	3 (3.95)	**0.8785**	2 (2.63)	**0.9374**
Ocular symptoms	11 (14.47)	5 (6.58)	4 (5.26)	**0.0947**	2 (2.63)	0.0318
Other	10 (13.16)	5 (6.58)	4 (5.26)	**0.1687**	3 (3.95)	**0.1280**

Abbreviations: NA= not available.

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2.2. Blood Leukocytes Biological Age

The mean values and standard deviations of blood leukocytes DNAmAge, AgeAcc, and TL for all HCWs (n=76) are reported in Table 5. Simple linear regression analyses confirmed that blood leukocytes DNAmAge positively correlated with chronological age (Figure 1A, r=0.9433, p<0.0001), and blood leukocytes TL negatively correlated with chronological age (Figure 1B, r=-0.3217, p=0.0046). Increased blood leukocytes DNAmAge, but not TL, was associated with the duration of COVID-19 infection (Figure 2A, r=0.0618, p=0.596; Figure 2B, r=0.2378, p=0.0386). Additionally, subjects with greater biological age, detected by DNAmAge and TL, showed lower WAI (Figure 3A, DNAmAge r=-0.5169, p<0.0001; Figure 3B, TL r=0.2828, p=0.0194).

Table 5. Mean values and standard deviations of blood leukocytes DNAmAge, AgeAcc, and TL of all n=76 HCWs.

	Age	***Blood* leukocytes DNAmAge (years)**	***Blood leukocytes AgeAcc (years)***	***Blood leukocytes TL (T/S)***
Mean± SD	46.00±12.88	-2.59±3.47	1.12±4.37	1.20±0.06

Abbreviations: DNAmAge= DNA methylation Age; AgeAcc= Age acceleration; TL= telomere length.

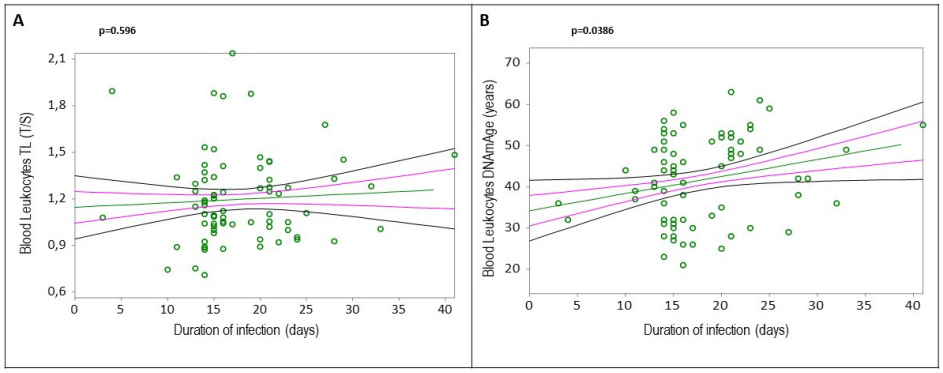

Figure 2. Correlation curves between blood leukocytes TL (A) or DNAmAge (B) and duration of infection (days) in n = 76 HCWs COVID19 survivors. In (A), a simple linear regression plot shows the correlation between blood leukocyte TL and days of infection [correlation coefficient (r) = 0.0618; two-sided p = 0.596], while in (B), simple linear regression linear regression plot showing the correlation between blood leukocyte DNAmAge and days of infection [correlation coefficient (r) = 0.2378; two-sided p = 0.0386]. Mean, standard error (SE), and 95% coefficient intervals (CI) are represented as green, pink, and black lines, respectively.

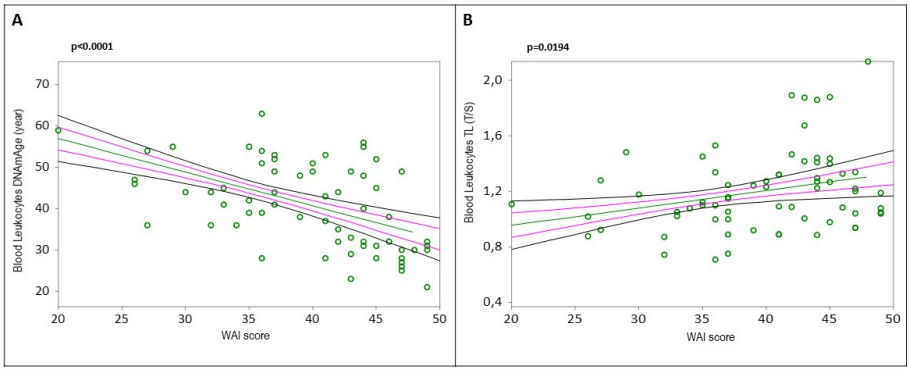

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Figure 3. Correlation curves between blood leukocytes DNAmAge (A) or TL (B) and work ability index (WAI) in n = 76 HCWs COVID19 survivors. In (A), a simple linear regression plot shows the correlation between blood leukocyte DNAmAge and WAI score [correlation coefficient (r) = 0.5169; two-sided $p < 0.0001$], while in (B), simple linear regression linear regression plot showing the correlation between blood leukocyte TL and WAI score [correlation coefficient (r) = 0.2828; two-sided $p = 0.0194$]. Mean, standard error (SE), and 95% coefficient intervals (CI) are represented as green, pink, and black lines, respectively. Table 1. Demographic and clinical characteristics of study population (n=76 HCWs).

Table 1. Demographic and clinical characteristics of study population (n=76 HCWs).

Variable	Mean±SD	N subjects	%
Age [years]	44.64±11.75		
Gender [n (%)]			
M		22	28.95
F		54	71.05
Marital status [n (%)]			
Not married		31	40.79
Married		40	52.63
Divorced		5	6.58
Widower		0	0.00
Years of education [years]	16.67±5.57		
BMI [Kg/m2]	24.48±4.01		
Systolic blood pressure [mmHg]	123.55±13.74		
Diastolic blood pressure [mmHg]	77.43±10.55		
EMPLOYMENT ANAMNESIS			
Professional position: [n (%)]			
Healthcare assistant		19	25.00
Nurse		30	39.47
Doctor		19	25.00
Resident		0	0.00
Other		8	10.53
Total years of work [years]	19.74±12.65		
Years of work in the current job [years]	9.78±9.23		
Performance of night shifts [n (%)]		43	56.58
Frequency of night shifts/month			
0		33	43.42
From 1 to 4		13	17.11
>5		30	39.47
Work ability - WAI (n=68) [n (%)]			
Poor (7-27)		5	7.35
Moderate (28-36)		15	22.06
Good (37-43)		5	33.82
Excellent (44-49)		25	36.76
PHYSIOLOGICAL ANAMNESIS AND LIFESTYLE			
Chronic diseases [n (%)]			

