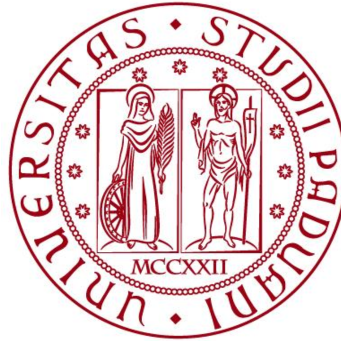
UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI BIOLOGIA

Corso di Laurea magistrale in Biologia Sanitaria



TESI DI LAUREA

A PILOT STUDY ON EFFECTIVENESS OF VISUAL ARTS MEDIATION IN COGNITIVE ACTIVATION THERAPY (CAT) ON BIOLOGICAL AGEING AND COGNITIVE/FUNCTIONAL STATUS

Relatore: Prof.ssa Sofia Pavanello
Dipartimento di Scienze Cardio-Toraco-Vascolari e Sanità Pubblica

Correlatore: Dott.ssa Manuela Campisi
Dipartimento di Scienze Cardio-Toraco-Vascolari e Sanità Pubblica

Laureanda: Isotta Farinon

ANNO ACCADEMICO 2022/2023

INDEX

1. ABSTRACT 2
2. ABBREVIATIONS 3
3. INTRODUCTION 4
3.1 Epidemiology of ageing 4
3.2 Chronological versus biological aging 5
3.3 Active ageing 6
3.4 Factors involved in ageing 9
3.5 Oxi-Inflamm-Ageing 11
3.6 Characteristics of the aged cell 14
3.7 Biological ageing indicators: LT and DNAmAge 25
3.8 Reversing Ageing 26
3.8.1 Pharmacological and no-pharmacological therapies 27
3.9 Dementia 30
3.9.1 Dementia and biomarkers of ageing 31
4. OBJECTIVES 32
5. MATERIALS AND METHODS 33
5.1 Study Design 33
5.2 Cognitive assessment 34
5.3 Analytical procedures 35
5.3.1 Extraction and quantification of DNA from blood 35
5.3.2 Telomere Lengths 36
5.3.3 DNAmAge 38
5.3.4 Statistical Analysis 46
6. RESULTS 47
6.1 Characteristics of study subjects 47
6.2 Telomere length analysis 50
6.3 Epigenetic ageing analysis 54
6.4 Cognitive tests results 60
7. DISCUSSION 65
8. CONCLUSION 71
9. RIASSUNTO IN ITALIANO 73
10. BIBLIOGRAPHY 75

1. ABSTRACT

Ageing is one of the most significant phenomenon of the 21st century and it’s also the greatest risk factor for the development of dementia.

In this pilot study we analyse whether visual arts-mediated Cognitive Activation Therapy (CAT), a structured and standardized rehabilitation protocol, influences biological ageing and cognition/functional status. Twenty patients affected by major neurocognitive disorder in the mild to moderate phase of the disease were recruited at the Regional Centre for Cerebral Aging (CRIC) and underwent a cycle of CAT lasting 30 days. The biological ageing was determined on blood samples by evaluation of biological ageing indicators, epigenetic age (DNAmAge) and leucocyte telomere length (LTL), at the beginning and at the end of CAT.

Promising results emerged, revealing CAT's potential in influencing biological ageing. CAT extended telomere length, potentially slowing ageing through stress reduction, enhanced cellular health, and overall well-being. Gender-specific LTL differences were noted, attributed to biological, genetic, and hormonal factors. DNAmAge correlated significantly with age, validating the model's epigenetic age estimation accuracy. Unexpectedly, ΔDNAmAge correlated positively with therapy duration, requiring further investigation. Cognitive tests demonstrated CAT's positive impact on visuo-constructional ability, visual memory, object and space perception, functional communication skills, and overall functional capabilities.

2. ABBREVIATIONS

CAT: Cognitive Activation Therapy;

CRIC: Regional Centre for the Study and Treatment of Cerebral Aging

DNAmAge: DNA Methylation Age

LTL: Leucocyte telomere length;

ΔLTL (T1-T0): Difference between leucocyte telomere lengths at the beginning of the treatment (T0) and at the end (T1).

ΔDNAmAge (T1-T0): Difference between DNAmAge at the beginning of the treatment (T0) and at the end (T1).

3. INTRODUCTION

3.1 Epidemiology of ageing

Ageing is one of the most significant phenomenon of the 21st century with important consequences on all sectors of society.

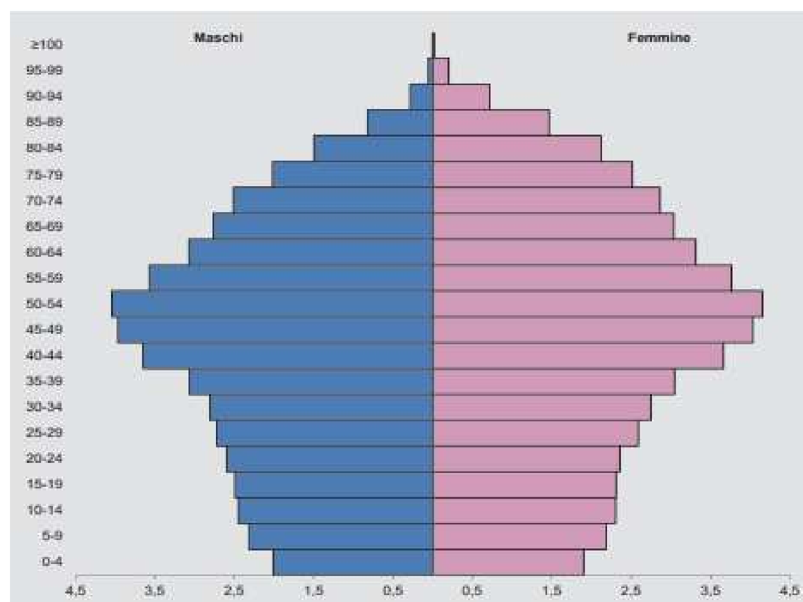
According to The World Health Organization (WHO, 2022) 1 in 6 people in the world will be aged 60 years or over by 2030. In fact, WHO estimated that the number of individuals that were aged 60 or over in 2020 is expected to rise from 1 billion to 1.4 billion by 2030 and to 2.1 billion by 2050. Furthermore, the number of persons aged 80 years or older is expected to triple between 2020 and 2050 to reach 426 million (WHO, 2022). This phenomenon, called Grey (silver) tsunami, is due to an improvement of hygiene conditions, lifestyle and to a great progress made in the fields of modern medicine and experimental research. In addition to the continuous increase in the survival of the older ages, in Italy there is a steady decline in fertility. The situation is graphically represented by the population pyramid, characterised by a base, corresponding to the youngest age groups, which is particularly contracted and a tip, representing the population at the oldest ages, enlarged. The pyramid also makes clear the advantage enjoyed by women in terms of survival, with the contingents of the female population at older ages being larger than those of their male peers (Figure 1).

On 1 January 2022, the old-age index was 187.9% (estimated data), growing compared to the previous year (182.6%). The old-age index, given by the ratio of the population aged 65 and over to the population aged under 15, is the indicator that best summarises the degree of aging of the population.

After declining in 2020, life expectancy at birth. i.e. the average number of years an individual expects to live, returned to increase in 2021, reaching 80.1 years for males and 84.7 for females.

In 2021 the decline in births continued: live births, which in 2020 reached 404,892, dropped for the first time under the threshold of four hundred thousand (399.431). The birth rate remained 6.8 births per thousand inhabitants.

The total fertility rate (TFT), i.e. the average number of children per woman, fell further down from 1.27 in 2019 to 1.24 children per woman on average in 2020.
In 2021, deaths fell by more than 30,000 compared to the previous year, and stand at a value of 709,035 (-4.2 per cent). The mortality ratio from 12.5 per thousand in 2020 dropped to 12.0 in 2021.
The combination of these dynamics makes Italy one of the of the oldest countries in the world, with 187.9 people aged 65 and over for every 100 people under the age of 15 years on 1 January 2022 (Istat, 2022).
Figure 1: Population pyramid by age and age for Italy in 2022.



3.2 Chronological versus biological aging

Ageing is an individual, natural and biologically complex process characterised by a gradual decrease in physical and mental capacity to respond appropriately to internal and/or external stressors. On top of that during ageing there is an impair maintenance of repair systems of various organs and this leads to decreased fertility and to an increased risk of disease (cancer, diabetes, cardiovascular diseases and neurodegenerative diseases are those most frequently associated with ageing) and death (López-Otín et al, 2013).

However, the rate of ageing is not the same in all human beings. Ageing is thought to be reliant upon a balance between exposure and resiliency, and as a result, there is a heterogeneity within and between species. In fact, some individuals have a more rapid physiological senescence than others (Hayflick, 2007).

In particular, a study conducted in 2015 by an international team of researchers coordinated by Prof. Daniel W. Belsky selected a group of volunteers in the city of Dunedin (New Zealand) born between 1972 and 1973 to analyse their biological age at 26, 32 and 38 years in terms of blood pressure, metabolism, kidney, lung and liver function, cholesterol, immune system, dental health, telomere length, coordination, balance and cognitive skills. The results showed that the average ageing rate in the analysed sample is 1.2 years per year; some showed early ageing, others showed late ageing and, finally, some subjects did not age at all (Belsky et al., 2015).

The biological age doesn't coincide with chronological age. Consequently, chronological age (CA) may not be a reliable indicator of body's rate of physiological and mental decline, but it can be used as a proxy for the rate of ageing (Levine et al., 2013). CA, also, makes it possible to estimate the various demographic indicators that characterise a given population.

The analysis of biological age, therefore, can be used to evaluate the different speed of ageing which occurs in the body of different subjects and it may facilitate future development of preventative interventions with implications for health and longevity.

3.3 Active ageing

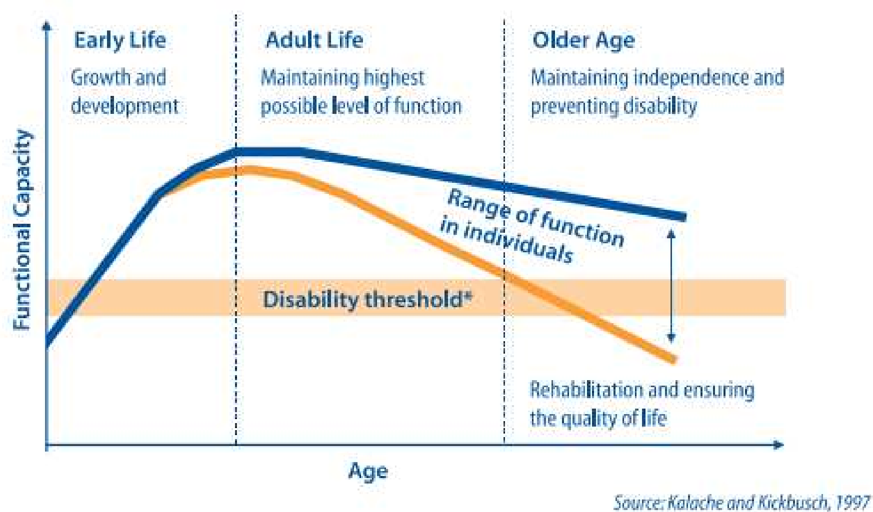
As previously mentioned, in the last century, we have seen a dramatic global rise in the number of older persons who are living markedly longer than their predecessors. However, the older we become, the more susceptible we are to a series of age-related pathologies and also ageing presents us with a multitude of debilitating consequences, among which there are disabilities, cognitive decline, and loss of social relationships. For this reason, an important social goal shared by all European countries is to promote active and healthy ageing. In 2002, the World Health

Organization has adopted the term "*active ageing*" (AA) to express the process for achieving this vision.

According to the WHO, "active ageing is the process of optimizing opportunities for health, participation and security in older to enhance quality of life as people age (WHO, 2002)".

As we already said, older people are not one homogeneous group and individual diversity tends to increase with age. Therefore, interventions that create supportive environments and promote healthy choices are important at all stages of life.

Figure 2: Maintaining functional capacity over the life course

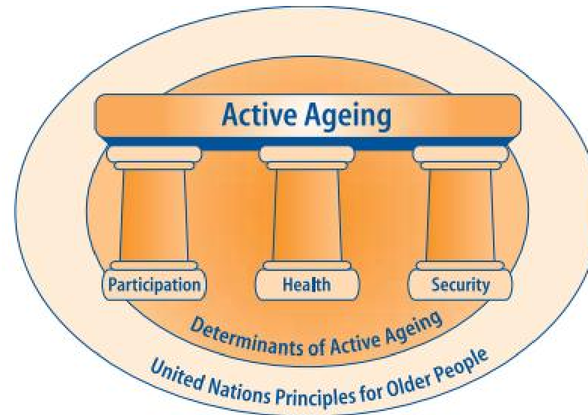


Functional capacity (such as ventilatory capacity, muscular strength, and cardiovascular output) increases in childhood and peaks in early adulthood, eventually followed by a decline (Figure 2). The rate of decline, however, is largely determined by factors related to adult lifestyle – such as smoking, alcohol consumption, levels of physical activity and diet – as well as external and environmental factors. The gradient of decline may become so steep as to result in premature disability. However, the acceleration in decline can be influenced and may be reversible at any age through individual and public policy measures.

The policy framework for active ageing is guided by the *United Nations Principles for Older People* (Figure 3, the outer circle): independence, participation, care, self-fulfilment and dignity.

The policy framework requires action on three basic pillars: Health, Security and Participation.

Figure 3: The three pillars of a policy framework for Active Ageing



Regarding *Health*:

1. Prevent and reduce the burden of excess disabilities, chronic disease and premature mortality;
2. Reduce risk factors associated with major diseases and increase factors that protect health throughout the life course;
3. Develop a continuum of affordable, accessible, high quality and age-friendly health and social services that address the needs and rights of women and men as they age.
4. Provide training and education to caregivers.

Regarding *Participation*:

1. Provide education and learning opportunities throughout the life course.
2. Recognize and enable the active participation of people in economic development activities, formal and informal work and voluntary activities as they age, according to their individual needs, preferences and capacities.
3. Encourage people to participate fully in family community life, as they grow older.

Regarding *Security*:

1. Ensure the protection, safety and dignity of older people by addressing the social, financial and physical security rights and needs of people as they age.
2. Reduce inequities in the security rights and needs of older women.

3.4 Factors involved in ageing

Ageing is an extremely complex multifactorial process. In fact, the inter-individual variability that characterises ageing is influenced by several genetic and environmental factors that interact to determine the typical events of ageing.

Regarding the genetic factors influencing ageing, due to the great development of genome analysis, numerous genes involved in this process could be identified and this large number led to the creation of the Human Genomic Resources database (http://genomics.senescence.info/) containing the list of these genes.

Polymorphisms or mutations at the level of these genes can induce all those molecular and cellular changes involved in the ageing process or slow down the advancement of ageing. For example, mutations in genes LMNA and BANF1 encoding protein components of nuclear lamina cause accelerated aging syndromes such as the Néstor-Guillermo progeria syndrome (NGPS) and the Hutchinson-Gilford syndrome (HGPS) (López-Otín et al, 2023). Furthermore, polymorphisms or mutations in the genes coding for GH, IGF-1 and insulin receptor appear to result in longevity-associated effects, while increased expression of $p16^{Ink4a}$, $p19^{Arf}$ or p53 may be regarded as a beneficial compensatory response aimed at preventing the proliferation of damaged cells and, thus, cellular senescence. However, when damage is widespread, the tissue's regenerative capacity may be exhausted and, under these extreme conditions, the $p16^{Ink4a}$, $p19^{Arf}$ and p53 responses may become deleterious and accelerate ageing (López- Otín et al., 2013).

Regarding the environmental factors influencing ageing, they include the combination of environmental and occupational exposure, lifestyle and socio-economic factors. Many studies had demonstrated the important role of the environment on biological ageing; however the development of tools for analysing the exposome is still at a standstill compared to the development of methods allowing the analysis of genes that appear to play a role in ageing.

One of these studies was conducted in the Laboratory of Occupational Medicine of the Department of Cardio-Thoracic-Vascular Sciences and Public Health of the University of Padua that shows how biological ageing advances more rapidly following exposure to environmental agents (smoke, alcohol, air pollutants) and

occupational exposure (PAH, Polycyclic Aromatic Hydrocarbons). Exposure to these types of agents, in conjunction with alterations in the inflammatory responses implicated in the ageing process, favours the development of oxidative stress that contributes to a more rapid shortening of telomere length, thereby increasing the risk of the onset of age-associated diseases, especially chronic degenerative diseases such as cancer (Pavanello et al, 2010).

An emerging problem in the working environment concerns the irregular circadian rhythms assumed by night workers. It's thought that circadian rhythms may be associated with premature ageing and that their effects may lead to the development of chronic diseases, such as obesity, cardio-vascular diseases and metabolic diseases. Therefore, it has been recognised that the night shift may affect several age-associated diseases, although the mechanisms involved have not yet been fully elucidated. The most plausible hypothesis, so far, is to consider inflammation and the resulting biological ageing as the possible pathophysiological mechanism influencing the increased risk of disease onset in night workers, by assessing plasma levels of long pentraxin 3 (PTX3), an essential component of humoral immunity involved in the regulation of inflammation, and LT in leucocytes, which is considered a reliable biomarker of ageing.

The association between PTX3 and LT was positive: during the regulation of inflammatory processes, PTX3 is able to protect telomeres from accelerated shortening and ensure genetic stability; in contrast, a reduction in PTX3 levels does not result in this protective function. Furthermore, the association between PTX3 and CRP (c-reactive protein) was evaluated and found to be negative, meaning that low PTX3 levels are related to increased CRP levels. This results in an increased systemic inflammation in night workers who are therefore more susceptible to premature ageing (Pavanello et al., 2017).

Also socio-economic factors are not to be underestimated. They included social and psychological distress, presence of illness or disability, income, education, which can have a negative impact on the individual.

In a study of 1,552 female twins aged 18–75 years, participants belonging to lower SES category (Socioeconomic Status) were found to have shorter TL relative to peers in higher SES, with the white blood cell telomeres in the lower SES category

being shorter on average by 140.3 base pairs after accounting for other risk factors such as body size, smoking and physical activity (Cherkas et al., 2006).
Regarding education, evidence from the National Health and Nutrition Examination Survey (NHANES) study showed that individuals completing high school education had relatively shorter telomeres than college graduates.
A low level of education is also associated with a higher risk of incidence of dementia and a higher risk of dementia-related death; education as a cognitive indicator, therefore, may have a protective effect against the risk of developing dementia, as it may provide a better neural network and compensatory mechanisms during the course of life.

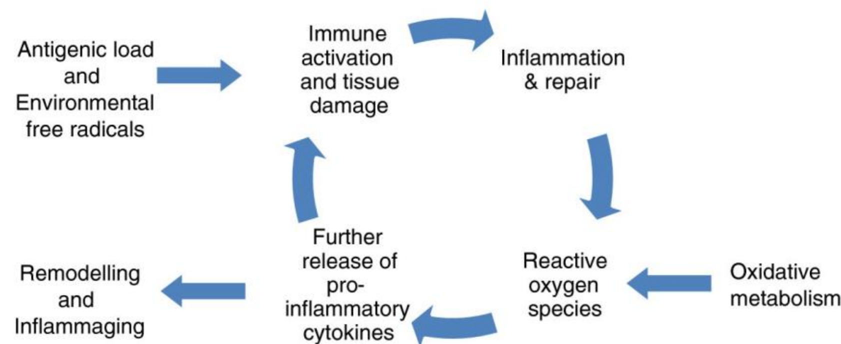
3.5 Oxi-Inflamm-Ageing

One of the most relevant processes involved in ageing is chronic inflammation. In fact, ageing is characterized by an increase in the concentration of pro-inflammatory molecules in the bloodstream leading to a state of low-grade, chronic inflammation at the systemic level. This phenomenon has been termed "inflammageing" (Franceschi et al., 2000).
According to Krabbe et al. (2004), ageing is accompanied by 2-4 fold increases in plasma/serum levels of inflammatory mediators such as cytokines and acute phase proteins. In particular, in a recent study, principal component analysis was used to investigate 19 biomarkers including pro- and anti- inflammatory cytokines, cytokine receptors, chemokines and C-reactive protein (CRP) in a group of Italian subjects of different age and this study observed that STNF-RI, STNF-RII, IL-6, TNFα, hsCRP, IL-18 and IL-1 RA were strongly correlated with age.
The results of this study also indicated that inflammageing does not simply reflect an increase of pro-inflammatory markers but an overall activation of inflammatory systems that probably also promotes a concomitant rise in the levels of anti-inflammatory mediators. This process may result in different outcomes depending on the nature of the stimulation, the pre-existing physiological reserve, the immune background and exposure to infections (Morrisette-Thomas et al., 2014).
The chronic inflammatory stress is established when there is an imbalance between pro-inflammatory compounds and anti-inflammatory compounds in

favour of the former. This could happen due to the persistence of the antigenic challenge caused by both clinical and subclinical infections as well as exposure to noninfective antigens, or weakening of the regulatory systems of the immune response (Fülöp et al., 2019).

The inflammation respond is characterized by mononuclear immune cell infiltration (monocytes, macrophages, and lymphocytes) to different tissues where these cells produce excessive reactive oxygen species (ROS) and pro-inflammatory mediators to conclude this situation but, at the same time, generate tissue damage and fibrosis. Therefore, a continued and active oxidant response by immune cells can lead to cellular damage due to ROS overproduction, which can also recruit other inflammatory cells leading to additional pro-inflammatory and oxidant production amplifying cellular damage. This results in a vicious cycle, driving immune system remodelling and favouring a chronic proinflammatory state where pathophysiological changes, tissue injury and healing proceed simultaneously. Over decades there is a slow and not clinically evident accumulation of irreversible cellular and molecular damage (Baylis et al., 2013) (Figure 4).

Figure 4: Cycle of inflammageing

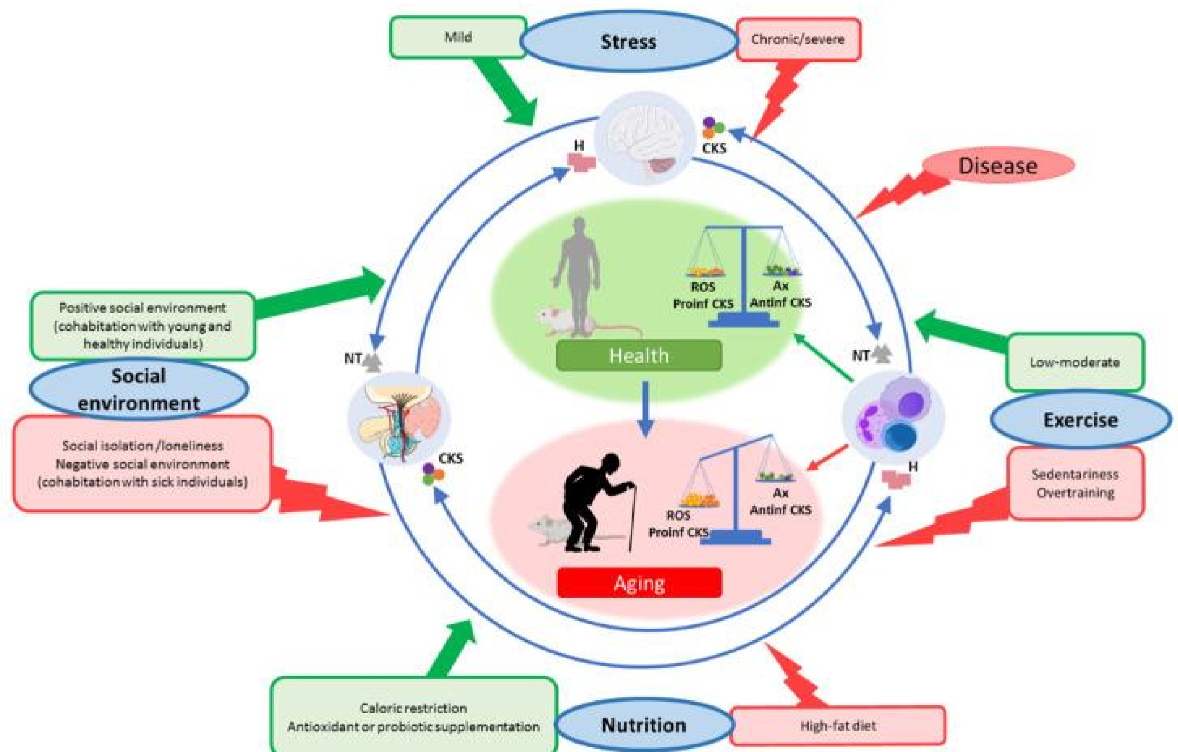


Indeed, oxidation and inflammation are associated and based on this link the oxi-inflamm-aging theory of ageing was processed (De la Fuente et al., 2009). According to this theory, ageing would be the consequence of chronic oxidative stress, associated with inflammatory stress, which would cause the deterioration of the function of all cells of the individual, but would have a greater impact on those of the homeostatic systems, that are the nervous, endocrine, and immune systems. This would explain the lower ability to maintain homeostasis that occurs with ageing and leads to increased morbidity and mortality.

Regarding the three systems, they are constantly exchanging information via neuroimmunoendocrine communication, which consists of neurotransmitters (NT), hormones (H), and cytokines (CKS). As living organisms, we are continuously exposed and adapted to different stressors. An individual reacts to a potentially health-threatening physical or mental stressor by activating interconnected neuroimmunoendocrine circuits. This pro-survival response allows the body to cope and deal with the challenge and re-establish homeostatic equilibrium, promoting health. However, if the individual perceives a noxious stimulus as too intense, or its duration as too long, he or she may fail to cope with it, maladaptation occurs, neuroimmunoendocrine parameters remain altered, accelerating the rate of aging and the onset of age-related diseases.

External factors, such as the social environment, nutrition, and exercise, can modify the oxidative and inflammatory stress of the organism and the function of immune, nervous, and endocrine cells, altering this communication, and consequently, modulating the ageing rate of an individual (Figure 5).

Figure 5: Dual effect of different lifestyle situations on oxi-inflamm-ageing.



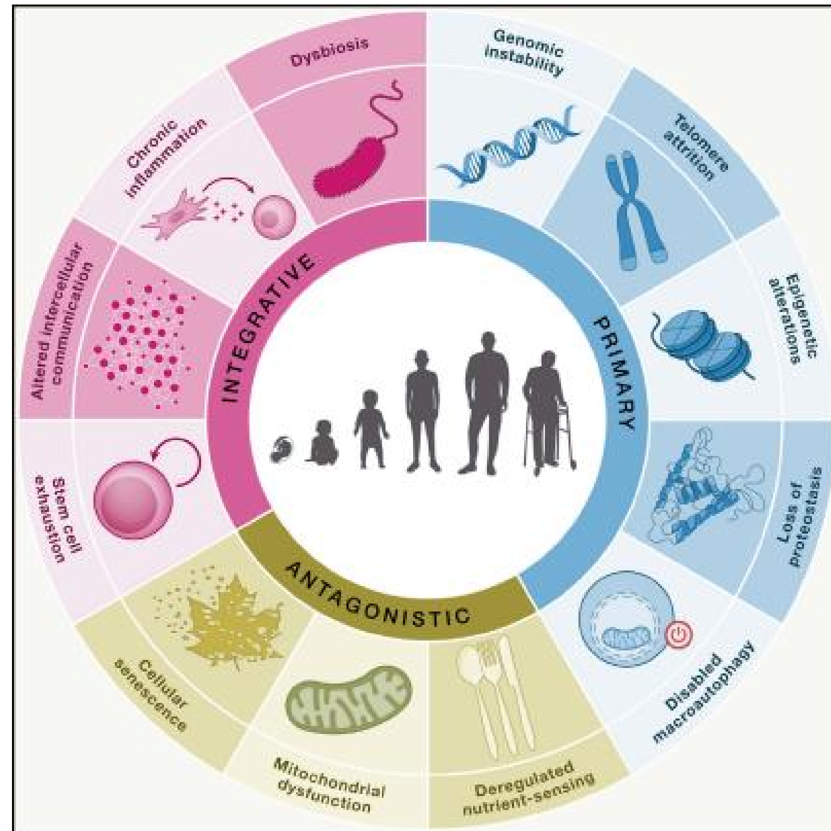
Furthermore, this theory introduced the involvement of the immune system in the greater or lesser oxidation and inflammation that occurs with ageing. Since immune cells need to produce oxidant and inflammatory compounds to carry out their defensive function, when uncontrolled, they may be responsible for the generation of oxidative-inflammatory stress that would not only cause their functional deterioration (immunosenescence) but could also increase these stresses in the body, accelerating the ageing process.

3.6 Characteristics of the aged cell

Nowadays, ageing is subjected to scientific scrutiny based on the ever-expanding knowledge of the molecular and cellular bases of life and disease. This year, 12 cellular and molecular hallmarks of ageing were identified and categorised into three categories (López-Otín et al., 2023) (Figure 6):

- *Primary hallmarks*. Those considered to be the primary causes of cellular damage, which, in majority, are reversible (genetic instability, telomere attrition, epigenetic alterations, loss of proteostasis and disabled macroautophagy).
- *Antagonistic hallmarks*. Those considered to be part of compensatory or antagonistic responses to the damage. These responses initially mitigate the damage, but eventually, if chronic or exacerbated, they become deleterious themselves (mitochondrial dysfunction, deregulated nutrient-sensing and cellular senescence).
- *Integrative hallmarks*. Those are considered to be the end result of the previous two groups of hallmarks and those responsible for the functional decline that characterises ageing, so they are no longer reversible (stem cell exhaustion, altered intercellular communication, chronic inflammation and dysbiosis).

Figure 6: The hallmarks of ageing.



Therefore, the aged cell is characterised by:

- *Genomic instability*. Genomic instability is defined as a process prone to genomic changes or an increased propensity for genomic alterations. During cell division, genomic instability is associated with the failure of parental cells to accurately duplicate the genome and precisely distribute the genomic material among the daughter cells.

  Genomic alterations that can cause genomic instability arise from the fact that DNA integrity and stability are pervasively threatened by exogenous chemical, physical, and biological agents, as well as by endogenous challenges, such as chromosome segregation defects, oxidative processes, spontaneous hydrolytic reactions and DNA replication errors. The genetic lesions caused by these extrinsic or intrinsic sources of damage are highly diverse and include point mutations, deletions, translocations, telomere shortening, single- and double-strand breaks, chromosomal rearrangements,

defects in nuclear architecture, and gene disruption caused by the integration of viruses or transposons.

Accordingly, organisms have evolved a complex array of DNA repair and maintenance mechanisms to deal with the damage inflicted to nuclear and mitochondrial DNA (mtDNA) and to ensure the appropriate chromosomal architecture and stability. These DNA repair networks lose efficiency with age, which accentuates the accumulation of genomic damage and the ectopic accumulation of DNA in cytosol.

Accumulation of mutations throughout life is one common denominator of ageing and it's a result of misbalance between DNA damage and repair. Once a certain level of DNA damage is reached, cells may undergo a wide range of phenotypic changes, from cell cycle arrest, apoptosis, or cellular senescence to malignant transformation.

- *Telomere shortening*. Telomeres are small portions of nucleoprotein material found at the ends of each chromosome; they are species-specific, non-coding, highly tandemly repeated nucleotide sequences associated with different proteins. They play an important role in the correct positioning of the chromosomes within the nucleus, chromosome replication and meiotic recombination.

  In addition, telomeres protect 5' and 3' ends of chromosome from aberrant recombination, degradation and incomplete replication, conferring genetic stability. This is closely derived from the structure of telomeres; in humans, they consist of hexanucleotide sequences 5'-TTAGGG-3' in the leader strand and 3'-CCCTAA-5' in the lagging strand that repeat for approximately 9-15 kb. The 3' end along the leader strand is free as it protrudes 50-300 nucleotides from the complementary strand and it's rich in G. Therefore, this end is referred to as G-strand overhang and tucks itself into the end of chromosome, creating a conformation called a T-loop that inhibits the action of exonucleases and termination-terminal fusion phenomena.

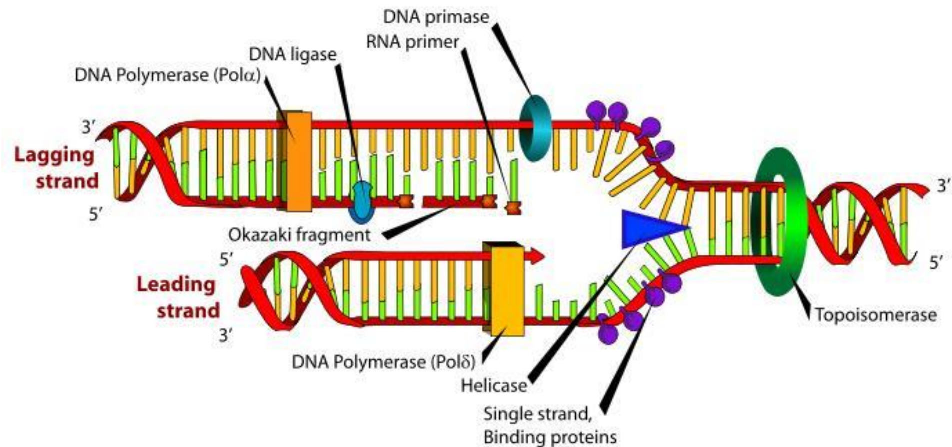
  With each cycle of cell replication, telomeres undergo a shortening as a result of the DNA replication mechanism. The enzyme DNA polymerase III has 5'→3' polymerising activity, so the 5'→3' strand or leader is replicated

continuously, while the 3'→5' or lagging strand is replicated discontinuously in the form of fragments (Okazaki fragments) that are joined together via the enzyme ligase. The synthesis of the fragments is possible thanks to primers synthesised by the primase enzyme. Then, this primers are degraded by DNA polymerase I, which has 5'→3' exonuclease activity; the empty space left by primer degradation is then filled by DNA polymerase I itself (Figure 7). This means that the primer used for the synthesis of the last fragment, at the 5' end of the lagging strand, is degraded and DNA polymerase I is unable to insert the missing nucleotides. At the end of the process, the 5' end of the lagging strand has about 50-100 fewer base pairs resulting in shortened telomeres.

To prevent this from happening, there is the enzyme telomerase, a telomere-specific reverse transcriptase that allows telomere sequences to be added to the ends of chromosomes. This enzyme, however, is not expressed in all cell types; in fact, it can be found in the germ line, but not in the somatic line, which is highly represented in the human organism. As replicative cycles progress, the lack of telomerase leads to a progressive and cumulative erosion of telomere sequences until a critical threshold of telomere length defined as the Hayflick limit (Hayflick et al., 1961). Reached it, a p53-dependent intracellular signal is produced, which blocks the cell cycle in the G1 phase and, therefore, cell proliferation, leading to cell senescence.

For this reason, telomere shortening is associated with ageing and the analysis of their length is used to monitor ageing, as telomeres act as 'molecular clocks' in that they indicate the number of replicative cycles the cell has undergone (López-Otín et al., 2013).

Figure 7: Strand lagging during DNA replication.



- *Epigenetic alterations*. Epigenetics is the study of stable genetic modifications that result in changes in gene function and expression without an alteration in the corresponding DNA sequence. The large variety of epigenetic changes that contribute to ageing include alterations in DNA methylation (DNAm) patterns, abnormal post-translational histone modifications and aberrant chromatin remodelling and deregulated function of non-coding RNAs (ncRNAs) (Lopez-Otín et al., 2023). In particular, DNAm is the most frequent; normally, it regulates gene expression and maintains genomic stability, so it is a dynamic process that changes over the lifespan of an individual and is strongly influenced by age and genetic and environmental factors. Alterations in DNAm can therefore be considered one of the main biomarkers associated with ageing.

  In humans, the DNA methylation process consists of covalent addition, i.e. the binding of a methyl group (-CH 3) at the C5 position of cytosine located in the 5'-CG-3' (CpG) sequence in a reaction catalysed by a family of DNA methyltransferase enzymes. The product of this reaction is 5-methyl cytosine (5mC) (Figure 8).

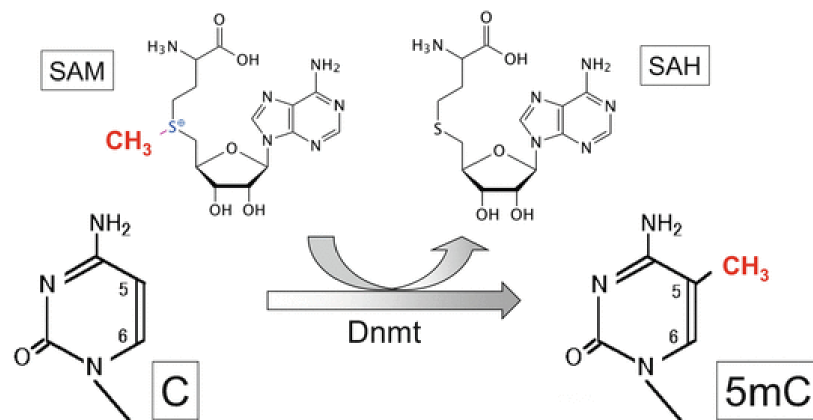
  The DNA methyltransferase (DNMT) enzymes that appear to be involved in the process are DNMT1, DNTM3a and DNMT3b; they are able to catalyse the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to cytosine present along the CpG sequences of DNA. DMTN1 is expressed in somatic cells, while DNTM3a and DNMT3b are involved in embryonic development.

CpG sequences are not highly represented along the genome, but are abundant in the promoter region of genes. Methylation is a process that generally results in inhibition of gene expression, whereby hypomethylation of CpG regions is associated with increased gene expression, whereas hypermethylation of CpG sequences at the promoter level results in gene silencing.

A subject's DNAm pattern accumulates multiple changes with the passage of time. Early studies described an age-associated global hypomethylation, but further analyses revealed that specific loci, including those of several tumor suppressor genes and Polycomb target genes, are hypermethylated with age. The levels of site-specific methylation can, then, predict the age of the individual in a range of two to four years (Horvath, 2013).

Unlike mutations, epigenetic alterations are, at least theoretically, reversible and thus offer the opportunity to identify new anti-ageing treatments (López-Otín et al., 2013).