 doi:10.20944/preprints202407.0377.v1

0		28	36.84
1		16	21.05
≥2		32	42.11
Tobacco habit [n (%)]			
Smoker		10	13.16
Ex-smoker		13	17.11
Non-smoker		53	69.74
Pack/years [(cigarettes/20) per years of smoking]	1.63±4.26		
Alcohol consumption [n (%)]		56	73.68
Alcohol consumption [u.a./die]	106.89±155.76		
Binge drinking habit [n (%)]		1	1.32
Meals with grilled meat or pizza cooked in a wood-fired oven/year [n of meals/year]	80.63±59.72		
Frequency of fruit meals/day [n (%)]			
<2		39	51.32
>2		37	48.68
Frequency of vegetable meals/day [n (%)]			
<2		32	42.11
>2		44	57.89
IPAQ score (n=69) [n (%)]			
<700		11	15.94
≥700; ≤ 2519		20	28.99
>2520		38	55.07
Indoor pollution*			
0		52	68.42
1		19	25.00
2		5	6.58
3		0	0.00
Living area [n (%)]			
Urban/ peripheral area		50	65.79
Rural area		26	34.21
Traffic in the living area [n (%)]			
Continuous intense for a good part of the day		23	30.26
Intermittent intense		30	39.47
Scarce or absent		23	30.26
BASIC BIOCHEMISTRY PARAMETERS			
Leukocytes (103/ml)	6.19±1.59		
Blood red cells (103/ml)	4.70±0.43		
Platelet count (103/ml)	267.08±57.13		
Neutrophils (103/ml)	3.41±1.18		
Lymphocytes (103/ml)	2.17±0.58		
Monocytes (103/ml)	0.51±0.13		
Eosinophils (103/ml)	0.27±0.97		

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Basophils (103/ml)	0.03±0.03
Hemoglobin (g/dl)	138.18±13.79
Glycemia (mg/dl)	92.12±14.31
Cholesterol (mg/dl)	194.00±14.27
Triglycerides (mg/dl)	93.11±42.18
HDL (mg/dl)	59.85±15.58
LDL (mg/dl)	123.04±30.74
Creatinine (mg/dl)	1.49±6.26
Bilirubin (umol/L)	9.60±10.58
LIVER FUNCTION	
AST/GOT (U/L)	23.23±6.85
ALT/GPT (U/L)	21.8±12.73
GGT (U/L)	9.60±10.58
INFLAMMATION	
PCR (mg/L)	5.19±3.23
LUNG FUNCTION	
FEV1 (L)	3.30±0.88
FEV1 (%)	101.76±13.54
FVC (L)	4.10±0.92
FVC (%)	95.56±12.68
FEV1/VC (%)	0.82±0.06
TLC (L)	5.55±1.26
TLC (%)	96.47±15.70
RV (L)	1.59±0.40
RV (%)	107.36±23.43
HEART RATE	
Mean HR	68.05±9.71
HEART RATE VARIABILITY	
nLF: 0.04 - 0.15 Hz	52.38±18.20
nHF: 0.15 - 0.40 Hz	47.31±18.41
LF/HF ratio	1.54±1.30
SDNN	35.16±25.45
RMSSD	36.89±34.19

*Sum of three variables: presence of a pellet or wood stove in the home used more than five times a year, extra-occupational exposure to Polycyclic Aromatic Hydrocarbons (PAH) and exposure to passive tobacco smoke. Abbreviations: ALT= Alanine Aminotransferase; AST= Aspartate Aminotransferase; BMI= Body mass index ; FEV1= Forced Expiratory Volume in the 1st second; FVC= Forced vital capacity; GGT= Gamma glutamyl transferase; HCWs= Health Care Workers; HDL= High-density lipoproteins; HR= Heart rate; IPAQ= International Physical Activity Questionnaires; LDL= Low-density lipoproteins; nHF= normalized high frequency; nLF= normalized low frequency; PCR= C-reactive protein; RMSSD= Root mean square of successive RR interval differences; RV= Residual volume; SDNN= Standard deviation of normal-to-normal R-R intervals; TLC= Total lung capacity; WAI= Work ability index.

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2.3. Determinants of Blood Leukocytes DNAmAge and TL

Multiple linear regression analysis (Table 6) indicated that an increase in blood leukocytes DNAmAge was determined by age (p<0.0001), being male (p=0.014), presence of chronic diseases (p=0.029), decline in lung function (FEV1, p=0.0014), and decrease in lymphocyte count (p=0.002). Decreased blood leukocytes TL was determined by age (p=0.003) and reduced lymphocyte count (p=0.033).

Table 6. Multiple regression analysis of the influence of age, gender, presence of chronic diseases, decline in lung function (FEV1) and lymphopenia on blood leukocytes DNAmAge / LTL.

		b	r	t value	P
Blood Leukocytes DNAmAge (years)	**Age**	b1 = 0.74399	r = 0.911647	t = 17.884209	**<0.0001**
	Gender (male)	b2 = 2.703646	r = 0.298937	t = 2.525593	**0.014**
	Chronic diseases (0=no; 1=yes)	b3 = 1.860584	r = 0.266824	t = 2.23213	**0.0291**
	FEV1 (L)	b4 = -2.219589	r = -0.382602	t = -3.338666	**0.0014**
	Lymphocytes (10^9/L)	b5 = -2.017434	r = -0.36957	t = -3.206587	**0.0021**
Blood Leukocytes TL (T/S)	**Age**	b1 = -0.011446	r = -0.363521	t = -3.146031	**0.0025**
	Gender (male)	b2 = 0.004352	r = 0.005765	t = 0.046482	0.9631
	Chronic diseases (0=no; 1=yes)	b3 = 0.131573	r = 0.218459	t = 1.804868	0.0757
	FEV1 (L)	b4 = -0.023505	r = -0.05008	t = -0.404266	0.6873
	Lymphocytes (10^9/L)	b5 = 0.120086	r = 0.261295	t = 2.182445	**0.0327**

Abbreviations: DNAmAge= DNA methylation Age; FEV1= Forced Expiratory Volume in the 1st second; LTL= leukocyte telomere length.

Multiple regression analysis (Table 7) showed that an increase in blood leukocytes DNAmAge correlated with a decrease in WAI (p=0.0015) and daytime work (p=0.0325), but not job position (p=0.4352). Blood leukocytes TL decreased with job positions involving direct patient contact (healthcare assistants, nurses, doctors, residents) (p=0.0295), but not with WAI (p=0.2268) or daytime work (p=0.1864).

Table 7. Multiple regression analysis of the influence of occupation or professional position, night shift work and work ability index (WAI) on blood leukocytes DNAmAge / TL.

		b	r	t value	P
Blood Leukocytes DNAmAge (years)	**Occupation (0=HA; 1=N; 2=D; 3=R; 4=T and A)**	b1 = -0.727891	r = -0.097697	t = -0.785337	0.4352
	Night shift work (0=no; 1=yes)	b2 = -5.367146	r = -0.263551	t = -2.185686	**0.0325**
	WAI	b3 = -0.617807	r = -0.382432	t = -3.311157	**0.0015**

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Blood Leukocytes TL (T/S)	**Occupation (0=HA; 1=N; 2=D; 3=R; 4=T and A)**	b1 = 0.064564	r = 0.268124	t = 2.226516	**0.0295**
	Night shift work (0=no; 1=yes)	b2 = 0.102616	r = 0.164683	t = 1.3357	0.1864
	WAI	b3 = 0.007125	r = 0.150818	t = 1.220504	0.2268

Abbreviations: HA=healthcare assistants, N=nurses, D=doctors, R=residents, T and A=technicians and administrators.