Figure 8: Cytosine methylation mechanism in DNA.

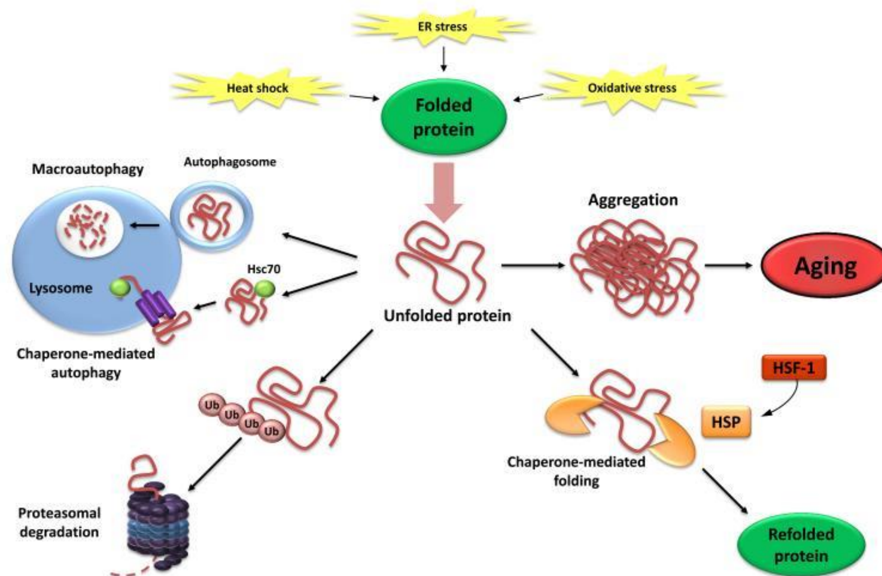


- *Loss of proteostasis*. Proteostasis or protein homeostasis is a physiological process involved in protein turnover and control of the quality of synthesised proteins; these are events that allow the cell to perform its functions correctly and consequently affect the tissues and the entire organism. In fact, when there are damaged or malfunctioning or even potentially toxic proteins, they are degraded and eliminated by various systems in the cell. When a protein is misfolded due to endogenous or exogenous stress, it is usually properly folded by Heat-Shock Proteins

(HSPs) or is degraded by the ubiquitin-proteasome system (UPS) following recognition of the protein by the chaperone HSC70, which transports it to the lysosomal surface for internalization and rapid intralysosomal degradation, or by the autophagy-lysosomal pathway (ALP), which engages the damaged protein in an autophagosome that then fuses with the lysosome. When neither of these pathways is able to restore the correct folding of the protein or its elimination, there will be an accumulation of the proteins that aggregate with each other, thus toxic effects may occur (López-Otín et al., 2013) (Figure 9).

Accumulation of misfolded, oxidized, glycated, or ubiquitinylated proteins that often form aggregates as intracellular inclusion bodies or extracellular amyloid plaques are the result of an impaired protein homeostasis or proteostasis, which are associated with ageing and several age-related morbidities, such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease and cataract (López- Otín et al., 2023). In fact, it has been shown that the proteostasis process is altered with age. With advancing age, stress-induced cytosolic chaperone synthesis declines and, in addition, the ubiquitin-proteasome and autophagic-lysosomal systems tend to be less efficient.

Figure 9: Protein homeostasis mechanisms.



- *Disabled macroautophagy*. Macroautophagy involves the sequestration of cytoplasmic material in two-membrane vesicles, the autophagosomes, which later fuse with lysosomes for the digestion of luminal content. This process has the important function of maintaining a well-controlled balance between anabolism and catabolism. In fact, autophagy is not only involved in proteostasis but also affects non-proteinaceous macromolecules (such as ectopic cytosolic DNA, lipid vesicles, and glycogen) and entire organelles (including dysfunctional mitochondria targeted by ‘‘mitophagy,’’ and other organelles leading to ‘‘lysophagy,’’ ‘‘reticulophagy,’’ or ‘‘pexophagy’’), as well as invading pathogens (‘‘xenophagy’’).

  An age-related decline in autophagy constitutes one of the most important mechanisms of reduced organelle turnover. Also genes and proteins that participate in the autophagic process are also involved in alternative degradation processes such as LC3-associated phagocytosis of extracellular material, and the extrusion of intracellular waste (e.g., dysfunctional mitochondria) in the form of exospheres for their subsequent removal by macrophages. That said, there is strong evidence that the core process of autophagy is relevant to ageing.
- *Mitochondrial dysfunction*. According to the theory developed by Harman, the progressive mitochondrial dysfunction that occurs with ageing causes an increase production of ROS (Harman, 1956). The excessive production of ROS results in oxidative stress, which can induce alterations in the structure of the lipids that make up cell membranes (lipid peroxidation) with consequences on fluidity, structural alterations in proteins and alterations in nucleic acids. Nucleic acids are particularly susceptible to the action of free radicals, hence the increased risk of occurrence of mutations and deletions in nuclear and mitochondrial DNA. Mitochondrial DNA (mtDNA), compared to nuclear DNA (nDNA), has a diminished capacity to repair DNA damage and a reduced amount of histone proteins, so that it is more prone to suffer the toxic action of free radicals. Therefore, oxidative stress causes further mitochondrial deterioration and global cellular damage (López-Otín et al., 2013).

On the other hand, the production of ROS by mitochondria represents a survival signal in response to stress of various kinds aimed at compensating for the damage associated with ageing, but with advancing age, instead of providing benefits, it contributes to the generation of damage at the molecular and cellular level.

In addition, with ageing, mitochondrial function deteriorates due to multiple intertwined mechanisms including the accumulation of mtDNA mutations, deficient proteostasis leading to the destabilization of respiratory chain complexes, reduced turnover of the organelle, and changes in mitochondrial dynamics. This situation compromises the contribution of mitochondria to cellular bioenergetics, enhances the production of ROS, and may trigger accidental permeabilization of mitochondrial membranes causing inflammation and cell death.

- *Deregulated nutrient-sensing*. There are several metabolic pathways that allow for the transformation and elimination of nutrients introduced with the diet; among the most prominent is the intracellular signal transduction pathway mediated by insulin, a hormone produced by the β-cells of the islets of Langerhans in the pancreas that allows glucose to enter the cells, thus lowering the blood glucose level.

  The growth factor IGF-1 is produced by hepatocytes as a result of the synthesis of the hormone GH by the pituitary gland; IGF-1 has a signal transduction pathway similar to that of insulin, so much so that it is referred to as the insulin and IGF-1 signalling (IIS) pathway. It has been seen how polymorphisms or mutations that lead to a reduction in GH, IGF-1 receptor and insulin function are associated with a longer lifespan in both humans and model organisms (Barzilai et al., 2012). Subjects without such polymorphisms or mutations will inevitably undergo more rapid ageing, which is therefore characterised by a decrease in GH hormone synthesis and, consequently, of the growth factor IGF-1; insulin resistance is observed, which leads to a reduction in the biological effects of insulin at physiological concentrations, and also an increase in pro-inflammatory

cytokines produced by fat cells acting at the insulin receptor, leading to further insulin resistance.

- *Cellular senescence*. This is a mechanism capable of blocking the cell cycle and thus cell proliferation as a result of various stimuli, including shortening of telomere length, DNA damage, exposure to free radicals or activation of oncosuppressor genes. In young individuals, this phenomenon is able to prevent the development of tumours, while in adult individuals there is an accumulation of senescent cells that eventually contribute to ageing.
  However, cellular senescence is a beneficial process for the organism, since the proliferation of damaged cells is inhibited and they are thus made visible to the immune system; it is necessary, however, to have an effective cell repair system that includes clearance of senescent cells and recall of progenitor cells that restore cell numbers.
  With advancing age, these turnover systems may become inefficient or the regenerative capacity of progenitor cells may be exhausted, resulting in the accumulation of senescent cells that leads to ageing (López-Otín et al., 2013). In fact, in humans, senescent cells accumulate in multiple tissues at different rates, from 2- to 20-fold when comparing young (<35 years) to old (>65 years) healthy donors, mainly affecting fibroblasts, endothelial cells, and immune cells, although all cell types can undergo senescence during ageing. Another evidence for the causal role of cellular senescence in ageing is that continued genetic or pharmacological elimination of senescent cells extends the healthspan and longevity of naturally aged mice (Xu et al., 2018).
- *Stem cell exhaustion*. Stem cells are undifferentiated cells capable of giving rise to different mature cell types through cell differentiation, although they divide by mitosis very slowly while maintaining their undifferentiated state. They are very active during embryonic development, during which the cell types that will go on to form the various tissues are generated. Once development in the adult is complete, stem cells become quiescent, but are able to divide and allow regeneration of tissue damaged by trauma or disease. In this sense, one can distinguish labile tissues, i.e. with high cell

turnover (skin, liver, red blood cells, etc.), and perennial tissues characterised by little or no cell turnover (voluntary muscles, heart, nervous tissue).

One of the most obvious features of ageing is that there is a reduced tissue renewal at steady state, as well as with impaired tissue repair upon injury, with each organ having its own strategy for renewal and repair. Therefore, there is a decrease in the regenerative power of tissues (López-Otín et al, 2013); very often, a decrease in haematopoiesis is observed, resulting in a decreased production of adaptive immunity cells in a process known as immunosenescence and an increased incidence of anaemia and myeloid neoplasms, as well as a reduction in bone marrow function.

- *Altered intercellular communication*. Normally, cells within an organism interact with each other to coordinate processes such as growth, differentiation and metabolism; this interaction can be physical (communicating junctions) or chemical (chemical messengers).

  Ageing is coupled to progressive alterations in intercellular communication that compromise homeostatic and hermetic regulation. Thus, ageing is characterised by deficiencies in neural, neuroendocrine, and hormonal signalling pathways, including adrenergic, dopaminergic, and insulin/IGF-1 based and renin-angiotensin systems, as well as sex hormones commensurate with the loss of reproductive functions. There is an increase in inflammatory reactions, a decrease in immune defences against pathogens and precancerous cells and a change in the composition of the peri- and extracellular environment (López-Otín et al, 2013).
- *Dysbiosis*. The gut microbiome is a key factor in multiple physiological processes such as nutrient digestion and absorption, protection against pathogens, and production of essential metabolites including vitamins, amino acid derivatives, secondary bile acids, and short-chain fatty acids (SCFAs). The intestinal microbiota also signals to the peripheral and central nervous systems and other distant organs and strongly impacts on the overall maintenance of host health (López-Otín et al., 2023).

Bacterial diversity is established during childhood and it remains relatively stable during adulthood. However, the architecture and activity of this bacterial community undergoes gradual changes during ageing, finally leading to a general decrease in ecological diversity.

In fact, old healthy participants show continued drift toward a unique microbial composition, whereas this drift is reduced or absent in individuals in worse health. Several studies conducted on centenarian populations showed a reduction in core abundant taxa, such as *Bacteroides* and *Roseburia*, but also an increase in several genera such as *Bifidobacterium* and *Akkermansia*, which appear to have pro-longevity effects (Biagi et al., 2016).

3.7 Biological ageing indicators: LT and DNAmAge

In order to assess the biological age of a subject, it is possible to use indicators that measure both phenotypic aspects (cardiac, pulmonary, metabolic, renal, hepatic, immune and inflammation status) and molecular aspects (epigenetic, biological, transcriptomic, metabolic age). It seems that molecular indicators tend to alter with age and, what is more, allow new mechanisms underlying ageing to be identified, but there is no single indicator that can give us the exact biological age of an individual, so analysis usually consists of measuring several indicators.

Among the hallmarks mentioned above, one of the most widely used in determining biological ageing is telomere length analysis. In fact, all cells in our body have a biological clock at the level of telomeres, the functional repetitive DNA/protein complexes at the ends of chromosomes. Telomeres preserve the integrity of DNA which, without them, would be gradually lost with each cell division. A specific enzyme, telomerase, is involved in telomere synthesis after mitosis, but is only active in progenitor cells and in some diseases. Telomeres, therefore, become progressively shorter during each somatic cell division and their length measured in peripheral blood lymphocytes is considered an indicator of biological age (Frenck et al., 1998).

Another reliable biomarker of biological ageing is DNAmAge. The advent of high-throughput sequencing analyses at the epigenome level has led to the correct identification of a large number of genomic sites that are highly associated with age. These discoveries made it possible to create a very precise 'epigenetic clock' for age estimation with an average error of only 3.6 years, called the DNAmAge. The methylation-based biological age was mostly developed on DNA extracted from blood, which is a readily available source. Researchers have created multiple age prediction models, using various statistical methods, to determine a person's age based on age-dependent methylation alterations at certain CpG loci. The number of CpG sites used in the construction of these age prediction models varies from a few to 100 years. An attempt was made, however, to increase the practicality of age indicators and the use of as few loci as possible. Among the most widely used indicators is the model proposed by Zbieć-Piekarska et al. (2019), developed on five CpG sites. The difference between DNAmAge and chronological age, defined as AgeAcc, provides information on the speed of the epigenetic clock and is closely associated with age-related disorders, including cardiovascular disease and mortality risk.

3.8 Reversing Ageing

One of the main goals of ageing research is to find a treatment to reverse ageing. Over the years, a number of studies have been published which, through the use of indicators of ageing, analyse the effectiveness of certain therapies on the biology of ageing.

López-Otín himself, in his latest review published on the hallmarks of ageing (López-Otín et al., 2023) analysed for each indicator studied its possible use in reversing ageing. However, most of the studies reported were carried out on model organisms.

We have, therefore, searched in the PubMed database for articles published since the date the database was opened using the terms 'telomere length', 'DNAmAge', “No-Pharmacologic treatment” and “Pharmacological therapies” to identify studies aimed at analysing the effects of different therapies on two hallmarks of ageing, telomere length and DNA methylation, in blood samples.

3.8.1 Pharmacological and no-pharmacological therapies

After a recent thorough literature search, we found several longitudinal and cross-sectional studies analysing the effects of different drug treatments on the two bioindicators of ageing, telomere length and DNA methylation, analysed from blood samples. In particular, we identified a total of 115 articles concerning the effects of pharmacological treatments on TL and 268 on DNAmAge; of these we selected 51 and 14 respectively (Figure 10 and 11). Various drug treatments have led to positive effects such as telomere lengthening and a decrease in DNAmAge. Among these, for example, one has shown that after 60 daily Hyperbaric oxygen exposures on 35 healthy adults there is a elongation by over 20% of telomeres length of T helper, T cytotoxic, natural killer and B cells (Hachmo et al., 2020).

Figure 10: Research graph of the effects of pharmacological treatments on TL.

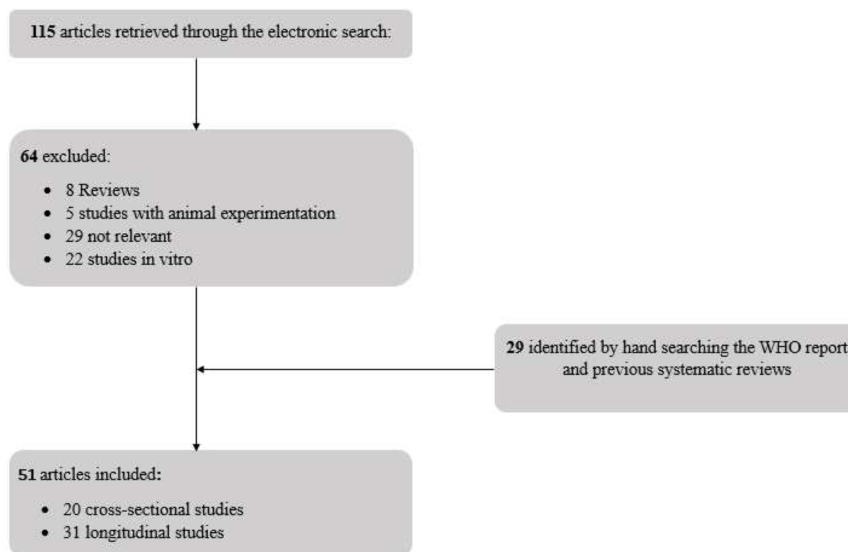
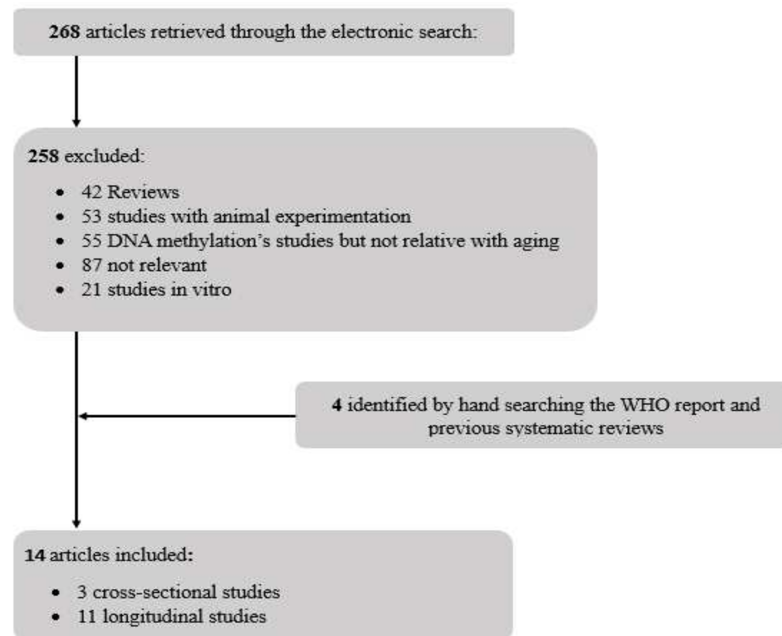


Figure 11: Research graph of the effects of pharmacological treatments on DNAmAge.



It was very promising the research into the effect of non-pharmacological treatments, such as yoga and meditation, on two biomarkers of ageing. We were able to identify a total of 49 articles concerning the effects of non-pharmacological treatments on TL and 285 on DNAmAge; of these, we selected 24 and 10 respectively (Figures 12 and 13). In particular, the majority of studies that demonstrated a positive effect on biomarkers of biological ageing concerned meditation, yoga and religiosity. Of particular interest is the study of Mahendran et al. (2018), who investigated telomere length from a peripheral blood lymphocyte (PBL) sample of 68 MCI (Mild Cognitive Impairment) subjects after 9 months of art therapy (AT). After the intervention, lymphocyte telomere length increased in the AT group at 9 months (mean change = 552; p = 0.003).