Multiple regression analysis of the influence of haemoglobin (g/dL), glycaemia (mg/dL), cholesterol (mg/dL), triglycerides (mg/dL), HDL (mg/dL), LDL (mg/dL), creatinine (mg/dL) and bilirubin (mg/dL) on blood leukocytes DNAmAge and TL (Table 8) revealed that higher DNAmAge was associated with lower hemoglobin (p=0.0163), higher glycemia (p=0.0078), and higher LDL (p=0.0015). Shorter TL was associated only with higher LDL levels (p=0.0506). Cholesterol, triglycerides, HDL, creatinine, and bilirubin were not determinants in biological aging indicators.

Table 8. Multiple regression analysis of the influence of haemoglobin (g/dL), glycaemia (mg/dL), cholesterol (mg/dL), triglycerides (mg/dL), HDL (mg/dL), LDL (mg/dL), creatinine (mg/dL) and bilirubin (mg/dL) on blood leukocytes DNAmAge / TL.

		b	r	t value	P
Blood Leukocytes DNAmAge (years)	**haemoglobin (g/dL)**	b1 = -0.205578	r = -0.300885	t = -2.484288	**0.0157**
	glycaemia (mg/dL)	b2 = 0.2006	r = 0.329513	t = 2.748063	**0.0078**
	cholesterol (mg/dL)	b3 = 0.006919	r = 0.022835	t = 0.179853	0.8579
	triglycerides (mg/dL)	b4 = 0.003211	r = 0.01292	t = 0.101744	0.9193
	HDL (mg/dL)	b5 = 0.063761	r = 0.098495	t = 0.779342	0.4387
	LDL (mg/dL)	b6 = 0.172631	r = 0.388744	t = 3.322289	**0.0015**
	creatinine (mg/dL)	b7 = 0.267526	r = 0.205048	t = 1.649598	0.1041
	bilirubin (mg/dL)	b8 = 0.022068	r = 0.028541	t = 0.224826	0.8229
Blood Leukocytes TL (T/S)	**haemoglobin (g/dL)**	b1 = 0.003138	r = 0.141452	t = 1.125107	0.2649
	glycaemia (mg/dL)	b2 = -0.00175	r = -0.089968	t = -0.711294	0.4796
	cholesterol (mg/dL)	b3 = -0.000016	r = -0.001611	t = -0.012687	0.9899
	triglycerides (mg/dL)	b4 = 0.001514	r = 0.177891	t = 1.42342	0.1596
	HDL (mg/dL)	b5 = -0.000176	r = -0.008083	t = -0.06365	0.9495
	LDL (mg/dL)	b6 = -0.003492	r = -0.245463	t = -1.993777	**0.0506**
	creatinine (mg/dL)	b7 = -0.0017	r = -0.039478	t = -0.311094	0.7568
	bilirubin (mg/dL)	b8 = -0.000485	r = -0.018608	t = -0.146549	0.884

Abbreviations: HDL=high density lipoprotein, LDL=low density lipoprotein.

Multiple regression analysis of the influence of mean HR and HRV Parameters (i.e., SDNN, RMSSD) and drugs affecting HRV (i.e., antidepressants, beta-blockers, calcium channel blockers, inhaled or oral beta-mimetics, theophylline, and alpha-adrenergic agonists) on blood leukocytes DNAmAge and TL (Table 9) indicated that increased DNAmAge correlated with low mean HR and drug use affecting HRV, but not with other HRV parameters. No significant correlations were found for TL.

Table 9. Multiple regression analysis of the influence of mean HR, HRV parameters (i.e., SDNN, RMSSD), and drugs affecting HRV (i.e., antidepressants, beta-blockers, calcium channel blockers, inhaled or oral beta-mimetics, theophylline, and alpha-adrenergic agonists) on blood leukocytes DNAmAge / TL.

		b	r	t value	P
Blood Leukocytes DNAmAge (years)	**SDNN**	b1 = -0.311272	r = -0.186422	t = -1.564708	0.1223

 doi:10.20944/preprints202407.0377.v1

	RMSSD	b2 = 0.14605	r = 0.114644	t = 0.95165	0.3446
	Mean HR	b3 = -0.403812	r = -0.356467	t = -3.14618	**0.0025**
	Drugs affecting HRV (0=no; 1=yes)	b4 = 8.905208	r = 0.297306	t = 2.567761	**0.0124**
Blood Leukocytes TL (T/S)	**SDNN**	b1 = 0.008358	r = 0.166005	t = 1.388176	0.1696
	MSSD	b2 = -0.006522	r = -0.167858	t = -1.404117	0.1648
	Mean HR	b3 = 0.005025	r = 0.154982	t = 1.293644	0.2002
	Drugs affecting HRV (0=no; 1=yes)	b4 = 0.154968	r = 0.176234	t = 1.476368	0.1445

Abbreviations: SDNN=standard deviation of normal-to-normal RR intervals, RMSSD= root mean square of successive RR interval differences, HR=heart rate.

Multiple linear regression analysis of the influence of leukocytes (10^9/L) and different blood cell counts, including neutrophils (10^9/L), lymphocytes (10^9/L), and monocytes (10^9/L) on blood leukocytes DNAmAge and TL (Supplementary Table S1) showed positive correlations between TL and neutrophils (p=0.0006) and lymphocytes (p=0.0046), and negative correlations with leukocytes (p=0.0344), but not with monocytes. DNAmAge was not determined by leukocytes, neutrophils, lymphocytes, or monocytes (p>0.05).

2.4. Biological Age of Blood Leukocytes, IS Cells, and NC

Supplementary Table S2 reports number of subjects and mean values of biological aging indicators i.e., DNAmAge, AgeAcc, TL, in blood leukocytes, NC, and IS cells. DNAmAge of blood leukocytes (Figure 1A, r=0.9433, p<0.0001), NC, and IS (Figure S2, A r=0.8015, p<0.0001; B, r=0.9279, p<0.0001) was positively correlated with chronological age, while TL of blood leukocytes (Figure 1B, r=-0.3217, p=0.0046), but not of IS (Figure S2 C, r=-0.2641, p=0.2897), was negatively correlated with chronological age. Insufficient DNA prevented TL analysis in NC samples.

Figure 4A shows that in a subset of 17 HCWs with all tissue samples, the IS DNAmAge was higher than blood leukocytes (p=0.0011) and NC (p=0.0003), and NC DNAmAge was lower than blood leukocytes (p=0.0028). Similarly, Figure 4B reports that IS TL was shorter than blood leukocytes in the same patients (p=0.05).

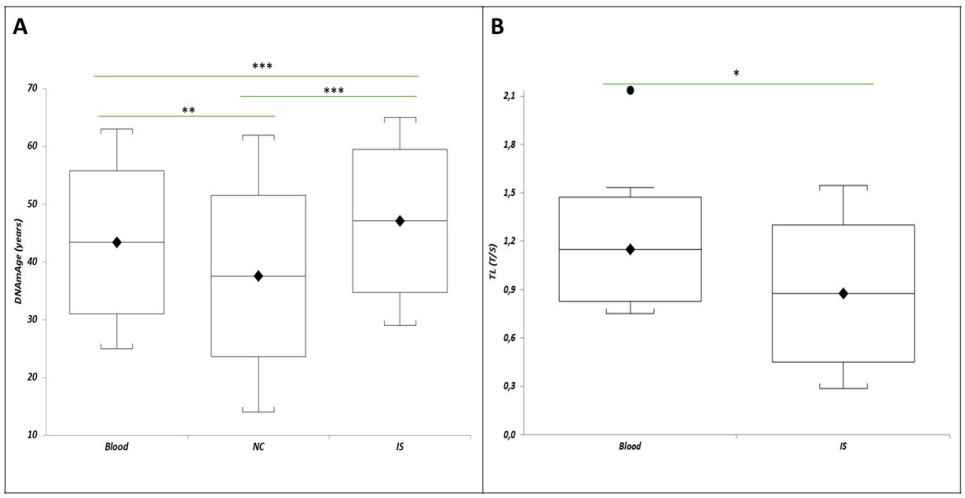

Figure 4. DNAmAge and TL of blood leukocytes, NC and IS cells in the subgroup of n = 17 HCWs COVID19 survivors. In (A), box plots show levels of DNAmAge (years) in blood leukocytes, NC and IS cells in the subgroup of n = 17 HCWs COVID19 survivors from whom all three tissue samples were collected. In box plots, the boundary of the box closest to the x-axis indicates the 25th percentile, the line within the box marks the mean, and the boundary of the box farthest from the x-axis indicates

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the 75th percentile. Whiskers (error bars) above and below the box indicate the 95 and 5th percentiles. The horizontal bar with asterisks indicates the significant comparison between blood leukocytes and paired NC of the same subject (*Paired *t*-test: mean 43.4 ± 12.9 years vs. mean 37.6 ± 14.0 years; p = 0.0028), NC and paired IS cells of the same subject (*Paired *t*-test: mean 37.6 ± 14.0 years vs. mean 47.1 ± 12.4 years; p = 0.0003), and blood leukocytes and paired IS cells of the same subject (*Paired t-test: mean 43.4 ± 12.9 years vs. mean 47.1 ± 12.4 years; p = 0.0011). In (B), box plots show levels of TL in blood leukocytes and paired IS cells of n = 17 HCWs COVID19 survivors. In box plots, the boundary of the box closest to the x-axis indicates the 25th percentile, the line within the box marks the mean, and the boundary of the box farthest from the x-axis indicates the 75th percentile. Whiskers (error bars) above and below the box indicate the 95th and 5th percentiles. The horizontal bar with an asterisk indicates the significant comparison between in blood leukocytes and paired IS cells TL of the same patient (*Paired *t*-test (n = 7): mean 1.15 ± 0.32 T/S vs. mean 0.88 ± 0.43 T/S; p = 0.05].

2.5. Correlations between Biological Aging Indicators

Simple linear regression analyses (Figure S3) showed positive correlations between DNAmAge of blood leukocytes and NC (r=0.8207; p<0.0001) and IS (r=0.9353; p<0.0001), but no correlation between TL of blood leukocytes and IS (r=-0.0222; p=0.9304).

2.6. Comparison of Biological Aging in HCWs and COPD Patients

Comparing HCWs (n=17) with COPD patients (n=7), HCWs showed greater biological aging in blood and IS cells, including higher AgeAcc (Table 10: blood leukocytes AgeAcc, p=0.0002; IS AgeAcc, p=0.012) and shorter TL (Table 10: predicted blood and IS TL, p<0.0001) one year after SARS-CoV-2 infection resolution.

Table 10. AgeAcc and Predicted TL Post COVID-19 subjects and COPD patients.

Post COVID-19 subjects					
N=17	**Age**	***Blood* AgeAcc (years)**	***IS AgeAcc (years)***	***Predicted Blood TL (T/S)***	***Predicted IS TL (T/S)***
Mean± SD	46.00±12.88	-2.59±3.47*§	1.12±4.37§	1.20±0.06*§	0.93±0.02§
COPD patients					
N=7	**Age**	***Blood* AgeAcc (years)**	***IS AgeAcc (years)***	***Predicted Blood TL (T/S)***	***Predicted IS TL (T/S)***
Mean± SD	72.43±6.00	-10.29±3.50*	-4.29±5.15	1.31±0.03*	0.97±0.01

Abbreviations: AgeAcc = Age acceleration; IS= Induced sputum cells; TL= Telomere Length. ****Paired t test:*** *Post COVID19 subjects.* Blood AgeAcc versus IS AgeAcc (Two sided p=0.0011). Predicted Blood TL (T/S) vs Predicted IS TL (T/S) (Two sided p<0.0001). *COPD patients.* Blood AgeAcc versus IS AgeAcc (Two sided p=0.0006). Blood TL versus IS TL (Two sided p<0.0001). §***Mann-Whitney U test:*** PostCOVID19 Blood AgeAcc versus COPD Blood AgeAcc (Two sided p=0.0002). PostCOVID19 IS AgeAcc versus COPD IS AgeAcc (Two sided p=0.012). PostCOVID19 Predicted Blood TL (T/S) vs COPD Predicted Blood TL (T/S) (Two sided p<0.0001). PostCOVID19 Predicted IS TL (T/S) vs COPD Predicted IS TL (T/S) (Two sided p<0.0001).

3. Discussion

This study offers groundbreaking insights on the biological aging, long-term sequelae, and their impact on work ability in pauci- and asymptomatic HCWs of the University Hospital of Padua, who survived COVID-19 during the first wave (February-May 2020). We also assessed the biological aging of blood leukocytes, NC, and IS cells in HCWs and compared them to a control group of COPD

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patients, who are considered a model group for 'aged lung' resulting from continuous inflammatory action affecting these patients [23].

3.1. PCS and Symptom Prevalence

At 12 weeks, PCS symptoms were more prevalent in women than men consistent with Ortona et al.'s findings that women are twice as likely as men to develop PCS until around the age of 60 [24]. Given our population's median age of 47.5 years and a peak age of 66 years, this trend is confirmed. Women make up about 71% of our study population and dominate the healthcare workforce, with the WHO reporting that women constitute 67% of the global health and social care workforce [25]. This demographic representation explains the higher prevalence of PCS among women in our study. Furthermore, the autoimmune hypothesis posits that women's stronger immune response, influenced by genetic and hormonal factors, leads to a higher incidence of PCS [24,26]. After 1 year, symptom prevalence was similar between genders, with symptoms decreased in women but persisted in men. This persistence is an ongoing area of investigation. Overall, 30% of HCWs reported persistent symptoms 1 year post-infection. This is in line with previous studies in paucisymptomatic individuals, reporting similar rates at one year [11,27,28], and even two years post-infection [29]. Persistent symptoms such as dyspnea, palpitations, peripheral neuropathy, anxiety, loss of concentration, and memory problems were observed approximately one year after infection, confirming earlier findings [14,27]. These underscore the need for ongoing research and tailored healthcare strategies to manage the long-term effects of PCS, particularly among HCWs, who represent our study cohort.