Figure 11: Research graph of the effects of no-pharmacological treatments on TL

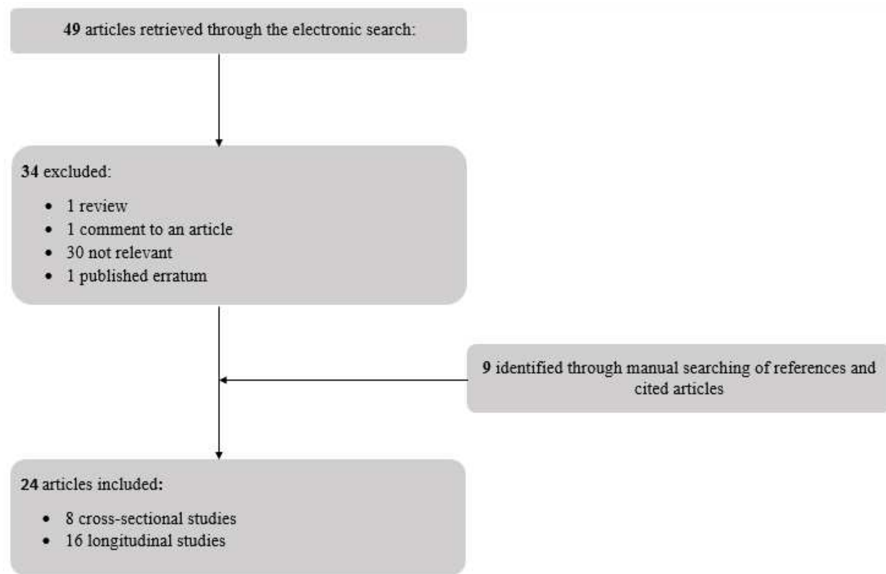
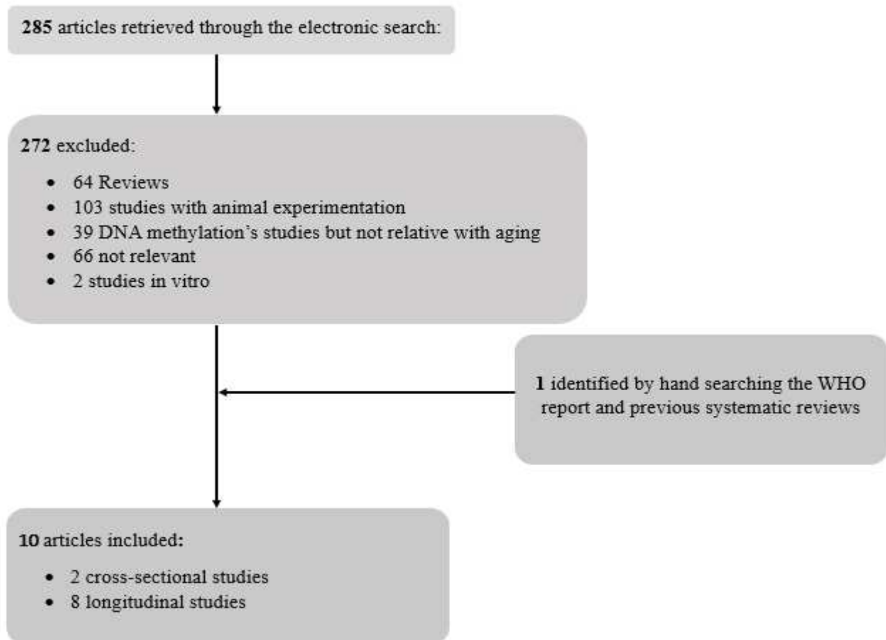


Figure 12: Research graph of the effects of no-pharmacological treatments on DNAmAge.



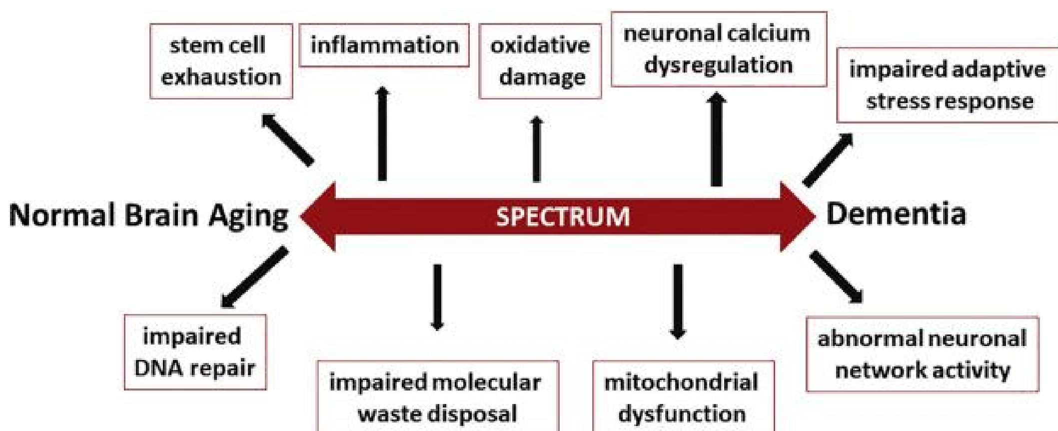
3.9 Dementia

Dementia is a clinical syndrome that gradually and irreversibly impairs cognitive functions (i.e. the ability to process thought) and functional abilities, as a result of which the person gradually loses his or her independence and quality of life.

Dementia is one of the leading causes of disability worldwide and carries a huge emotional, social and financial burden and, for this reason, in 2012 the World Health Organization (WHO) defined research in this area as a "global priority".

The greatest risk factor for dementia is age. In fact, neurodegenerative diseases like dementia are characterised by cellular and metabolic processes which accelerate during advanced age (Mattson, Arumugan, 2018). Mattson and Arumugam outlined the common characteristics of brain ageing which include reduced DNA repair, abnormal molecular waste disposal, mitochondrial dysfunction, oxidative damage, neuronal calcium dysregulation, impaired adaptive stress response, inflammation, stem cell exhaustion and abnormal neuronal network activity (Figure 14). All these characteristics are common to the brain ageing process and dementia suggesting that the two processes are linked on cellular and molecular levels.

Figure 13. The main hallmarks of normal brain ageing are also present in dementia.



The most common form of dementia is Alzheimer disease (AD) and it may contribute to 60–70% of cases. AD is clinically defined by a slowly progressing loss of cognitive functions, primarily memory impairment. The disease is

characterized by the aggregation and deposition of beta amyloid (Aβ) peptide in the form of neuritic plaques and hypophorylated tau protein in the form of intracellular neurofibrillary tangles.

Despite research efforts and numerous clinical trials, there are currently no treatments that can intervene in the irreversible causes of dementia. Regarding the pharmacological therapies used that can help manage symptoms, the most used are cholinesterase inhibitors like Donepezil, than NMDA receptor antagonists like memantine are used for severe Alzheimer disease and vascular dementia.

3.9.1 Dementia and biomarkers of ageing

Ageing is the primary risk factor for most neurodegenerative diseases, including Alzheimer disease (AD) and Parkinson disease (PD). Consequently, biological ageing may be a robust biomarker of dementia and several studies were able to find an association between biomarkers of ageing, in particular leukocyte telomere length (LTL), and dementia or Alzheimer's disease (AD).

For instance, shorter telomeres have been found in patients suffering from Alzheimer's disease (AD) (Fu et al., 2022), and for this reason it has been proposed as a valuable predictor of the AD incidence. Mendelian Randomization (MR) approaches were used to assess causality in observational studies that aimed to study the association between a shortening of telomere length an increased risk of developing AD. Many MR studies were able to describe potential causal effects of genetically predicted telomere length on the risk of AD.

Regarding the association between epigenetic age and dementia or Alzheimer's disease, seven studies have been published showing a lack of association between this biomarker of ageing and dementia (Zhou et al., 2022). Therefore, there are not sufficient evidences to indicate that current epigenetic aging clocks can be clinically useful biomarkers of dementia or cognitive aging.

4. OBJECTIVES

This pilot study was carried out thanks to the collaboration between the Regional Centre for Cerebral Ageing (CRIC) of the Padua Hospital, the Municipality of Padua, the Department of Cultural Heritage (DBC) of the University of Padua and the Laboratory of Genomics and Environmental Mutagenesis (Department of Cardio-Thoracic-Vascular Sciences and Public Health, University of Padua) with the aim of verifying the effectiveness of visual arts mediation in the use of a structured and standardised rehabilitation protocol, called Cognitive Activation Therapy (CAT), on cognitive/functional status and biological ageing.

Regarding the evaluation of treatment efficacy on biological ageing, we analysed genetic and epigenetic biological ageing indicators, DNA methylation and leucocyte telomere length, on blood samples from patients with a major neurocognitive disorder in the mild-to-moderate phase of the disease recruited by CRIC, at the beginning and end of the visual arts-mediated cognitive activation cycle.

5. MATERIALS AND METHODS

5.1 Study Design

A total of 31 patients with a major neurocognitive disorder in the mild to moderate phase of the disease (predominantly Alzheimer's disease) were recruited by the CRIC. Patients recruited were aged between 47 and 85 years and have an educational background between 5 and 18 years. The exclusion criteria were: severe hypovisus and the use of antipsychotics and neuroleptics.

Each patient underwent a cycle of visual arts-mediated Cognitive Activation Therapy (CAT) and a pre- and post-treatment neuropsychological and functional assessment.

Cognitive Activation Therapy is a non-pharmacological therapy for the cognitive-communicative activation of the person with cognitive impairment in the mild to moderate phase of the disease, developed within the Regional Centre for Cerebral Ageing (CRIC) since 2001 (D. Gollin et al., 2011).

The patients worked in 8 small groups homogeneous in terms of age, schooling and cognitive resources. Each group underwent a rehabilitation cycle that included 17 sessions of which 14 at the CRIC and 3 at the Eremitani Civic Museum in Padua. The total treatment period was 30 days.

CAT sessions at the CRIC, were bi-weekly and included:

- one hour of physical activity in a small group led by the physiotherapist;
- two hours of CAT mediated by visual arts, in small groups, conducted by the speech therapists with the intervention of students in training from the Department of Cultural Heritage as art experts.

The 3 CAT sessions at the Eremitani Civic Museum lasted one hour and half.

Of the 31 patients, 20 underwent blood sampling at the beginning and end of the drug treatment and visual arts-mediated cognitive activation cycle to asse biological ageing indicators, i.e. DNAmAge and Telomere length, in our Laboratory of

Genomics and Environmental Mutagenesis – Department of Cardio-Thoracic-Vascular Sciences and Public Health (DCTV) of the University of Padua.

They were studied longitudinally at two different times (each subject is longitudinal the control of him/herself). The data thus obtained made it possible to analyse:

1. the relationship between the indicators of biological ageing, telomeres (telomerase) and epigenetic age, and with age in patients with neurocognitive disorders of a different nature at the mild-moderate stage.
2. the effect of the visual arts-CAT mediated cycle on indicators of biological ageing.

5.2 Cognitive assessment

All patients underwent cognitive assessment by some standardized tests performed at CRIC at the beginning and at the end of the study such as:

- **Mini Mental State Examination (M.M.S.E.)**. A questionnaire, consisting of very simple questions and small graphical tasks, to probe various aspects of brain function, such as orientation, memory, attention, computational ability, ability to recall certain acquisitions, and language.
- **Digit Span Forward and Backward Tests**. The Digit Span is the unit of memory measurement in Neuropsychology and consists of two tests that investigate the "verbal" span. The Digits Forward, the memorization of digits forward, and the Digits Backward, the memorization of digits backward. The first was used to assess the short-term memory and the second to evaluate the working memory.
- **Forward and Backward Course Tests**. Tests measuring the "span" of visuospatial memory, i.e., the amount of visuospatial information that can be retained in recent memory. The first was used to assess the short-term memory, while the second to evaluate the working memory.
- **Attentional Matrices**. A test designed to investigate different aspects of visual attention: speed, detection ability, and the interaction between working memory and visual-attentive processes.

- **Visual Object Space Perception** (V.O.S.P.) **Battery**. It evaluates visuospatial function, while minimizing the interference of other cognitive functions.
- **Complex Figure Copy Test** (pre CAT: Rey, post CAT: Taylor). It is widely used to assess the visuo-constructional ability and visual memory of neuropsychiatric disorders, including copying and recall tests.

Assessment of quality of life and mood tone:

- **Quality of Life**. Quality of life (QOL) is a multidimensional concept that measures a person's wellbeing.
- **Geriatric Depression Scale.** The Geriatric Depression Scale (GDS) is a self-report measure of depression in older adults.

Functional assessment by.

- **Direct Assessment of Functional Status** (DAFS). The DAFS is a standardized observation-based checklist designed to assess functional capabilities of adults with Alzheimer's disease, dementia, and schizophrenia. It can be used for evaluation and re-evaluation to assess any decline and effect on function of pharmacological intervention.
- **Communication Activities of Daily Living - 2** (CADL-2)**.** CADL-2 assesses the functional communication skills of adults with neurogenic communication disorders.

5.3 Analytical procedures

In the subset of 20 patients, , DNA was extracted from blood sample and then DNAmAge and LTL were determined. Blood tubes arrived at the Genomics and Environmental Mutagenesis Laboratory with the request and sample registration forms.

5.3.1 Extraction and quantification of DNA from blood

The automatic procedure for DNA extraction was performed for all blood samples using an automated QIAcube System (Qiagen, Milano, Italy) for high-throughput

purification. The DNAeasy Blood and Tissue Kit (Qiagen, Milano, Italy) was used, following the manufacturer's instructions and customized protocol. In particular, 400 µl of whole blood for each sample were processed for DNA extraction.

A volume of 400 µl of blood was taken from each sample and transferred into Sample Tubes RB (2 ml), which were then placed in the QIAcube shaker with the cap open. As instructed in the QIAcube Customised Protocol Sheet, the columns and elution tubes contained in the QIAamp DNA Blood and Tissue Kit were placed in the Rotor Adapters and inserted into the QIAcube according to the instructions in the QIAcube Technical Information loading chart according to the number of samples. In the side compartments, the holder containing the bottles of the reagents required for the extraction (AL buffer, ethanol, AW1 buffer, AW2 buffer, AE buffer), the tips and, in position A, a 2 ml tube containing Proteinase K were inserted according to the instructions in the QIAcube Customised Protocol Sheet. Everything was checked to ensure that everything was correctly positioned and the QIAcube was started by selecting the 'Blood or body fluid' protocol.

The eluted DNA samples in previously labelled 1.5 ml tubes were quantified using QIAexpert Quantification System (Qiagen, Milano, Italy) and Quantus Fluorometer system (Promega, Italy).

The QIAxpert Slide-40 (Qiagen) was prepared for reading by inserting 2 µl of AE buffer (blank) into the first well and 2 µl of the samples to be analysed into the next 2 µl, up to a maximum of 15 samples per slide. In the main screen of the instrument, Measure was selected and the corresponding position was selected for the blank and the samples to be analysed. The '260dsDNA' measurement has been selected. The results was the amount of DNA (ng/µl) and the 260/280 and 260/230 ratios for each sample. The first ratio is used as an indicator of DNA purity. We obtained genetic material suitable for subsequent analytical procedures from a qualitive point of view. After the quantification was completed, the DNA samples were analysed.

5.3.2 Telomere Lengths

LTL was measured after DNA extraction from whole blood samples, by using quantitative Real-Time PCR. In this study we measured relative TL in genomic

DNA by estimating 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 pool sample. The single-copy gene employed in this assay was human β-globin (HBG) (Pavanello et al., 2010).

The instrument used for PCR is SteponePlus Real-Time PCR System (Applied Biosystems). For each series of analysis, in order to calculate the relative quantities of T and S in ng of samples to be examined, a standard curve consisting of 6 points was drawn and generated from a serially diluted pool of DNA Standard 1 was prepared from DNA pool such that the final concentration was 40 ng/µl; the other Standards were obtained by 1:2 scaled dilution.

The preparation of the 96-well plate for PCR, which consists of transferring 10 µl of reaction mix and 5 µl of DNA (5 ng/µl) in the 96-well plate, was performed in QIAgility (Qiagen, Milano, Italy). Each standard and each DNA sample was assayed in triplicate, as was the negative control (H_2O) to assess for possible contamination during plate preparation.

Two PCRs were set up for the two primer pairs Telg/Telc and HBG1/HBG2, described previously by Cawthon; the sequences are shown in Table 1.

Table 1: Tel and HBG primer sequences.

	Primers sequence
Telg	ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT
Telc	TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA
HBG1	GCTTCTGACACAACTGTGTTCACTAGC
HBG2	CACCAACTTCATCCACGTTCACC

Once the instrument was started up, the Mix was prepared and distributed in the wells. A volume of Standards, samples and H_2O was taken and placed in the wells according to the protocol settings. The plate was centrifuged before proceeding with PCR. The PCR reaction was performed using the SteponePlus Real-Time PCR System (Applied Biosystems, Monza, Italy) and includes several thermal cycles: the reaction conditions of the two PCRs differ in the sequence of primers used, their

annealing temperatures and the total number of cycles (Tables 2 and 3). Nevertheless, the thermal cycling profile for both amplicons began with incubation at 95°C for 210 min to activate the AmpliTaq DNA polymerase.

Table 2: Reaction conditions for telomere amplification.

	Temperature	**Time**	**Number of cycles**
Initial denaturation	95°C	10 min	1
Denaturation **Annealing**	95°C 49°C	15 s 15 s	2
Denaturation **Annealing** **Extension**	95°C 62°C 74°C	15 s 10 s 15 s	35

Table 3: Reaction conditions for HBG gene amplification.

	Temperature	**Time**	**Number of cycles**
Initial denaturation	95 °C	10 min	1
Denaturation **Annealing**	95°C 58°C	15 s 1 min	35

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 <25%. To examine the reproducibility of the T/S ratio, the test was repeated for 10% of the samples on different days.

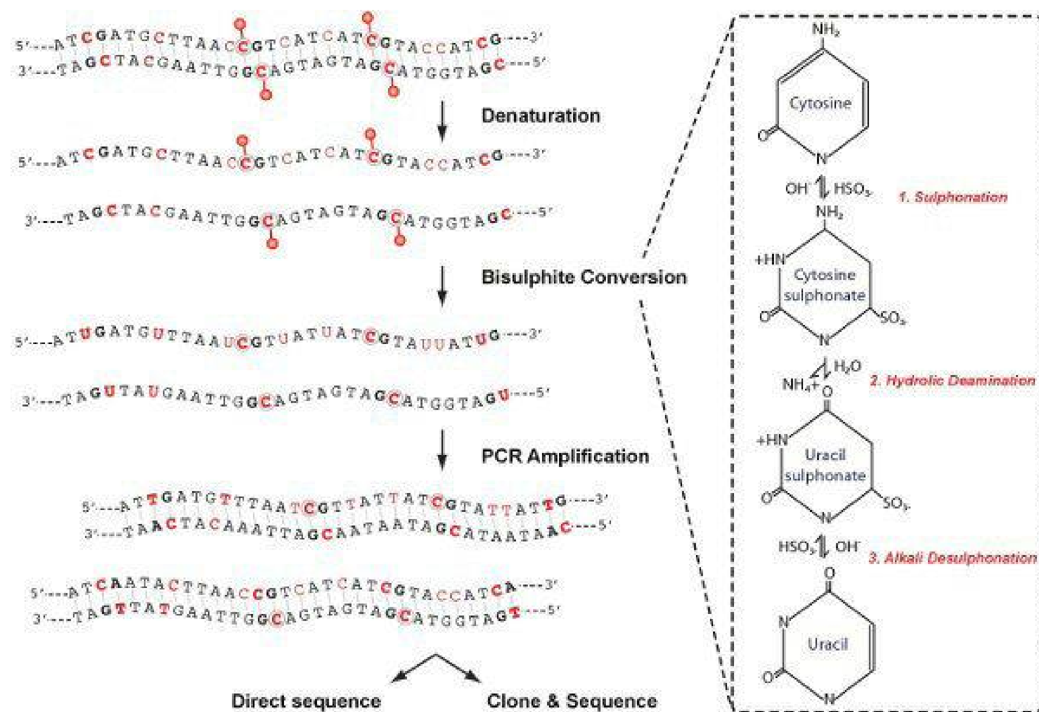
5.3.3 DNAmAge

DNAmAge, assessed on DNA extracted from blood samples, is determined by analysing the methylation level of selected markers using sodium bisulfite conversion and pyrosequencing. This method is based on determining the

methylation levels of a set of five selected markers (ELOVL2, C1orf132, KLF14, TRIM59 and FHL2) in genomic DNA.