3.2. Determinants of Increased Blood Leukocyte Dnamage

3.2.1. Sex-Related DNAmAge Differences

Male sex is significantly associated with increased DNAmAge, corroborating findings from Oblak et al. [30]. This aligns with the male-female health survival paradox, where males typically have shorter lifespans than female, who tend to experience higher rates of disability and poor health [31], supporting again the need for sex-specific health strategies.

3.2.2. Impact of SARS-CoV-2 Infection

Increased leukocyte DNAmAge correlates with the duration of SARS-CoV-2 infection (average 17 days) because prolonged infections lead to sustained inflammatory responses and cellular stress, which induce significant epigenetic changes [32–35]. This mechanism is similar to that observed in other viral infections like HIV [36–38] and SARS-CoV-2 infection [16], where extended viral presence exacerbates epigenetic modifications, thus increasing DNAmAge. This insight is crucial for developing post-infection management strategies, particularly for those with extended infection durations, to mitigate accelerated aging effects.

3.2.4. Chronic Diseases and DNAmAge

Our findings confirm that chronic diseases are linked to elevated DNAmAge, consistent with previous research on frailty [39], cancer [40], diabetes [41], cardiovascular diseases (CVD) [42], dementia [43], and the decreased lung function (FEV1) in COPD patients [21], a known consequence of aging [44–46]. This underscores the importance of managing chronic diseases to potentially slow down the biological aging process and improve overall health outcomes.

3.2.5. Lung Function and DNAmAge

We also found a correlation between the acceleration in DNAmAge and the decline in lung function measured by forced expiratory volume (FEV1), which is a well-documented consequence of aging [44–46]. This finding is consistent with our previous work on COPD patients [21]. Therefore, DNAmAge appears to be a reliable signature of the epigenetic aging chronic disease-related.

 doi:10.20944/preprints202407.0377.v1

3.2.6. Lipid Levels and DNAmAge

We identified a positive association between DNAmAge and LDL levels. Even if our finding contrasts with an unexpected inverse association reported by Ammous et al. [47], it aligns with the hypothesis on the detrimental health effects of these lipids [48,49], which are connected to an increased risk of CVD [50–52] and suggests that lipid management could be integral to mitigating accelerated aging. This highlights the need for comprehensive lipid monitoring and management strategies in PCS patients.

3.2.7. Blood Glucose and DNAmAge

Our study also found a positive correlation between DNAmAge and blood glucose, which showed consistent and stronger associations with CVD risk factors in patients with diabetes (Borg et al., 2011). This correlation underscores the critical role of glucose regulation in aging. High blood glucose contributes to oxidative stress and inflammation, leading to epigenetic changes that increase DNAmAge. Effective glycemic control is crucial for slowing biological aging, particularly in diabetic populations, as highlighted by recent research [53].

3.2.8. Work Capacity and DNAmAge

The higher DNAmAge found in daytime workers may be attributed to older age, as they transition to daytime roles due to the challenges of night shifts. Furthermore, HCWs with greater DNAmAge showed lower WAI scores, marking this as the first study to link leukocyte DNAmAge with WAI, consistent with the decline in work capacity due to aging and chronic diseases [54,55]. Work capacity, measured by WAI, refers to an individual's perception of the balance between work demands and their ability to cope with them [56], resulting from the interaction between psychosocial and physical work-related elements, mental and physical capabilities, and health issues [57,58]. Our finding calls for workplace interventions to support aging workers, such as job modifications and health promotion programs, to sustain their work ability and productivity.

3.2.9. Lymphocyte Counts and DNAmAge

We observed a negative correlation between DNAmAge and lymphocyte counts. Aging is known to reduce the number of B cells and CD4+ and CD8+ T cells, increasing memory T cells while decreasing naïve T cells [59]. This finding is supported by Zhang et al. [60], who noted variations in epigenetic age depending on lymphocyte subpopulations. Strategies to also support immune health could play a role in mitigating DNAmAge increases, especially in the aging workforce

3.2.10. Haemoglobin Levels and DNAmAge

An association between higher DNAmAge and lower haemoglobin levels was found, reflecting the intertwined nature of aging and haematological health. Lower haemoglobin levels are indicative of anemia, which is common in older adults and linked to increased biological aging. This condition is often linked to increased biological aging due to its association with chronic inflammation, oxidative stress, and decreased erythropoiesis [61]. Monitoring and addressing haemoglobin levels can be beneficial in managing age-related health risks and potentially mitigating accelerated biological aging.

3.2.11. HR, HRV and DNAmAge

We found a relationship between increased DNAmAge and low mean HR. Basal and non-basal HR decreases with age and elderly people are more prone to bradycardia [62], and even HRV decreasing with age [63]. DNAmAge is a marker of aging associated with pathological conditions [64]. Increased DNAmAge signifies accelerated biological aging, exacerbated by factors like chronic stress and infections. COVID-19 survivors show increased DNAmAge [19] and reduced mean HR one year after SARS-CoV-2 infection compared to the post-acute phase [65], highlighting the impact

 doi:10.20944/preprints202407.0377.v1

of infections on biological aging. Maintaining HR and HRV through interventions could mitigate accelerated aging.

Furthermore, we noted an association between drugs affecting HRV and higher leukocyte DNAmAge. Drugs such as beta-blockers, calcium channel blockers, inhaled or oral beta-mimetics, theophylline, and alpha-adrenergic agonists like phenylephrine infusion can interfere with HRV [65,66]. While limited research exists, some studies indicate antihypertensives like calcium channel blockers may reduce DNAmAge [67]. Therefore, our findings highlight the need for further investigation into the effects of other HRV-modifying drugs on DNAmAge and elucidate the underlying biological mechanisms. Further research is necessary to elucidate the biological mechanisms and optimize pharmacological strategies to minimize adverse effects on biological aging.

Lastly, we established the robust positive correlation between DNAmAge and chronological age, reinforcing the accuracy of our analysis and the predictive strength of our model. This consistency with established methodologies [68,69] underscores that DNAmAge is a reliable biomarker for biological aging.

3.3. Determinants of Shorter Blood Leukocytes TL

Our study established a negative correlation between leukocyte TL and chronological aging, consistent with existing literature such as the systematic review by Müezzinler et al. [70] across 124 cross-sectional studies, reported a similar negative correlation ($r = 0.3$).

3.3.1. WAI

HCWs with shorter leukocyte TL exhibit lower WAI, reflecting accelerated biological aging. Chronic job-related stress and inflammation accelerate telomere shortening, impairing cellular repair and function [71]. This relationship is biologically plausible as shorter TL indicates advanced cellular aging, which reduces physical and cognitive capacity, impacting work ability. The parallel with epigenetic age (DNAmAge) further supports this connection, highlighting the detrimental effects of occupational stress on aging markers and work capacity. Interventions to reduce stress could improve HCWs' health and work performance.