Conversion with sodium bisulfite causes the deamination of cytosines, but not that of methyl cytosines; this method exploits the chemical properties of sodium bisulfite, i.e. it is only able to bind unmethylated cytosines belonging to any DNA sequence at the level of the double bond C5=C6. The unmethylated cytosine that binds the bisulfite undergoes hydrolytic deamination and the product, the deaminated cytosine, is converted to uracil in the presence of alkaline pH, resulting in the release of the bisulfite residue. The sodium bisulfite-treated DNA is then amplified by PCR, in which uracil is replicated as thymine, allowing the methylation rate to be analysed using a direct sequencing method such as pyrosequencing (Figure 15).

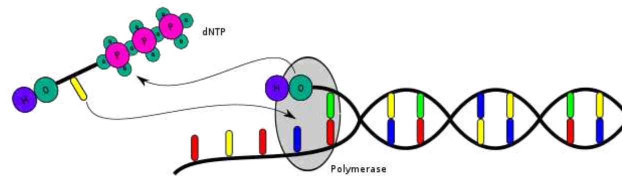
Figure 14: Steps for DNA methylation analysis.



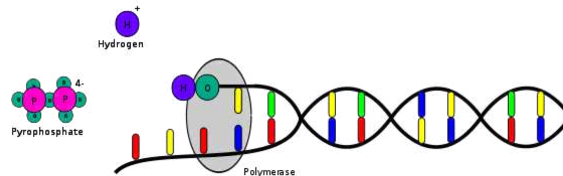
The pyrosequencing technique is based on the detection of pyrophosphates released during DNA synthesis and consists of several steps:

- Hybridization of the sequencing primer to the amplified single-stranded template DNA.
- Incubation of the template DNA with enzymes and substrates.
- Addition of the first deoxyribonucleotide triphosphate (dNTP) to the reaction; the DNA polymerase enzyme incorporates the nucleotide into the newly synthesised strand only if it is complementary to one or more bases in the template strand.
- Following nucleotide incorporation, pyrophosphate is released (PPi) to an extent equimolar to the amount of nucleotides incorporated (Figure 16).

Figure 15: Nucleotide incorporation and release of PPi- .



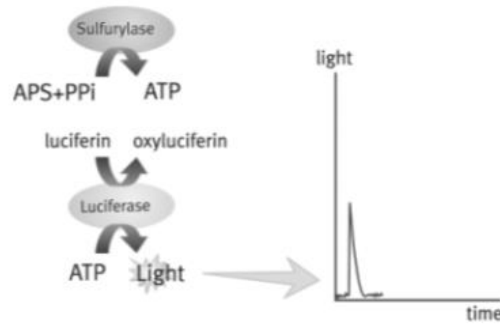
Polymerase integrates a nucleotide.



Hydrogen and pyrophosphate are released.

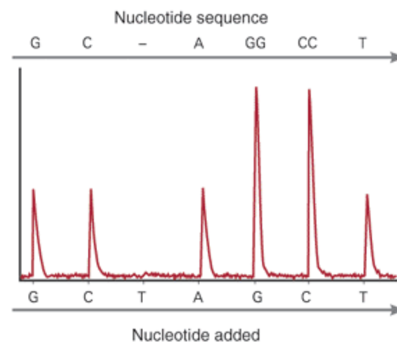
- The enzyme *adenosine triphosphate-phosphorylase*, in the presence of adenosine-5' phosphate, quantitatively converts PPi- to ATP.
- The enzyme *luciferase* converts luciferin to oxyluciferin using ATP, in a reaction that generates visible light proportional to the amount of ATP, so each light signal is proportional to the number of nucleotides incorporated; the light is detected by a photomultiplier that returns a peak in a graph (Figure 17).

Figure 16: Light generation and corresponding peak in the graph.



- The *apyrase* enzyme continuously degrades unincorporated nucleotides and excess ATP; when degradation is complete, another nucleotide is added.
- Nucleotides are added to the reaction one at a time and, when the complementary strand to the mould is fully synthesised, the nucleotide sequence is determined by software that returns graphs in which each incorporated base is displayed as a peak (Figure 18).

Figure 17: Nucleotide sequence obtained in the graph.



This method has a high sensitivity (more than 0.01% of the methylated alleles present are detected, low coefficient of variation in experiments, better signal-to-noise ratio) allowing qualitative and quantitative DNAm analysis and accurate quantification of methylation sites.

Conversion with sodium bisulfite was performed using the Epitect Fast Bisulfite Conversion kit (Qiagen, Milano, Italy) according to the manufacturer's instructions. These involved the conversion of 1 to 500 ng of DNA per sample on a maximum reaction volume of 140 µl. The preparation of the reaction volumes for each sample was performed on QIAgility (Qiagen, Milano, Italy), an instrument that enables

automatic, rapid and high-precision preparation. The components for the conversion reaction and the corresponding volumes are shown in Table 4.

Table 4: Components for reaction with bisulfite (*: for concentrations of DNA $\leq$ 25 ng/μl, 40 μl of DNA is used).

Components	Volume (μl)
DNA	Variable * (max 40 μl)
H_20 RNase-Free	Variable *
Bisulfite Solution	85
DNA Protect Buffer	15
Total Volume	**140**

At the end of the sample preparation, it was observed if the DNA Protect Buffer changed from green to blue after the addition of Bisulfite Solution, indicating sufficient mixing and the correct pH for the conversion reaction, so that the conversion could be performed using the Rotor-Gene instrument (Qiagen, Milano, Italy).

The prepared samples were labelled, vortexed and fixed on the support that was then inserted into the Rotor-Gene (Qiagen, Milano, Italy), then the instrument was started by selecting the 'Fast 15 min Conversion' protocol. For each sample, the reaction was conducted on a total volume of 140 μl. The reaction conditions for amplification are shown in Table 5.

Table 5: Conditions of the conversion reaction with bisulphite.

Step	Time	Temperature
Denaturation	5 min	95°C
Incubation	15 min	60°C
Denaturation	5 min	95°C
Incubation	15 min	60°C
Hold	Undetermined	20°C

Following the conversion reaction, the Clean Up step was carried out in the Qiacube (Qiagen Milano, Italy) using the Epitect Fast Bisulfite Conversion kit (Qiagen Milano, Italy). The reagents (Buffer BL, Buffer BW, Buffer BD, Buffer EB, ethanol 96-100%) and the material (columns, RB tubes, adapters, elution tubes) had to be prepared according to the instructions in the Protocol Sheet. for the 'Clean Up' protocol. at the end of the procedure, the DNA samples were eluted in the Elution Buffer (EB). Quantification of the DNA obtained as a result of the bisulfite conversion reaction was performed using QIAxpert by selecting 'A260 ssDNA' and using EB as a blank.

Following the bisulfite conversion, amplification of the selected markers was performed using the SteponePlus Real-Time PCR System instrument (Applied Biosystems). Before proceeding with PCR, the 96-well plate was prepared with the reaction mix in QIAgility (Qiagen, Milano, Italy), while the samples were added manually at the end of the reaction. For each sample, the reaction was prepared for each of the 5 markers using the primers contained in the AgePlexMono kit (Biovectis, Warszawa, Poland). Details of the sites and sequences to be analysed are given in Table 6.

Table 6: Markers, AgePlex sequences to be analysed, CpG sites and location. (*:Genome Reference Consortium Human genome build 38).

Marker	Analysis Sequence	CpG site	Position (GRCh38 *)
ELOVL2	CCRTAAACRTTAAACCRCCRCRCRAAACCRAC	C7	Chr6: 11044661
C1orf132	AAATCTACRCAAACRACRATAAATAATCC	C1	Chr1: 207823681
TRIM59	GGTTTGGYGYGGGAYGAGGYGAAGYGTYGG TGGTYGAYGGTTTTTGAGGAATTATTTTTATTT	C7	Chr3: 160450199
KLF14	TYGYGTTTTTTTTTTGTYGGYGAGTTAGGTA	C1	Chr7: 130734355
FHL2	AGTTATYGGGAGYGTYGTTTTYGGYGTGGG	C2	Chr2:

	TTTTYGGGYGYGAGTTTYGGAYGAGGTT TGGG		10539928 8

For the PCR reaction, a total reaction volume of 25 µl was prepared for each sample (as shown in Table 7):

Table 7: Components for the amplification reaction of selectable markers (*: approx. 10-20 ng of converted DNA).

Component	**Volume (µl)**
PyroMark PCR Master Mix*	12,5
CoralLoad	2,5
$MgCl_2$ 3 mM	1
AgePlex PCR primers	2
DNA	Variabile* (max 7 µl)
H_2O RNase-Free	Variabile (max 25 µl)
Total Volume	**25**

* PyroMark PCR Master Mix contains 1x PyroMark PCR Buffer, dNTP and the enzyme HotStarTaq DNA Polymerase, which is thermostable and therefore able to withstand the high temperatures required for DNA denaturation.

PCR was performed on SteponePlus Real-Time PCR System (Qiagen, Milano, Italy) by selecting the "AgePlexMono" protocol. The conditions of the amplification reaction are shown in Table 8.

Table 8: Reaction steps and conditions relating to the amplification of selected markers.

	Temperature	**Time**	**Number of cycles**
Initial denaturation	95 °C	10 min	1
Denaturation	94°C	30s	40-45
Annealing	54-56°C	60s	
Extension	72°C	60-90 s	
Final extension	72°C	10 min	1

Once the PCR reaction was complete, each of the amplification products contained one of five methylation markers to be sequenced separately.

The pyrosequencing was fully automated with the PyroMark Q48 Autoprep instrument (Qiagen, Milano, Italy), which used Pyromark Q48 Advanced Reagents (Qiagen, Milano, Italy), containing Denaturation Solution (DS), Enzyme (E), Substrate (S), Annealing Buffer (AB) and nucleotides. Before starting with the sample loading phase, the run file for the Pyromark Q48 Autoprep Software assay had to be prepared with the sample layout, then the file was transferred to the PyroMark Q48 and selected. The instrument indicated for each cartridge position the reagent and the corresponding volume to be added according to the number of samples to be analysed, so the reagents were loaded, the cartridges closed and the instrument waited for Priming to take place. At the end of Priming, following the indications in the display, a new disc (PyroMark Q48 Discs) was inserted and the wells in the disc were loaded, proceeding as indicated by the run file. 3 µl of beads were loaded first, followed by approximately 10 µl of sample per well according to the run file. When all samples were loaded, the instrument was started. The pyrosequencing primers (AgePlexMono kit, Biovectis) were manually loaded when the instrument indicated that loading was possible. 2 µl of primer per sample were added according to the assay set up. When the run was completed, it was automatically saved to a USB stick. Analysis of the run file was performed automatically using Pyromark Q48 Autoprep software, which generates pyrograms.

Methylation levels are, therefore, expressed as the percentage of cytosines methylated at the CpG sites considered. The methylation percentages obtained for each of the five markers (ELOVL2-C7, C1orf132-C1, TRIM59-C7, KLF14-C1 and FHL2-C2) were entered into an online system (www.agecalculator.ies.krakow.pl) to estimate DNAmage in years.

All samples were analysed 3 times for each marker to check the reproducibility of the results and the mean was used for statistical analysis.

All samples were analysed on two different days and the coefficient of variation (CV) in replicate pyrosequencing cycles was 0.5%.

5.3.4 Statistical Analysis

Statistical analysis was performed using the software StatsDirect 3. The results were considered significant for p-values ≤ 0.05.

To analyse the main characteristics of the patients, we performed descriptive statistic that allowed us to determine all patients' characteristics at baseline (T0) and at follow up (T1), including interval variables (mean ± SD), categorical variables (number, %) and p-values comparing the two groups.

The paired t-test was used to assess both LT and DNAmAge. The ***Paired t-test*** assesses the presence or absence of a significant difference between the mean of the two measurements observed at the baseline (T0) and at follow up (T1) for all patients. A simple linear regression test was used to assess possible correlations between both LT and DNAmAge with certain clinical parameters

We used ***Multiple Linear Regression*** to examine the influence of other independent variables such as gender, days of treatment and education on both LT and DNAmAge (dependent variables) of all study subjects.

6. RESULTS

6.1 Characteristics of study subjects

The characteristics of the subjects n=20 who underwent a visual arts-mediated cognitive activation cycle in addition to the pharmacological treatments indicated for their condition that had been stabilised for at least three months are shown in Table 9. The age of the participants presents mean age of 77,8 years and the gender distribution is quite uniform with 55% of men and 45% of women.

As shown in Table 9, there are eight different types of clinical diagnosis within the study population including Alzheimer's disease (AD), Mild Cognitive Impairment (MCI), Lewy body dementia (LBD), Mixed dementia (MD), Frontotemporal degeneration (FTD), Primary progressive aphasia (PPA), Normal Pressure hydrocephalus (NPH) and Traumatic brain injury (TBI). Most patients suffer from Alzheimer's disease (35%) and the second most common disease among project participants is Mild Cognitive Impairment, MCI (25%).

LTL, DNAmAge, Blood parameters, Treatments, Cognitive Activation Therapy (CAT) as Days of Treatment and Hours of treatment are showed for 20 subject. Non difference in LTL and DNAmAge at the beginning of the study (T0) and at the follow up (T1) are showed.

Table 9: Main characteristics of n=20 patients at the beginning of the study (T0) and at the follow up (T1), including interval variables (mean ± SD), categorical variables (number, %) and p-values.

Variables	T0	T1	cut-off PC	*P; P-trend*
Demographic data				
Age (years)	77,8 (±6,2)	77,8 (±6,2)		
Gender				
Male (n, %)	11 (55 %)			
Female (n, %)	9 (45 %)			
Education (years)	10.3 (± 4.67)			
Physical activity (n, %)	19 (96.8%)			
Diagnosis				
MCI (n, %)	5 (25%)			
AD (n, %)	7 (35%)			
LBD (n, %)	1 (5%)			
MD (n, %)	3 (15%)			
FTD (n, %)	1 (5%)			
PPA (n, %)	1 (5%)			
NPH (n, %)	1 (5%)			
TBI (n, %)	1 (5%)			
DNAmAge (n=20)	63.55 (± 5.31)	64.05 (± 5.48)		0.3622
LTL (n=20)	1.11 (± 0.30)	1.18 (±0.26)		0.1248
Blood parameters				
Leucocytes (10^3/ml)	6.26 (± 2.44)		4.40 - 11.00	
Blood red cells (10^3/ml)	4.36 (± 0.61)		4.31 - 5.10	
Hemoglobin (g/dl)	128,13 (± 25.23)		123 - 153	
Platelet count (10^3/ml)	235.12 (± 67.61)		150 - 450	
Neutrophils (10^3/ml)	3.68 (± 1.78)		1.80 - 7.80	
Lymphocytes (10^3/ml)	1.79 (± 0.53)		1.10 - 4.80	
Monocytes (10^3/ml)	0.58 (± 0.29)		0.20 - 0.96	
Eosinophils (10^3/ml)	0.20 (± 0.14)		0.00 - 0.50	
Basophils (10^3/ml)	0.04 (± 0.07)		0.00 - 0.20	

Cholesterol (mg/dl)	166.65(± 46,27)			
Triglycerides (mg/dl)	94.82 (± 32.01)			
Low-density lipoprotein (mg/dl)	91.69 (± 44.32)			
High-density lipoprotein (mg/dl)	60.06 (± 18.26)			
Glycated hemoglobin (mmol/mol)	42.27 (± 7.58)			
Creatinine (mg/dl)	0.87 (± 0.22)			
Urea (mmol/L)	6.13 (±2.73)		2.50 - 7.50	
Bilirubin (mg/dl)	0.04 (± 0.18)		/	
Total protein	71.14 (± 13.03)		64 - 83	
Aspartate aminotransferase (U/L)	24.12 (± 4.96)		10 - 35	
Omocisteina (u--mol/L)	12.73 (± 2.67)		0.00 - 15.00	
APOE (SNPs)				
E3/E3 (n, %) (1)	10 (62,5 %)			
E3/E4 (n, %) (2)	3 (18,75%)			
E2/E3 (n, %) (3)	0 (0 %)			
E2/E4 (n, %) (4)	1 (6.25 %)			
E4/E4 (n, %) (5)	2 (12,5 %)			
Treatments				
Cognitive Activation Therapy (CAT)				
Days of Treatment	43,45 (± 12.1)			
Hours of treatment	30.03 (± 3.49)			
Physiotherapy				
Days of Treatment	8.4 (± 3.28)			
Hours of treatment	8.4 (± 3.28)			

*Paired t tests two sided

In Table 9 days of treatment 43,45 days (SD 12,1), the high standard deviation of days of treatment may be related to the fact that three out of 20 participants stopped the cognitive activation treatment at 16 days.

6.2 Telomere length analysis

Table 10 shows not significant difference of telomere length in the peripheral blood leucocytes (LTL) before and after cognitive activation therapy (p-value= 0,1248). However, if we exclude participants who underwent 16 days of therapy, the difference in leucocyte telomere length at T1 and T0 becomes significant (p= 0,0269).

Table 10: Main characteristics of n=20 patients at the start of the study (T0) and at follow-up (T1), including interval variables (mean ± SD), categorical variables (number, %) and p-values.

Variables	T0	T1	p §
Age (years)	78.14 (±6.26)	78.31 (±6.27)	
Gender			
Male (n, %)	11 (55 %)	11 (55 %)	
Female (n, %)	9 (45 %)	9 (45 %)	
LTL (T/S)	1.11 (±0.30)	1.18 (±0.26)	0.1248
Treatment (days)		43.45 (±12.16)	

*Paired t tests two sided

Simple linear regression analysis was used to assess the influence of age and gender on the difference between LTL at the beginning (T0) and at the end (T1) of the treatment [ΔLTL (T1-T0)]. The influence of age on ΔTL was not significant (p= 0,6942), whereas the influence of gender was significant (p= 0,0267). In particular, the ΔLTL of men was higher than women's ΔTL as shown in Chart 1.

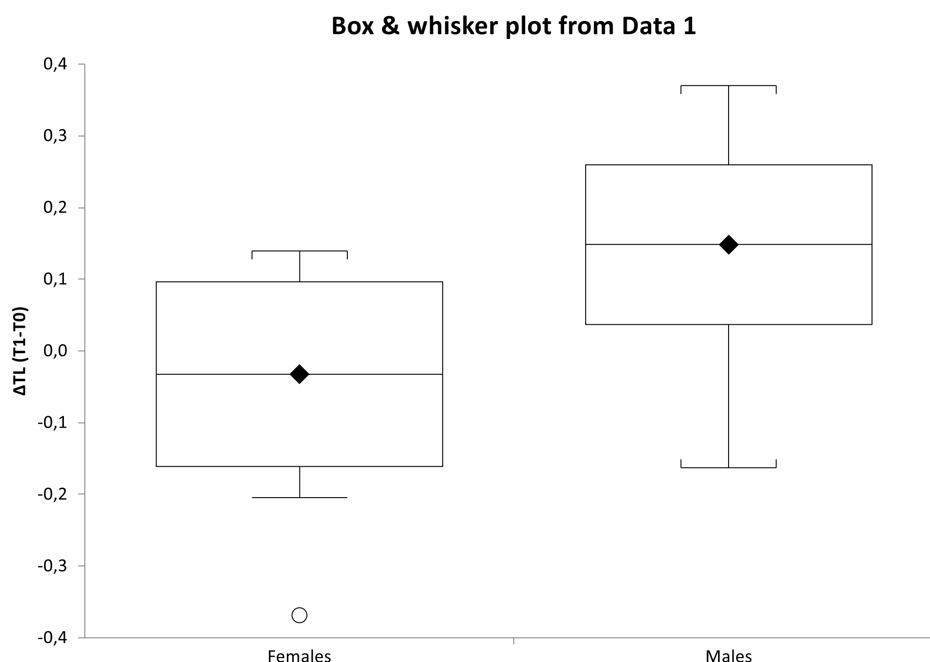


Chart 1. Box and Whisker plot: mean and 95 % Interval confidence and range of ΔTL (T1-T0) (T/S) in Females (9 patients) and Males (11 patients).

If we separated the study population into two groups, men and women, in Table 11 the Paired t-test shown that T1 LTL are higher than T2 in men but not in women. Women's LTL is longer than men's leucocyte telomere length at the beginning of the study (T0) (p= 0,0074) and even at the follow-up (T1) (p= 0,049), but the difference of women's telomere length at T0 and T1 is not significant. Men's telomere length is shorter than women's at the beginning and end of treatment, but the difference is significant with an increase in TL after therapy.

Table 11: Leucocyte telomere lengths (mean ± SD) at the start of the study (T0) and at the follow-up (T1) of men and women, including p-values comparing the measurement at T0 and T1 in the two groups.

Groups	T0	T1	*p* *
Men			
LTL (mean ± DS)	0,95 (± 0,18)	1, 10 (± 0,24)	0,0142
Women			
LTL (mean ± DS)	1,32 (± 0,30)	1,28 (± 0,27)	0,578

*Paired t tests two sided

Furthermore, we performed simple linear regression analysis to evaluate the influence of independent variables (red blood cells) on LTL at T1 in the study population. We found a positive correlation (p= 0,063) between LTL and red blood cells shown in Chart 2. Although the correlation is not significant, it's evident that as telomere length increases, there is an increase in the number of red blood cells.

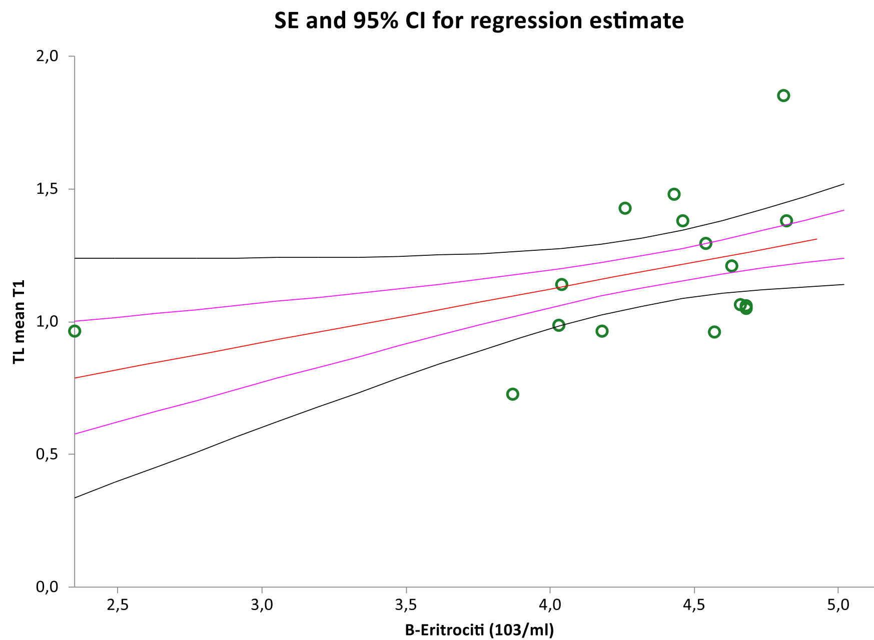


Chart 2. Simple linear regression: TL mean T1 and erythrocytes on 20 participants (p=0,063).

Equation: TL mean T1 = 0,20251 B-Eritrociti (103/ml) + 0,313007

Standard Error of slope = 0,100838

95% CI for population value of slope = -0,012421 to 0,41744

Correlation coefficient ® = 0,460327 (r_2 = 0,211901)

95% CI for r (Fisher's z transformed) = -0,02609 to 0,770497

t with 15 DF = 2,00827

Two sided P = 0,063

Power (for 5% significance) = 44,69%

Correlation coefficient is not significantly different from zero

We also performed a multiple regression analysis to study the influence of erythrocytes and leukocytes on LTL at follow-up (T1) by excluding the three patients who did not complete therapy.

As shown in Table 12, erythrocytes, unlike leucocytes, are positively and significantly correlated with telomere length (p= 0,022). Thus, as the number of erythrocytes increases, telomere length increases, slowing down ageing.

Table 12. Multiple Linear Regression on the influence of erythrocytes and leukocytes on LTL at the follow-up (T1) in 17 patients.

Variables	b	r	t	p
Erythrocytes (10^3/ml)	b1 = 0,298769	r = 0,626184	t = 2,663695	**P = 0,022**
Leukocytes (10^3/ml)	b2 = 0,043406	r = 0,416954	t = 1,521443	P = 0,1564

*Paired t tests two sided

We also analysed, through the use of linear regression analysis, the effect of treatment duration (days) on the difference in LTL between T1 and T0 [ΔLTL (T1-T0)]. In Chart 3, a positive correlation (p=0.052) is observed, indicating an increase in ΔLTL (T1-T0), a slowdown in ageing, as the number of treatment days increased.

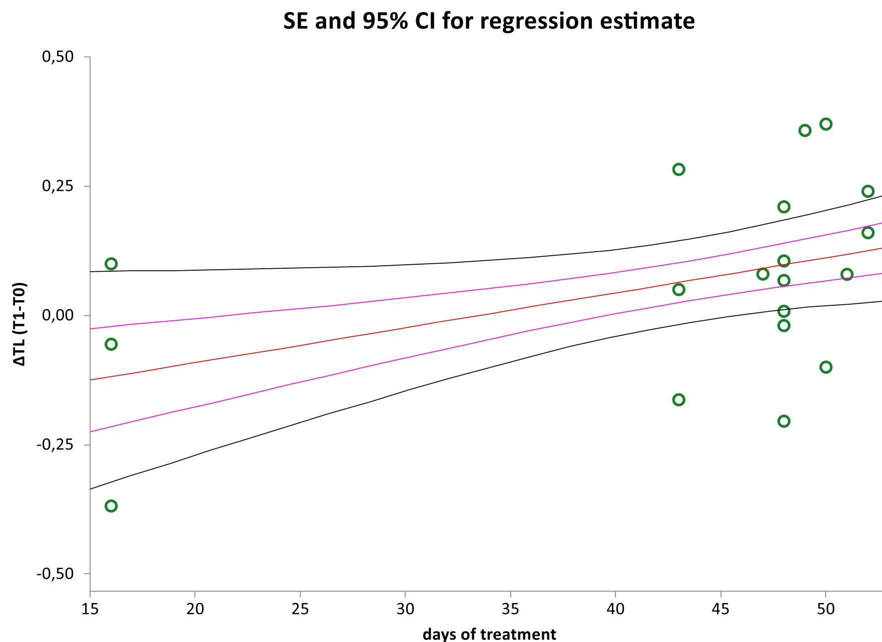


Chart 3. Simple linear regression: ΔTL (T1-T0) and days of treatment evaluated on 20 participants (p=0,052).

Equation: ΔTL (T1-T0) = 0,006758 days of treatment – 0,226671

Standard Error of slope = 0,003244

95% CI for population value of slope = -0,000058 to 0,013573

Correlation coefficient ® = 0,440705 (r_2 = 0,194221)

95% CI for r (Fisher's z transformed) = -0,002256 to 0,739088

t with 18 DF = 2,082936

Two sided P = 0,0518

Power (for 5% significance) = 48,24%

Correlation coefficient is not significantly different from zero

A multiple linear regression was also performed to assess the influence of days of treatment and years of education at ΔLTL. The results shown in Table 13 show that the days of treatment associated with years of education is a determinant of ΔLTL.

Table 13. Multiple Linear Regression of the days of treatment and education's influence on ΔLTL.

Variables	b	r	t	p
Treatment (days)	b1 = 0,00823	r = 0,526178	t = 2,551216	**P = 0,0207**
Education (years)	b2 = 0,013847	r = 0,371211	t = 1,648318	P = 0,1176

*Paired t tests two sided

6.3 Epigenetic ageing analysis

As Table 14 shows, there is no difference between DNAmAge before and after the treatment (p= 0,3622) in the 20 patients, and the result remains the same even when excluding the three patients who had a 16-day treatment (p= 0,4624).

Table 14: Main characteristics of n=20 patients at the start of the study (T0) and at follow-up (T1), including interval variables (mean ± SD), categorical variables (number, %) and p-values.

Variables	T0	T1	p [§]
Age (years)	78.14 (±6.26)	78.31 (±6.27)	
Gender			
Male (n, %)	11 (55 %)	11 (55 %)	
Female (n, %)	9 (45 %)	9 (45 %)	
DNAmAge	63.55 (± 5.31)	64.05 (± 5.48)	0.3622
Treatment (days)		43.45 (±12.16)	

*Paired t tests two sided

Through Simple linear regression analysis, we assessed the correlation between DNAmAge and age, confirming a significant positive correlation (p= 0.0077) between the biomarker of ageing and age, as shown in Chart 4.

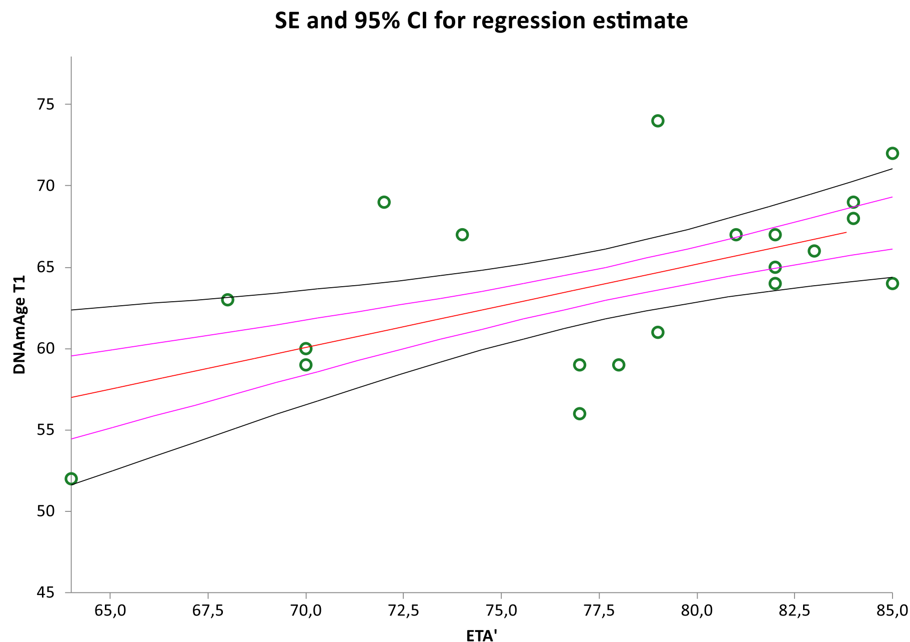


Chart 4. <u>Simple linear regression</u>: DNAmAge mean T1 and Age on 20 patients (p= 0,0077).

Equation: DNAmAge T1 = 0,510394 Age + 24,341357

Standard Error of slope = 0,170023

95% CI for population value of slope = 0,15319 to 0,867598

Correlation coefficient ® = 0,577596 (r_2 = 0,333618)

95% CI for r (Fisher's z transformed) = 0,181455 to 0,812455

t with 18 DF = 3,001919

Two sided P = 0,0077

Power (for 5% significance) = 76,04%

Correlation coefficient is significantly different from zero

In contrast to telomere length analysis, we did not find a significant correlation with gender. Thus, there is no influence of gender on epigenetic age.

We performed Simple linear regression analysis to evaluate the influence of clinical parameters on DNAmAge. As shown in Chart 5, although not significant, we found a negative association between DNAmAge and the count of leucocytes (p= 0,0668). Thus, as the number of leucocytes increases, the epigenetic age decreases.

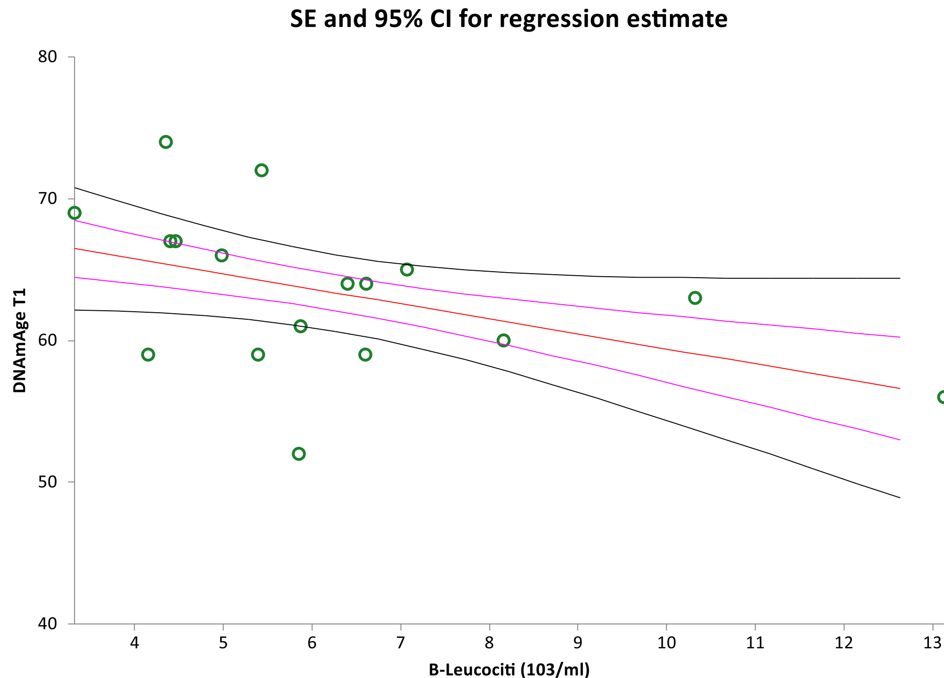


Chart 5. Simple linear regression: DNAmAge mean T1 and Leucocytes (10^3/ml) on 20 patients (p= 0,0668).

Equation: DNAmAge T1 = -1,056842 B-Leucocytes (103/ml) + 69,973121

Standard Error of slope = 0,534754

95% CI for population value of slope = -2,196642 to 0,082959

Correlation coefficient ® = -0,454525 (r_2 = 0,206593)

95% CI for r (Fisher's z transformed) = -0,767498 to 0,033421

t with 15 DF = -1,976314

Two sided P = 0,0668

Power (for 5% significance) = 43,63%

Correlation coefficient is not significantly different from zero

A further interesting non-significant correlation we found between DNAmAge and clinical parameters is with total cholesterol (p = 0,134), as shown in Chart 6. Excluding the three patients with only 16 days of therapy, as total cholesterol (mg/dl) increases, there is an increase in epigenetic age.

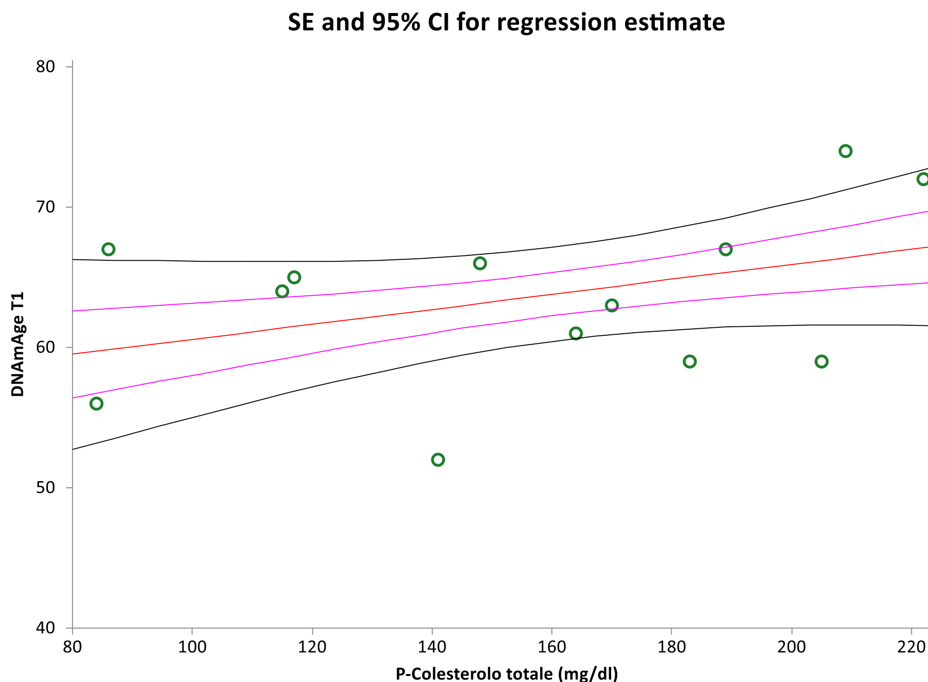


Chart 6. Simple linear regression: DNAmAge and Total Cholesterol (mg/dl), on 17 patients (p=0,134).

Equation: DNAmAge T1 = 0,053587 P-Colesterolo totale (mg/dl) + 55,214362

Standard Error of slope = 0,033343

95% CI for population value of slope = -0,019062 to 0,126235

Correlation coefficient (r) = 0,420853 (r_2 = 0,177117)

95% CI for r (Fisher's z transformed) = -0,141272 to 0,777761

t with 12 DF = 1,607134

Two sided P = 0,134

Power (for 5% significance) = 30,79%

Correlation coefficient is not significantly different from zero

Simple linear regression analysis assessed the influence of treatment days on the difference between DNAmAge at the beginning (T0) and at the end of the therapy (T1) [ΔDNAmAge (T1-T0)]. Unlike telomere length, there is not a significant correlation between epigenetic age and treatment days on 20 patients (p= 0,7133) as shown in Chart 7.