3.3.2. LDL Levels and Cardiovascular Disease

Our research supports the well-documented association between shorter TL and CVD, alongside elevated LDL levels as a major risk factor [72]. The observed correlation between shorter leukocyte TL and higher LDL levels contributes another piece to this controversial area, aligning with some previous studies [73–77], while contradicting others [76,78–80]. This underscores the need for further investigation into the interplay between lipid levels and TL.

3.3.3. Blood Leukocyte TL and Job Position

The observation that blood leukocyte TL is decreased in HCWs involved in direct patient contact (assistants, nurses, doctors, residents) can be explained by a few key mechanisms. HCWs face high levels of chronic stress due to long hours, high workload, and emotional strain [81]. Chronic stress increases cortisol levels, which can lead to oxidative stress and inflammation. Elevated cortisol from chronic stress increases oxidative stress, producing reactive oxygen species (ROS) that damage cells and DNA, including telomeres [71]. Frequent exposure to pathogens (viruses, bacteria) in healthcare settings triggers immune responses, increasing leukocyte replication and further contributing to telomere shortening [82–84]. Overall, the combination of chronic stress, oxidative stress, inflammation, and frequent exposure to pathogens leads to accelerated telomere shortening in HCWs involved in direct patient contact.

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3.3.4. Lymphocyte Numbers

We observed a relationship between TL shortening and a decrease in lymphocyte numbers. Although lymphocytes experience a faster rate of age-dependent TL shortening than granulocytes [85], recent studies have shown significant reductions in lymphocyte numbers among healthy COVID-19 survivors [86]. Furthermore, our analysis revealed that TL shortening was associated with an increased total leukocyte count, primarily driven by monocytes. This is in line with findings of generalized low-grade inflammation, T lymphocyte senescence, and increased monocyte activation in individuals with long-COVID [87]. Mean leukocytes TL is considered an indicator of biological aging [88]. Our findings, linking TL shortening to lymphocyte reduction, expand on this understanding, suggesting novel mechanisms underlying TL dynamics in relation to immune cell populations and chronic stress responses.

These innovative results provide new insights into the determinants of leukocyte TL, advancing our comprehension of biological aging and its interaction with chronic disease, stress, and occupational factors.

3.4. Biological Age of the Blood Leukocytes, IS Cells, and NC Determined by DNAmAge AND TL

Our study uniquely analyzed DNAmAge and TL in blood leukocytes, IS cells, and NC from the same cohort of 17 HCWs who survived COVID-19. This comprehensive comparison revealed that IS cells exhibit a higher biological age than both blood leukocytes and NC. Specifically, IS cells demonstrated higher DNAmAge and shorter TL, while NC had a lower DNAmAge compared to blood leukocytes, indicating different aging rates within the same individuals.

3.4.1. Tissue-Specific Aging Rates

The accelerated aging observed in IS cells compared to blood leukocytes and NC confirms that different tissues and organs age at varying rates within the same individuals. This aligns with previous findings on heart, kidney, and COPD patients [21,89,90], showing tissue-specific aging rates [68]. The study also suggests that cells in the deeper airways of COVID-19 survivors are more susceptible to epigenetic changes than those in more superficial airways and blood leukocytes [21].

3.4.2. COVID-19 Impact on DNAmAge and TL

Our study supports existing research showing that COVID-19 significantly alters DNA methylation profiles, particularly in critically ill patients [91,92]. These epigenetic changes persist even after recovery, suggesting long-term impacts on gene expression and cellular function [34,93–95]. Additionally, shorter telomeres are associated with severe COVID-19 and lingering post-COVID-19 conditions, supporting the hypothesis that telomere attrition plays a role in the pathology of COVID-19 [96–98]. Telomere shortening can lead to cellular aging and reduced regenerative capacity, contributing to the severe and long-lasting effects observed in COVID-19 patients [96,99,100]. Our findings agree with other studies that have documented accelerated biological aging in various tissues due to COVID-19 [16,19,101], emphasizing the need for further research into the long-term effects of the virus on cellular health and aging.

3.4.3. Biological Implications of Telomere Shortening in IS

Telomere shortening, a marker of biological aging, occurs with increased cell division and DNA replication. Our study found that IS cells, the primary target of SARS-CoV-2, showed significant telomere shortening, supporting previous findings in alveolar epithelial cells of COVID-19 patients [101]. This suggests that SARS-CoV-2 infection accelerates telomere shortening due to an enhanced proliferative response to regenerate alveolar injury, potentially leading to long-term lung fibrosis [101,102].

 doi:10.20944/preprints202407.0377.v1

3.4.4. Epigenetic Aging in IS cells, NC and Implications for Surrogate Tissue Use

While the change in methylation profile is evident in the blood of COVID-patients and survivors [16,19,103], there is no data yet on the other target tissues. However, epigenetics, linking environmental and genetic factors [104], is recognized as the basis of inflammation [105], which underpins several lung diseases like COPD, cancer, and COVID-19 [106]. The lung is the primary target of SARS-CoV-2 infection, causing diffuse alveolar damage, apoptotic epithelial cells, interstitial inflammation and activated T-cell responses resulting in a cytokine storm [107], mainly through host immune dysregulation, increased inflammation and/or hyperinflammation. This similarity and overlap in the pathogenetic mechanism between lung disease and COVID-19 [106], coupled with the results of our previous study in COPD patients showing accelerated lung aging [21], supports our finding that IS cells are older than blood leucocytes.

Interestingly, NC were found to be biologically younger than IS cells and blood leukocytes. This could be due to their role as the initial entry point for SARS-CoV-2 [108], acting as a gateway to the lower respiratory tract and triggering systemic inflammation upon viral replication [109], or as a gateway to the central nervous system [110,111].

A strong correlation was found between the DNAmAge of IS cells, NC, and blood leukocytes, suggesting that blood leukocytes could serve as a surrogate for studying lung and airway aging. However, caution is advised as there was a noted six-year difference in DNAmAge between lung tissue and blood leukocytes in post-COVID-19 patients, mirroring findings in COPD patients [21].

3.5. Comparison of Biological Aging (AgeAcc and TL) in HCWs and COPD Patients

Given the numerous overlaps between COPD and COVID-19 [106], we compared a group of HCWs recruited approximately one year after SARS-CoV-2 infection with a group of COPD patients as a positive control group. COPD patients are considered a suitable positive control group because they exemplify accelerated biological aging due to chronic inflammation and oxidative stress [21,112–115].

Our results revealed that the blood leukocytes and IS cells of HCWs are biologically older than those of COPD patients, as determined by AgeAcc and predicted TL. This indicates that COVID-19 may induce more pronounced epigenetic changes and telomere attrition than COPD.

3.6. Limitations and Strengths

The current study has several limitations. Firstly, the lack of a control group of COVID-19-free, age-matched subjects is a significant limitation. This was due to the difficulty in recruiting HCWs approximately one year after infection during the first wave (February-May 2020), when workloads were high and reinfections were common, leading to the exclusion of these subjects from the study. To address this, we compared our HCWs population (n=17), for whom we had all three tissues available, with a small positive control group of COPD patients (n=7) known for accelerated lung aging compared to blood leukocytes [21,112,115].