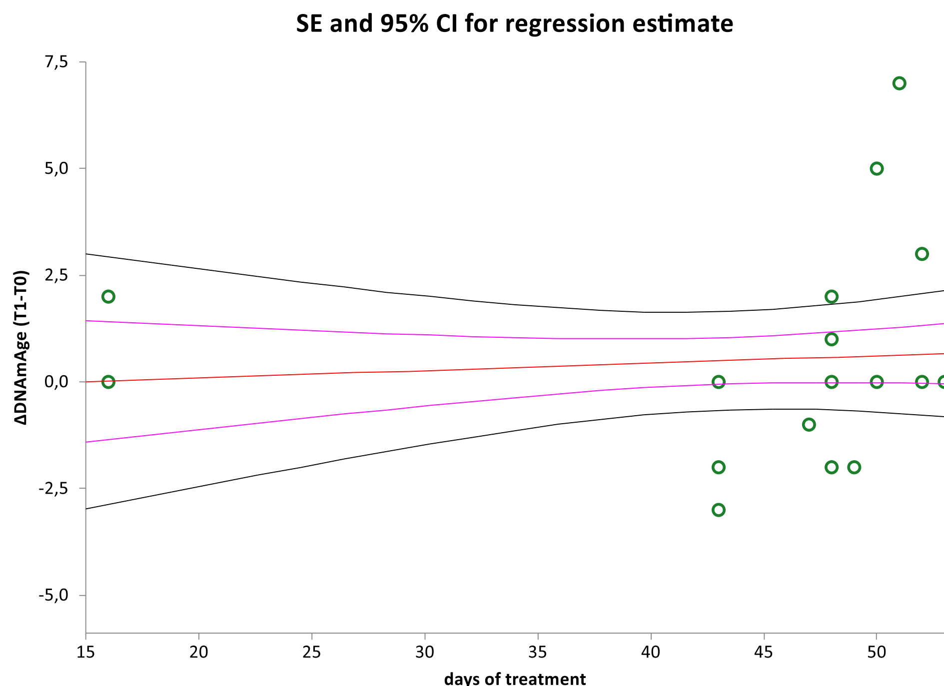


Chart 7. Simple linear regression: ΔDNAmAge (T1-T0) and days of treatment (days) on 20 patients (p=0,7133).

Equation: ΔDNAmAge (T1-T0) = 0,017266 days of treatment – 0,250218

Standard Error of slope = 0,046252

95% CI for population value of slope = -0,079906 to 0,114438

Correlation coefficient ® = 0,087651 (r_2 = 0,007683)

95% CI for r (Fisher's z transformed) = -0,36919 to 0,510376

t with 18 DF = 0,373308

Two sided P = 0,7133

Power (for 5% significance) = 5,44%

Correlation coefficient is not significantly different from zero

By excluding the three patients with only 16 days of therapy, there is a positive and significant correlation between ΔDNAmAge (T1-T0) and days of treatment (p= 0,035) as shown in Chart 8. Unlike telomere length analysis where as the number of treatment days increases there is a slowing down of ageing, in this case as the number of treatment days increases there is an increase in ageing.

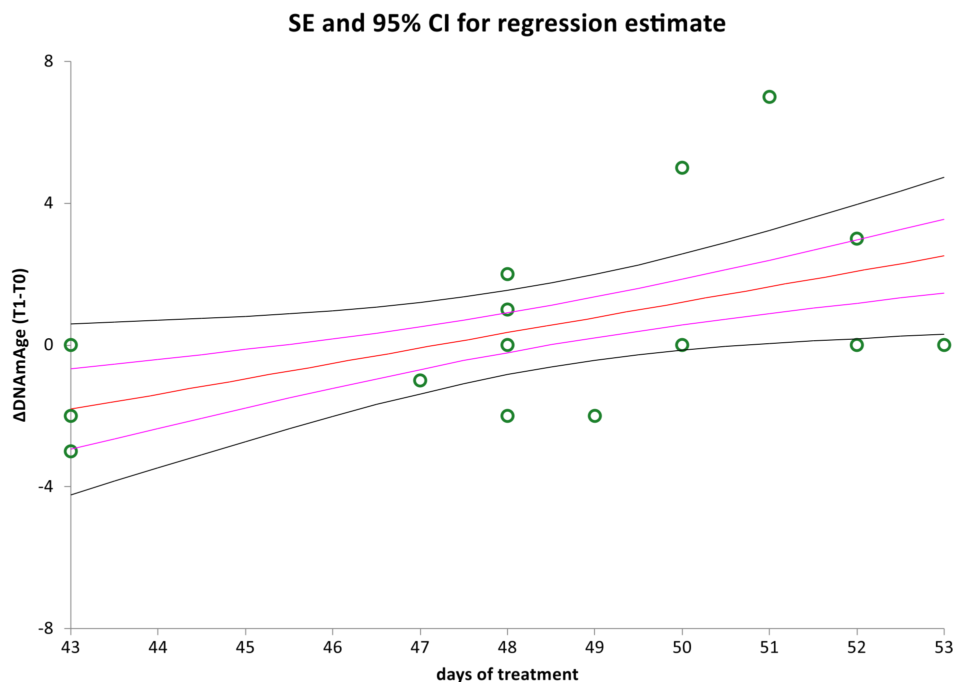


Chart 8. Simple linear regression: ΔDNAmAge (T1-T0) and treatment days (T1-T0) evaluated on 17 patients (p=0,035).

Equation: ΔDNAmAge (T1-T0) = 0,432337 days of treatment – 20,408733

Standard Error of slope = 0,186824

95% CI for population value of slope = 0,03413 to 0,830543

Correlation coefficient ® = 0,512922 (r_2 = 0,263089)

95% CI for r (Fisher's z transformed) = 0,042838 to 0,797064

t with 15 DF = 2,314137

Two sided P = 0,0352

Power (for 5% significance) = 54,75%

Correlation coefficient is significantly different from zero

6.4 Cognitive tests results

The results of the twelve cognitive tests performed on the 20 patients at the Regional Centre for the Study and Treatment of Cerebral Aging (CRIC) are shown in Table 20.

To assess the effectiveness of the cognitive activation therapy, we performed Paired t tests of the results of each cognitive test at the beginning (T0) and the end (T1) of the treatment.

As shown in Table 15, five of the twelve cognitive tests are significant. In particular, a significant positive change was noted in the results of the Rey–Osterrieth complex figure (ROCF) test ($p< 0,0001$), Progressive Silhouettes and Position of Visual Object and Space Perception Battery (VOSP) ($p <0,0001$ and $p=0,0349$ respectively), Communication Activities of the Daily Living (CADL 2) test ($p <0,0001$) and Direct Assessment of Functional Status (DAFS) test ($p <0,0001$).

Table 15. Cognitive tests' results of n=20 patients at the beginning of the study (T0) and at the follow up (T1), including interval variables (mean ± SD), categorical variables (number, %) and p-values comparing the two groups.

Variables	T0	T1	cut-off PC	*P; P-trend*
Cognitive test/tools				
Screening				
Mini Mental State Examination (MMSE)				
PG	23.4 (± 2.74)	24.63 (± 2.81)		0,1031
PC	22.66 (± 2.56)	23.86 (± 2.35)	≤ 24	0,1666
Clock Test	16.4 (± 2.21)	15.74 (± 4.73)	≤ 18	0,421
Short-term memory				
Digit Span Forward				
PG	4.7 (± 0.80)	4.97 (± 0.81)		0,0551
PC	5.16 (± 0.79)	5.33 (± 0.85)	< 4.26	0,0551
Pathologic (n, %)	2 (10%)	0 (0%)		
Borderline (n, %)	2 (10%)	2 (10.53%)		
Non Pathologic (n, %)	16 (80%)	17 (89.47%)		
Forward Corsi				
PG	4.47 (± 1.12)	4.71 (± 1.16)		0,5292
PC	4.95 (± 1.17)	5.20 (± 1.19)	< 3.46	0,5292
Pathologic (n, %)	1 (5 %)	0 (0%)		
Borderline (n, %)	5 (25 %)	1 (5.88%)		
Non Pathologic (n, %)	14 (70 %)	16 (84.21%)		
Attentional skills				
Attentional matrices				
PG	43.75 (± 9.65)	45.95 (± 9.54)	/	0,3418
PC	45.15 (± 9.99)	47.46 (± 10.73)	≤ 30	0,3418
Pathologic (n, %)	1 (5%)	2 (10.52%)		
Borderline (n, %)	1 (5%)	0 (0%)		
Non Pathologic (n, %)	18 (90%)	17 (89.47%)		

Trial making test A				
PG	74.5 (± 32.52)	68.05 (± 25.60)	/	0,2746
PC	43.2 (± 38.02)	36 (± 34.78)	≥ 94	0,2464
Pathologic (n, %)	1 (5%)	0 (0%)		
Borderline (n, %)	2 (10%)	3 (15.79%)		
Non Pathologic (n, %)	17 (85%)	16 (84.21%)		
Trial making test B				
PG	278.29 (± 98.36)	289.06 (± 80.36)	/	0,5577
PC	199.82 (± 103.97)	207.5 (± 87.29)	≥ 283	0,5888
Pathologic (n, %)	3 (17.65%)	3 (16.67%)		
Borderline (n, %)	7 (41.18%)	7 (38.89%)		
Non Pathologic (n, %)	7 (41.18%)	8 (44.44%)		
Trial making test B - A				
PG	206.59 (± 84.43)	220.28 (±80.56)	/	0,406
PC	157.35 (± 79.73)	169.61 (± 77.23)	≥ 187	0,406
Pathologic (n, %)	8 (47.06%)	8 (44.44%)		
Borderline (n, %)	2 (11.76%)	3 (16.67%)		
Non Pathologic (n, %)	7 (41.18%)	7 (38.89%)		
Working Memory				
Digit Span backward				
PG	3.48 (± 0.63)	3.41 (± 0.95)	/	0,6499
PC	3.86 (± 0.75)	3.81 (± 1.11)	< 2.65	0,6499
Pathologic (n, %)	1 (5%)	1 (5.26%)		
Borderline (n, %)	3 (15%)	4 (21.05%)		
Non Pathologic (n, %)	16 (80%)	15 (78.95%)		
Backward Corsi				
PG	4 (± 1.11)	4 (± 0,89)	/	0,5445
PC	4.55 (± 1.18)	4.57 (± 0,98)	< 3.17	0,6065
Pathologic (n, %)	3 (15%)	2 (12.50%)		
Borderline (n, %)	1 (5%)	0 (0%)		
Non Pathologic (n, %)	16 (80%)	14 (87.50%)		
Constructive skills				
Copy of complex geometric figure				

PG	28.26 (± 4.41)	31.68 (± 4.19)	/	**P<0,0001**
PC	28.50 (± 4.09)	33.48 (± 4.05)	pre < 28 post < 27.66	**P<0,0001**
Pathologic (n, %)	8 (42.11%)	1 (5.26%)		
Borderline (n, %)	7 (36.84%)	2 (10.53%)		
Non Pathologic (n, %)	4 (21.05%)	16 (84.21%)		
Visual-perceptual / visual-spatial skills				
Visual Object and Space Perception Battery (VOSP)				
Screening	19.25 (± 0.72)	19.53 (±0.70)	≤ 15	0,2623
Pathologic (n, %)	0 (0%)	0 (0%)		
Borderline (n, %)	0 (0%)	0 (0%)		
Non Pathologic (n, %)	20 (100%)	19 (100%)		
Incomplete Letters	18.1 (± 1.29)	18.78 (±1.39)	≤ 17	0,0723
Pathologic (n, %)	2 (10%)	1 (5.26%)		
Borderline (n, %)	0 (0%)	0 (0%)		
Non Pathologic (n, %)	18 (90%)	18 (94.74%)		
Silhouettes	14.9 (± 4.63)	15.47 (± 4.45)	≤ 16	0,6416
Pathologic (n, %)	9 (45%)	8 (42.11%)		
Borderline (n, %)	2 (10%)	1 (5.26%)		
Non Pathologic (n, %)	9 (45%)	10 (52.63%)		
Obj Decision	14.4 (± 3.70)	16.05 (±2.07)	≤ 15	**0,0594**
Pathologic (n, %)	6 (30%)	2 (10.53%)		
Borderline (n, %)	3 (15%)	2 (10.53%)		
Non Pathologic (n, %)	11 (55%)	15 (78.95%)		
Progressive Silhouettes	12.25 (± 2.99)	11.05 (±2.53)	≥ 14	**P< 0,0001**
Pathologic (n, %)	2 (10%)	0 (0%)		
Borderline (n, %)	1 (5%)	2 (10.53%)		
Non Pathologic (n, %)	17 (85%)	17 (89.47%)		
Dot	9.9 (± 0.31)	9.63 (± 0.76)	≤ 8	0,1105
Pathologic (n, %)	0 (0%)	1 (5.26%)		
Borderline (n, %)	0 (0%)	0 (0%)		
Non Pathologic (n, %)	20 (100%)	18 (94.74%)		
Position	18.95 (± 1.39)	19.53 (± 0.77)	≤ 18	**0,0349**
Pathologic (n, %)	4 (20%)	1 (5.26%)		

Borderline (n, %)	2 (10%)	0 (0%)		
Non Pathologic (n, %)	14 (70%)	17 (89.47%)		
Number	8.47 (± 2.03)	8.53 (± 2.01)	≤ 7	0,848
Pathologic (n, %)	4 (21.05%)	3 (15.79%)		
Borderline (n, %)	1 (5.26%)	1 (5.26%)		
Non Pathologic (n, %)	14 (73.68%)	15 (78.95%)		
Cube	8.5 (± 2.06)	8.84 (± 1.26)	≤ 6	0,461
Pathologic (n, %)	3 (15%)	3 (15.79%)		
Borderline (n, %)	0 (0%)	1 (5.26%)		
Non Pathologic (n, %)	17 (85%)	15 (78.95%)		
Mood state				
Geriatric Depression Scale (GDS)	3.4 (± 3.10)	3.26 (± 2.28)	≥6	0,6875
Quality of life (QoL)				
Patient	34.75 (± 6.16)	35.26 (± 6.30)		0,2791
Caregiver	39.0 (± 5.24)	39.06 (± 4.11)		0,6215
Functional test/tools				
Functional communication				
Communication Activities of the Daily Living (CADL 2)	88.71 (± 5.17)	95.68 (± 4.03)		**P< 0,0001**
Direct assessment of functional status				
Direct Assessment of Functional Status (DAFS)	67.25 (± 6.77)	75.79 (± 6.07)	≤ 68	**P< 0,0001**

§ Paired t tests two sided

7. DISCUSSION

With the goal of assessing the efficacy of visual arts-mediated cognitive activation therapy on patients with a neurocognitive disorder in the mild to moderate phase and by analysing the two biomarkers of ageing, leucocytes telomere length and DNAmAge, this pilot study allowed us to obtain promising results on the effectiveness of a non-pharmacological therapy on biological ageing.

In this study, we found that, by excluding participating who underwent only 16 days of therapy, the difference in LTL at T1 and T0 becomes significant. This means that there is a lengthening of telomere length after CAT therapy and consequently a slowing down of ageing. This result could be attributed to various biological mechanisms related to stress reduction, improved cellular health, and overall well-being.

1. *Reduction in Oxidative Stress*: Chronic stress and oxidative stress are known to accelerate telomere shortening. CAT, which involves engaging in cognitive and creative activities, can lead to a reduction in stress levels. Lower stress means reduced production of reactive oxygen species (ROS) that can damage cells and their components, including telomeres. By mitigating oxidative stress, CAT could contribute to the preservation or even lengthening of telomeres.
2. *Enhanced Cellular Repair*: Engaging in cognitive and creative activities through CAT might stimulate cellular repair mechanisms. Cellular repair processes are crucial for maintaining telomere length. When cells are exposed to stressors, they activate repair pathways, which could indirectly contribute to the maintenance or elongation of telomeres.
3. *Stimulation of Telomerase Activity*: Telomerase is an enzyme that can add telomeric DNA sequences to the ends of chromosomes, effectively elongating telomeres. Telomerase activity is typically more active in stem cells and germ cells but is often suppressed in most somatic cells as a natural aging process. Certain non-pharmacological interventions, including stress reduction and increased physical activity, have been associated with increased telomerase activity. It's possible that the positive impact of CAT

on overall well-being and stress levels could stimulate telomerase activity, leading to telomere elongation.

4. *Neuroendocrine Regulation*: Engaging in cognitive and creative activities can activate various brain regions and stimulate the release of neurochemicals, such as endorphins and dopamine, which are associated with pleasure and positive emotions. These neurochemicals can have downstream effects on the endocrine system. Hormones released during pleasurable experiences may have positive effects on cellular health, potentially influencing telomere maintenance and length.

It's important to note that while these mechanisms offer plausible explanations for the observed effects, the relationship between cognitive therapy, telomere length, and ageing is complex and likely involves multiple pathways. The specific biological processes involved in telomere lengthening as a result of CAT would require further research and investigation to be fully understood.

Furthermore, treatment duration is found to be a determinant of the lengthening of LTL. In fact, an increase in ΔLTL (T1-T0) is positively correlated to the days of treatment, hence as the number of days of treatment increases, telomere length increases and ageing slows down.

Another interesting result we obtained is the correlation between telomere length and erythrocytes. The increased number of erythrocytes is a determinant in rising LTL at T1 (post-treatment) after Cognitive Activation Therapy (CAT) and this result could be attributed to several interconnected biological mechanisms:

1. *Improved Blood and Oxygen Supply*: Cognitive Activation Therapy involves engaging in cognitive and creative activities, which can stimulate brain function and neural connectivity. Improved brain activity could lead to better blood flow and oxygen supply to brain tissues. This could enhance overall vascular health, potentially leading to a positive impact on erythrocyte production and lifespan.
2. *Reduced Stress and Inflammation*: Engaging in CAT might help reduce stress and chronic inflammation, both of which have been linked to telomere shortening. A decrease in stress hormones and inflammatory markers could

indirectly support erythrocyte production and maintenance, potentially influencing telomere dynamics.

3. *Erythropoietin (EPO) Production*: Cognitive stimulation and increased brain activity have been associated with the release of various neurotrophic factors and molecules. One such molecule is erythropoietin (EPO), which plays a crucial role in regulating erythrocyte production in bone marrow. The positive effects of CAT on brain activity might lead to the production of EPO, subsequently promoting erythrocyte production and possibly contributing to improved LTL.
4. *Enhanced Cellular Repair Mechanisms*: Engaging in cognitive activities can lead to the activation of various cellular repair mechanisms. These mechanisms are essential for maintaining cellular health and function. An improved cellular environment could influence erythrocyte production, which might, in turn, have a positive impact on telomere dynamics.
5. *Healthy Lifestyle Changes*: Participation in CAT might lead to positive lifestyle changes, such as increased physical activity, improved sleep, and better dietary habits. These changes can influence overall health, including haematopoiesis (the process of blood cell formation). Enhanced haematopoiesis could potentially contribute to the increased number of erythrocytes and the associated positive effect on LTL.
6. *Neuroendocrine Regulation*: Engaging in cognitive activities can stimulate the release of various neurochemicals and hormones. Some of these hormones might indirectly influence erythropoiesis and overall hematopoietic function. Hormones related to brain activity could have downstream effects on bone marrow function, promoting erythrocyte production.
7. *Erythropoiesis*: According to De Meyer et al. (2008), telomere biology has an effect on erythropoiesis. In particular, he argues that if the telomere length of haematopoietic stem cells (HSCs) is short, and with advancing age there is a depletion of the HSCs' LTL, then there will be a greater amount of senescent erythroid progenitor cells and consequently fewer red blood cells.