The ongoing pandemic and restrictions imposed by the University Hospital of Padua on techniques involving droplets and airborne exposure, such as spirometry and the induced sputum technique, limited the number of IS samples collected. Despite the limited number of subjects (n=17) for whom we have all three tissues (IS cells, NC, and blood leukocytes), our sample size estimate indicates it is sufficient to obtain statistically significant results. Another limitation is the inability to analyze TL in NC samples due to insufficient DNA after performing DNAmAge analysis.

Our study has several strengths. Firstly, it provides a comprehensive assessment of biomarkers of biological aging, both genetic (TL) and epigenetic (DNAmAge), related to various parameters, including inflammation, basic hematochemical biomarkers, lung function indicators, and data on demographics, lifestyle, work, and physiological history. This study involved the collaboration of many healthcare professionals, including clinicians and researchers.

To our knowledge, this is the first study to determine the biological aging of post-COVID-19 subjects across three different tissues collected from the same individual, comparing blood leukocytes

 doi:10.20944/preprints202407.0377.v1

with SARS-CoV-2 target tissues (IS cells and NC). We found a strong correlation between the DNAmAge of IS cells and NC with that of blood leukocytes, suggesting the potential use of blood as a surrogate indicator of the biological age of IS cells and NC, although further investigations are needed.

Another strength of our study is the use of a validated non-invasive airway sampling technique, the induced sputum technique, to study biological age indicators in IS cells. This technique could be valuable for future research on lung biological aging, not only in COPD patients but also in other conditions.

4. Materials and Methods

4.1. Study Design

This study is a cross-sectional study in which the established clinical protocol has been applied to examine SARS-COV-2 positive healthcare workers (HCW) of the University –Padova Hospital approximately 1 year after the diagnosis of SARS-CoV-2 infection. The inclusion criteria were to be SARS-COV-2 positive HCW of the University –Padova Hospital, do not have COVID-19 vaccination, and not have had COVID-19 reinfection in the 12 months preceding the clinical visit. Study population consists of the n=76 among the n=144 HCW of the first wave (February-May, 2020). They were enrolled during the health surveillance activity foreseen according to Legislative Decree 81/2008 at the Occupational Medicine Unit approximately 12 months after the diagnosis of SARS-CoV-2 infection with molecular swab. Paucisymptomatic and asymptomatic HCWs, who did not have symptoms at the time of the swab and who continued to remain asymptomatic during the entire isolation period (at least 14 days), were included in the study population. The local Ethics Committee approved the study (288n/AO/22) in accordance with the principles of the Declaration of Helsinki.

All participants were informed of the purpose of the study and provided a written informed consent. Subjects unwilling to participate to the study were excluded. At enrolment each study participant was characterized by an ad hoc structured questionnaire to collect information on, among others, demographics, lifestyle, medical history, environmental and occupational exposure. The assessment of medium-long term effects in HCW who have had confirmed COVID-19, irrespective of whether they were hospitalized or had a positive SARS-CoV-2 molecular swab, were performed according to Guidelines of Managing the long-term effects of COVID-19 [116]. All patients underwent a clinical examination including respiratory function tests, cardiac assessment with evaluation of heart rate variability (HRV), administration of the work ability index (WAI) questionnaire to assess their work ability. For each patient, the biological samples were also collected for basic biochemistry tests, immunological profile and biological aging analyses. Table 1 summarizes the characteristics of the study population, including demographic variables, lifestyle, occupational history, basic biochemistry parameters, liver function, inflammation, lung function and HRV. Table 2 reports all data on the course of SARS-CoV-2 infection. The biological aging of HCWs was compared with that of a positive control group for biological aging parameters, including n=7 patients with chronic obstructive pulmonary disease (COPD) who gave their final consent to participate in the study [21], which had already been approved by the Ethics Committee (3849/AO/16) in accordance with the principles of the Declaration of Helsinki.

Table 2. Data relating to the course of SARS-CoV-2 infection (n=76 HCWs).

Variable	Mean±SD	N subjects	%
Duration of SARS-CoV-2 infection [days]	17.81±6.03		
Diagnosis of SARS-CoV-2 pneumonia			
Yes		4	5.26
No		72	94.74
Hospitalisation for COVID-19 [n (%)]		5	6.58
Drug therapy during acute infection [n (%)]			

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Antibiotics	14	18.42
Inhaled Corticosteroids	2	2.63
Systemic Corticosteroids	4	5.26
NSAID or paracetamol	49	64.47
Hydroxychloroquine	8	10.53
Tocilizumab	1	1.32
Antiviral	2	2.63
Anticoagulant	3	3.95
Other	7	9.21
None	22	28.95

Abbreviations: NSAID= Non-steroidal anti-inflammatory drug.

4.2. Information Acquired through Questionnaires

An ad hoc structured questionnaire [117] was administered during interviews to elicit information on: demographics (age, sex, marital status) 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, MET) at work; work injury], and medically relevant complaints including cardiovascular disease, musculoskeletal disorder, spinal disc hernia, gastrointestinal disease, endocrine disease, diabetes, respiratory disease and tumors. The Charlson comorbidity index, 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 [118]. Smoking history (current active smokers, former-smokers, never smokers) and pack-years [(number of cigarettes smoked per day/20) × number of years smoked] were also recorded, as well as the habitual alcohol consumption (yes/no), alcohol intake (units of drink/day, each unit being approximately 10–12 g alcohol intake) and binge drinking (>4 drink-units/day, i.e., more than 40 g alcohol/day). Physical activity in leisure time was estimated according to the International Physical Activity Questionnaire (IPAQ score).

4.3. Work Ability Assessment

The Work Ability Index (WAI), a self-assessment questionnaire consisting in 7 domains, was used as previously described [118]. WAI ranged from 7 to 49 points; four categories were identified to describe WAI levels into “poor” (score 7–27), “moderate” (score 28–36), “good” (score 37–43), and “excellent” (score 44–49) work ability as a function of the total WAI score. This is a valuable tool to identify any imbalances between what is required (performance requirements) and what you are able to give (individual potential) [119].

4.4. Respiratory FUNCTION TESTS

ALL LUNG FUNCTION MEASUREMENTS, Including forced expiratory volume in 1 second (FEV1), forced vital capacity (FVC), forced expiratory flows at different lung volumes), total lung capacity (TLC), residual volume (RV), were measured using a spirometer (Jaeger MasterScreen PFT, PRO, Viasys Sanità, Firenze, Italy) according to the guidelines/recommendations of the American Thoracic Society/European Respiratory Society (ATS/ERS) [120]. Predicted normal values from the Communaute Europeenne du Carbon et de l’Acier (CECA) were used [121].

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4.5. Assessment of Autonomic Cardiac Balance and HRV Parameters

ECG was recorded during periodical health checks. Blood pressure was also measured with an Omron 705IT electronic device (Omron Healthcare Europe, The Netherlands) while the patient was lying quietly for at least 5 min, according to the recommendations of the 2023 European Society of Hypertension [122]. Subjects were instructed to avoid smoking, and to stop coffee and alcohol intake for 2 h and 48 h, respectively. They should have had sufficient (at least 8 h) rest and must not have worked the night shift before the test was performed. Blood pressure was measured once with a sphygmomanometer while the patient was lying calmly. HRV was assessed by an ECG performed in a supine position under physiologically stable conditions and using a device connected to the patient via two electrodes. HRV data were acquired by a Bluetooth acquisition system (BT16 Plus, FM, Monza, Italy). ECG was recorded for at least 5 min between 9 a.m. and 2 p.m., at rest and under ideal temperature conditions. HRV was analyzed using Kubios HRV software (ver. 3.3) [123]. Normal and aberrant complexes were identified and all adjacent intervals between normal beats over 5 min intervals were considered. We analyzed the spectral components (HRV frequency domain variables) as the absolute values of power (ms2). Power spectral density was analyzed with an autoregressive modeling-based method (AR spectrum), using the default value for the model order, i.e., 16. The main spectral components were very low frequency (VLF), low frequency (LF), high frequency (HF), and the LF/HF ratio. The area under the curve of the spectral peaks within the frequencies 0.01–0.4, 0.01–0.04, 0.04–0.15, and 0.15–0.40 Hz were defined as the total power (TP), very low-frequency power (VLF), low-frequency power (LF), and high-frequency power (HF), respectively. In order to normalize LF and HF, we used the total power within the frequency range of 0.01–0.4 Hz. The normalized low-frequency power (nLF = LF/TP) corresponds to an index of combined sympathetic and vagal modulation [124] as well as a baroreflex index [125,126], while the normalized HF power (nHF = HF/TP) represents an index of vagal activity. The low/high-frequency power ratio (LF/HF) is thus an index of sympathovagal balance. Time domain measures included the standard deviation of normal-to-normal RR intervals (SDNN), the root mean square of successive RR interval differences (RMSSD)".

4.6. Samples Collection and IS Procedure

For each patient, blood samples were collected for basic biochemistry, immunological profile and biological aging analyses (i.e., TL and DNAmAge). During medical examination, the procedure of sputum induction and nasal swab were carried out for each patient to collect both a sample of airways cells and nasal epithelium cells, respectively, for biological aging analyses.

IS procedure was performed according to a standard protocol, and the induced sputum sample was processed as previously described [21].

4.7. Basic Biochemistry Analyses

Data of basic biochemistry included number of blood red cells, platelets and white cells, lymphocytes, monocytes, neutrophils, basophils, eosinophils, hematocrit, hemoglobin, blood glucose, triglycerides, cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL), and c-reactive protein (CRP), interleukin 6 (IL-6), alanine aminotransferase (ALT), aspartate aminotransferase (AST), Gamma-glutamyl transferase (gamma-GT), ferritin, total bilirubin, protein profile and creatinine. All the analyses were performed at the Laboratory Medicine Unit (AOUP).

4.8. DNA Extraction (from Biological Samples)

DNA was extracted from whole blood samples using the QIAamp DNA Mini Kit (Qiagen, Milano, Italy) on a QIAcube System (Qiagen, Milano, Italy) for automated high-throughput DNA purification, according to a customized protocol as previously described [21].

DNA extraction was also carried out from the IS and nasal cells collected, by the automated QIAcube System (Qiagen, Milano, Italy) utilizing QIAamp DNA Mini Kit (Qiagen, Milano, Italy) according to a customized protocol developed for highly viscous samples as previous described [21].

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DNA was quantified and checked for quality using QIAxpert Quantification System (Qiagen, Milano, Italy).

4.9. DNAmAge Analysis and AgeAcc Estimation

DNAmAge was determined by analysing the methylation levels of five selected markers (ELOVL2, C1orf132, KLF14, TRIM59 and FHL2) in genomic DNA using bisulfite conversion and Pyrosequencing® methodology on PyroMark Q48 Autoprep (QIAGEN, Milano, Italy), as previously described [127]. The methylation levels of were expressed as a percentage of methylated cytosines at the 5 CpG sites considered and were used for estimation of DNAmAge as previously reported [127]. All samples were analysed 3 times for each marker to verify the reproducibility of our results, and their average was used in statistical analyses. All samples were analysed in two different days and coefficient of variation (CV) in replicate pyrosequencing runs was 0.5 %. AgeAcc was computed as the discordance between the DNAmAge of blood leukocytes and IS and NC and the subjects' chronological age.

4.10. TL Analysis

TL was determined using quantitative real-time PCR after DNA extraction from both whole blood and IS samples [128]. This assay determines the ratio of telomere repeat copy number (T) to a single nuclear copy gene (S) in experimental samples relative to the T/S ratio of a reference pooled sample to determine measure TL in genomic DNA. Human (beta) globin (hbg) was the single-copy gene used. The PCR runs were performed in triplicate using a StepOnePlus Real-Time PCR System (Applied Biosystems, Milano, Italy), and the average of the three T/S ratio measurements was considered in the statistical analyses. To assess measurement reproducibility, 20 % of samples were replicated on separate days, and the CV for the average T/S ratio was accepted if less than 10%.

4.11. Statistical Analyses

Univariate and multivariate methods were used selecting the appropriate models. The analysese were performed using the statistical software StatsDirect and Rstudio. Regarding the analysis of biological age, our hypothesis of an accelerated lung and in nasal epithelium by COVID-19 infection were converted in a model with two final outcomes: TL and DNAmAge as previously described [21]. The biological aging of HCWs after 1 year from COVID-19 was compared to that of COPD patients as a positive control group for biological aging parameters. For this comparison we used the AgeAcc, i.e., the difference between DNAmAge and chronological age, and TL predicted computed by regressing TL measurements on chronologic age for each subject.

5. Conclusions

This innovative study's approach, involving the assessment of biological aging across multiple tissues, provides valuable insights into how different tissues age at varying rates within the same individuals. It also underscores the significant impact of SARS-CoV-2 on biological aging, revealing that HCWs exhibit accelerated aging in blood leukocytes and IS cells compared to COPD patients, even one year after SARS-CoV-2 infection. These findings highlight the need for targeted interventions to mitigate the long-term effects of COVID-19 on cellular aging and health also in pauci- and asymptomatic COVID-19 survivors who represent the general population.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: Percentage of subjects with and without persistent symptoms at 1 year after SARS-CoV2 infection, out of a total of n=76 HCWs; Figure S2: Correlation curves between NC and IS DNAmAge (A) or IS TL (B) with chronological age of HCWs COVID19 survivors; Figure S3: Correlation curves between blood leukocytes DNAmAge and NC DNAmAge (A) or IS DNAmAge (B), and blood leukocytes TL and IS TL (C) of HCWs COVID19 survivors; Table S1: Multiple linear regression analysis of the influence of leukocytes (109/L) and different blood cell counts, including neutrophils (109/L), lymphocytes (109/L), and monocytes (109/L) on blood leukocytes DNAmAge / TL; Table S2: Number of different tissues (blood, nasal cells,

induced sputum cells) samples collected from our HCWs population, biomarkers of biological aging analyzed (DNAmAge, AgeAcc and TL), and measurements determined (mean(±SD)).

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the local Ethics Committee approved the study (288n/AO/22) in accordance with the principles of the Declaration of Helsinki.

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Data Availability Statement: The datasets presented in this study are available from the corresponding author upon reasonable request.

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