It's important to note that while these mechanisms offer plausible explanations for the observed correlation, the relationship between erythrocyte count, cognitive therapy, and telomere length is complex and likely involves multiple interacting factors. Further research is needed to confirm these findings and gain a more comprehensive understanding of how cognitive therapy might influence biological processes leading to changes in erythrocyte count and telomere dynamics.
Leucocyte telomere length also correlates with the gender of the patient. As can be seen from the results obtained, women have a significantly longer telomere length than men at both the beginning (T0) (p= 0,0074) and end (T1) (p= 0,049) of therapy. The observed difference in leucocyte telomere length between women and men is in agreement with several studies, as supported by the review by Gardner et al. (2014), and can be attributed to a combination of biological, genetic, and hormonal factors. Telomeres are protective caps at the end of chromosomes that naturally shorten with each cell division and as a result of oxidative stress. Longer telomeres are generally associated with healthier cellular ageing and longevity.

One reason women tend to have longer telomeres than men is linked to sex hormones, particularly oestrogen. Oestrogen is a female sex hormone that has been shown to have a positive influence on telomere maintenance. There is evidence to suggest that oestrogen may stimulate the activity of telomerase, an enzyme responsible for lengthening telomeres. This is because telomerase contains an oestrogen-responsive element, which means that oestrogen could potentially encourage the addition of telomeric repeats to the ends of chromosomes, thus preserving telomere length.
Genetics also play a role in determining telomere length. Telomere length has a hereditary component, which means that individuals may inherit their telomere length tendencies from their parents. It's possible that certain genetic variations more commonly found in women contribute to longer telomeres.
Furthermore, women generally experience a slower rate of biological ageing compared to men, which may partly explain the longer telomeres. Biological ageing involves a combination of factors such as inflammation, oxidative stress, and cellular damage. The hormonal and genetic factors that contribute to longer telomeres in women could also be related to their overall slower rate of biological ageing.

It's important to note that while these factors contribute to the observed differences in telomere length between genders, individual variation exists, and not all women will have longer telomeres than all men. The relationship between gender, hormones, genetics, and telomere length is complex and continues to be an area of active research.
Nevertheless , the difference in leucocytes telomere length before and after therapy [ΔLTL (T1-T0)] of men was significantly longer than that of women (p= 0, 0142), which was not significant. This result shows us that men benefited more from CAT than women, a result that has not yet been found in other trials and would be interesting to explore with further longitudinal studies.

With regard to the analysis of the second biomarker of ageing, unlike LTL, DNAmAge was found to be significantly correlated with age (p= 0.0077). This result is a quality validation of our analysis, confirming the extreme accuracy for epigenetic age estimation of this model. The lack of correlation of age with LTL may be due to the restricted age range of the patients. In fact, the strength of the association of LTL with age is highly dependent on the age range of the population.

A surprising result we obtained concerns the correlation between DNAmAge and treatment duration. By excluding the three patients who had only 16 days' therapy, we obtained a significant positive correlation between ΔDNAmAge (T1-T0) and the duration of therapy. This means that as days of treatment increase, we have an acceleration of ageing. This result surprised us, as it is an opposite result to that obtained by telomere length analysis. Despite this, the difference of DNAmAge before and after cognitive activation therapy was found to be non-significant compared to that of telomere length. That said, longitudinal studies in larger population size are therefore required to validate and further characterize the effectiveness of cognitive activation treatment on epigenetic age.

Regarding the effectiveness of visual arts-mediated cognitive activation therapy on patients with a neurocognitive disorder in the mild to moderate phase, five cognitive tests were significant:

- *Rey–Osterrieth complex figure (ROCF) test* = at the beginning of therapy (T0) of the 20 patients who took this cognitive test, 8 (42.11% of participants) were

pathological. At the end of therapy only one was pathological (5.26% of the total participants). This mean that there was an improvement of the visuo-constructional ability and visual memory after the visual arts-mediated cognitive activation therapy.

- *Progressive Silhouettes of Visual Object and Space Perception Battery (VOSP) test* = at the beginning of therapy (T0) of the 20 patients who took this cognitive test, 2 (10% of participants) were pathological. At the end of therapy none were pathological. Progressive silhouettes test from Visual Object and Space Perception Battery (VOSP) assess object perception, thus there was an improvement on this kind of perception that concern the occipito-temporal neural circuit, in particular patients were able to identify different object silhouettes.
- *Position of Visual Object and Space Perception Battery (VOSP) test* = at the beginning of therapy (T0) of the patients who took this cognitive test, 4 (20% of participants) were pathological. At the end of therapy only one was pathological (5.26% of the total participants). Position test from Visual Object and Space Perception Battery (VOSP) assess space perception, thus there was an improvement on this kind of perception that concern occipito-parietal neural circuit.
- *Communication Activities of the Daily Living (CADL 2) test* = We found a significant and positive change in outcomes before (T0) and after (T1) therapy, this means that there was an improvement in the functional communication skills after the treatment.
- *Direct Assessment of Functional Status (DAFS) test* = We found a significant and positive change in outcomes before (T0) and after (T1) therapy, this means that there was an improvement in the functional capabilities of participants.

8. CONCLUSION

Ageing is a complex biological process characterised by a progressive inability to adapt to internal and external stimuli, increasing the risk of disease and death. When we think about good practices for healthy ageing, the connection with art is not immediate. Our first thought runs to nutrition or sporting activity, but modern cognitive neuroscience shows us how much and how the aesthetic experience and enjoyment of beauty contribute to our well-being and health, for healthy ageing. This pilot study aimed to assess the efficacy of visual arts-mediated Cognitive Activation Therapy (CAT) on patients with mild to moderate neurocognitive disorders by analysing the biomarkers of ageing, leucocyte telomere length (LTL), and DNAmAge. The study yielded promising results, indicating the effectiveness of a non-pharmacological therapy in influencing biological ageing.

Specifically, we observed a significant lengthening of telomere length after CAT therapy, suggesting a potential slowing down of ageing. This result could be attributed to several biological mechanisms, including the reduction of oxidative stress, enhanced cellular repair, stimulation of telomerase activity, and neuroendocrine regulation.

Moreover, the correlation between telomere length and erythrocyte count provided insights into the interconnected biological processes that may contribute to this phenomenon.

Furthermore, we observed gender-related differences in telomere length, with women having longer telomeres than men. This difference is influenced by a combination of biological, genetic, and hormonal factors.

The study also highlighted a surprising correlation between DNAmAge and treatment duration, suggesting the need for further research in this area.

In terms of cognitive outcomes, CAT demonstrated significant improvements in various cognitive tests, including visuo-constructional ability, visual memory, object and space perception, functional communication skills, and overall functional capabilities.

While the mechanisms underlying the observed effects require further investigation, this study contributes to our understanding of the potential benefits

of non-pharmacological interventions in influencing biological ageing and cognitive function. Future longitudinal studies with larger populations will be crucial to validate and further elucidate these findings.

9. RIASSUNTO IN ITALIANO

L'invecchiamento è uno dei fenomeni più significativi del XXI secolo con conseguenze importanti su tutti i settori della società. L'Organizzazione Mondiale della Sanità ha stimato che, se nel 2020 gli individui con un'età maggiore di 60 anni erano 1 miliardo, nel 2030 saranno 1.4 miliardi e nel 2050 2.1 miliardi. Questo fenomeno chiamato «*Grey tsunami*» costituisce un problema in ambito occupazionale, poiché l'invecchiamento si associa ad una insorgenza di malattie croniche, anche disabilitanti, che limitano le capacità lavorative degli individui. L'invecchiamento è un processo individuale, naturale e biologicamente complesso, caratterizzato da una graduale diminuzione della capacità fisica e mentale di rispondere in modo appropriato a fattori di stress interni e/o esterni. Durante l'invecchiamento si verifica un'alterazione dei sistemi di riparazione di vari organi, che porta a una diminuzione della fertilità e a un aumento del rischio di malattie. Tra queste patologie troviamo anche la demenza, la quale è una sindrome clinica che compromette in modo graduale ed irreversibile le funzioni cognitive e le capacità funzionali; a causa di ciò la persona perde progressivamente la propria indipendenza e peggiora la propria qualità di vita.

Tuttavia non tutti invecchiamo allo stesso modo e ci sono individui che lo fanno più rapidamente di altri, perciò l'età anagrafica non può essere un indicatore affidabile del declino fisiologico. Per valutare l'età biologica di un soggetto, è possibile utilizzare indicatori che misurano sia aspetti fenotipici (stato cardiaco, polmonare, metabolico, renale, epatico, immunitario e infiammatorio) sia aspetti molecolari (età epigenetica, biologica, trascrittomica e metabolica).

Tra gli indicatori più utilizzati per determinare l'invecchiamento biologico abbiamo l'analisi della lunghezza dei telomeri (TL) e l'analisi dell'età epigenetica (DNAmAge).

In questo studio pilota abbiamo analizziamo l'efficacia della Cognitive Activation Therapy (CAT) mediata dalle arti visive sull'invecchiamento biologico e sullo stato cognitivo/funzionale.

Venti pazienti affetti da disturbo neurocognitivo maggiore nella fase lieve-moderata della malattia sono stati reclutati presso il Centro Regionale per lo Studio e la Cura

dell'Invecchiamento Cerebrale (CRIC) e sottoposti a un ciclo di CAT della durata di 30 giorni.

L'invecchiamento biologico è stato eseguito su campioni di sangue all'inizio e alla fine della CAT, valutando la DNAmAge e la lunghezza telomerica leucocitaria (LTL) come indicatori di invecchiamento biologico.

Lo studio ha dato risultati promettenti, validando l'efficacia di una terapia non farmacologica nell'influenzare l'invecchiamento biologico.

In particolare, abbiamo osservato un significativo allungamento di LTL dopo la terapia, indicante un potenziale rallentamento dell'invecchiamento. Questo risultato potrebbe essere attribuito a diversi meccanismi biologici, tra cui la riduzione dello stress ossidativo, una maggiore riparazione cellulare, la stimolazione dell'attività della telomerasi e la regolazione neuroendocrina.

Si è anche osservata una correlazione tra la LTL e la conta degli eritrociti e delle differenze tra le lunghezze telomeriche tra i generi. In particolare, le donne presentano telomeri più lunghi degli uomini; una differenza che potrebbe essere influenzata da una combinazione di fattori biologici, genetici e ormonali.

Lo studio, inoltre, ha anche evidenziato una sorprendente correlazione tra DNAmAge e durata del trattamento, suggerendo la necessità di ulteriori ricerche in questo settore.

In termini di risultati cognitivi, la CAT ha dimostrato miglioramenti significativi in vari test cognitivi, tra cui l'abilità visivo-costruttiva, la memoria visiva, la percezione degli oggetti e dello spazio, le capacità di comunicazione funzionale e le capacità funzionali generali.

Sebbene i meccanismi alla base degli effetti osservati richiedano ulteriori indagini, questo studio contribuisce alla comprensione dei potenziali benefici degli interventi non farmacologici nell'influenzare l'invecchiamento biologico e la funzione cognitiva. Futuri studi longitudinali su popolazioni più numerose saranno fondamentali per convalidare e chiarire ulteriormente questi risultati.

10. BIBLIOGRAPHY

1. Barzilai N., Huffman D.M., Muzumdar R.H., Bartke A. 2012. The critical role of metabolic pathways in aging. Diabetes, 61:1315-1322.
2. Baylis D., Bartlett D.B., Patel H.P., Roberts H.C. 2013. Understanding how we age: insights into inflammaging. Longev Healthspan., 2:8.
3. Belsky D. W., Caspi A., Houts R. et al. 2015. Quantification of biological aging in young adults. PNAS, 112 (30) E4104-E4110.
4. Biagi, E., Franceschi, C., Rampelli, S., Severgnini, M., Ostan, R., Turroni, S., Consolandi, C., Quercia, S., Scurti, M., Monti, D., et al. (2016). Gut microbiota and extreme longevity. Curr. Biol. 26, 1480–1485. https://doi.org/10.1016/j.cub.2016.04.016.
5. Cherkas LF, et al. The effects of social status on biological aging as measured by white-blood-cell telomere length. Aging Cell. 2006;5:361–365.
6. D. Gollin, C. Ruaro, A. Peruzzi, E. Talassi, A. Ferrari, A. Codemo, A. La Sala, and C. Gabelli. Improvement of functional status in alzheimer's disease patients after cognitive activation therapy (CAT). Non-Pharmacological Therapies in Dementia, 2011. ISSN: 1949-484X Volume 3, Number 1.
7. De la Fuente M., Miquel J. An update of the oxidation-inflammation theory of aging: The involvement of the immune system in oxi-inflamm-aging. Curr. Pharm. Des. 2009;15:3003–3026. doi: 10.2174/138161209789058110.
8. De Meyer T, De Buyzere ML, Langlois M, Rietzschel ER, Cassiman P, De Bacquer D, Van Oostveldt P, De Backer GG, Gillebert TC, Van Criekinge W, Bekaert S; Asklepios Investigators. Lower red blood cell counts in middle-aged subjects with shorter peripheral blood leukocyte telomere length. Aging Cell. 2008 Oct;7(5):700-5. doi: 10.1111/j.1474-9726.2008.00419.x. Epub 2008 Jul 28. PMID: 18665909.
9. Franceschi C, Bonafè M, Valensin S, et al. Inflammaging. An evolutionary perspective on immunosenescence. Ann N Y Acad Sci. 2000;908:244–254.

10. Frenck R.W. Jr, Blackburn E-H., Shannon K.M. 1998. The rate of telomere sequence loss in human leukocytes varies with age. Proc Natl Acad Sci U S A., 95(10):5607-10.
11. Fu J., Ji X., Liu J., Chen X., Shang H. Meta-analysis of the connection between Alzheimer disease and telomeres. *Alzheimer Dis Assoc Disord.* 2022;36:73–79. doi: 10.1097/WAD.0000000000000468.
12. Fülöp T., Larbi A., Witkowski J.M. Human Inflammaging. Gerontology. 2019; 65:495–504. doi: 10.1159/000497375.
13. Gardner, M. , Bann, D. , Wiley, L. , Cooper, R. , Hardy, R. , Nitsch, D. , Martin-Ruiz, C. , Shiels, P. , Sayer, A. A. , Barbieri, M. , Bekaert, S. , Bischoff, C. , Brooks-Wilson, A. , Chen, W. , Cooper, C. , Christensen, K. , De Meyer, T. , Deary, I. , Der, G. , Ben-Shlomo, Y. (2014). Gender and telomere length: Systematic review and meta-analysis. Experimental Gerontology, 51, 15–27. 10.1016/j.exger.2013.12.004
14. Harman D. 1956. Aging: a theory based on free radical and radiation chemistry. J Gerontol, 11:298–300.
15. Hayflick L. 2007. Biological aging is no longer an unsolved problem. Ann N Y Acad Sci., 1100:1-13.
16. Hayflick L. P S Moorhead. The serial cultivation of human diploid cell strains. 1961. Exp Cell Res. 1961 Dec;25:585-621. doi: 10.1016/0014-4827(61)90192-6.
17. Horvath S. 2013. DNA methylation age of human tissues and cell types. Genome Biol, 14:R115. doi: 10.1186/gb-2013-14-10-r115.
18. Istituto nazionale di statistica (Istat). 2022. Annuario Statistico Italiano.
19. K.S. Krabbe, M. Pedersen, H. Bruunsgaard. Inflammatory mediators in the elderly Exp. Gerontol., 39 (2004), pp. 687-699
20. Levine M.E. 2013. Modeling the rate of senescence: can estimated biological age predict mortality more accurately than chronological age? J Gerontol A Biol Sci Med Sci., 68(6):667-74.
21. Lòpez-Otìn C, Blasco MA, Partridge L, Serrano M, Kroemer G. Hallmarks of aging: An expanding universe. Cell. 2023 Jan 19; 186(2):243-278. Doi:10-1016/j.cell.2022.11.001 Epub 2023 Jan 3.PMID:36599349.

22. López-Otín C., Blasco M.A., Partridge L., et al. 2013. The hallmarks of aging. Cell, 153(6):1194-1217.
23. M.P. Mattson, T.V. Arumugam. Hallmarks of brain aging: adaptive and pathological modification by metabolic states. Cell Metab., 27 (2018), pp. 1176-1199
24. Pavanello S, Pesatori AC, Dioni L, et al. Shorter telomere length in peripheral blood lymphocytes of workers exposed to polycyclic aromatic hydrocarbons. Carcinogenesis. 2010;31:216–221.
25. Pavanello S., Stendardo M., Mastrangelo G., Bonci M., Bottazzi B., Campisi M., Nardini M., Leone R., Mantovani A., Boschetto P. 2017. Inflammatory Long Pentraxin 3 is Associated with Leukocyte Telomere Length in Night-Shift Workers. Front Immunol., 8:516.
26. Rathi Mahendran, Mihir Gandhi, Rajesh Babu Moorakonda, Jonathan Wong, Madhu Mathi Kanchi, Johnson Fam, Iris Rawtaer, Alan Prem Kumar, Lei Feng, and Ee Heok Kua. Art therapy is associated with sustained improvement in cognitive function in the elderly with mild neurocognitive disorder: findings from a pilot randomized controlled trial for art therapy and music reminiscence activity versus usual care 2018 Nov 9;19(1):615. doi: 10.1186/s13063-018-2988-6.
27. V. Morrisette-Thomas, A.A. Cohen, T. Fülöp, E. Riesco, V. Legault, Q. Li, E. Milot, F. Dusseault-Bélanger, L. Ferrucci. Inflamm-aging does not simply reflect increases in pro-inflammatory markers Mech. Ageing Dev., 139 (2014), pp. 49-57
28. World Health Organization (WHO) and Alzheimer's Disease International. Dementia: a public health priority. Geneva: World Health Organization; 2012. ISBN: 978 92 4 156445 8
29. World Health Organization. (WHO). Ageing and health. https://www.who.int/news-room/fact-sheets/detail/ageing-and-health
30. World Health Organization. (WHO). Active ageing : a policy framework. World Health Organization. 2002. https://apps.who.int/iris/handle/10665/67215
31. Xu, M., Pirtskhalava, T., Farr, J.N., Weigand, B.M., Palmer, A.K., Weivoda, M.M., Inman, C.L., Ogrodnik, M.B., Hachfeld, C.M., Fraser, D.G., et al.

(2018). Senolytics improve physical function and increase lifespan in old age. Nat. Med. 24, 1246–1256. https://doi.org/10.1038/s41591-018-0092-9.

32. Yafit Hachmo , Amir Hadanny, Ramzia Abu Hamed , Malka Daniel-Kotovsky , Merav Catalogna , Gregory Fishlev , Erez Lang , Nir Polak , Keren Doenyas , Mony Friedman , Yonatan Zemel , Yair Bechor , Shai Efrati. Hyperbaric oxygen therapy increases telomere length and decreases immunosenescence in isolated blood cells : a prospective trial. Aging, 12 (2020), p. 22
33. Zbieć-Piekarska R., Spólnicka M., Kupiec T., Parys-Proszek A., Makowska Ż., Pałeczka A., Kucharczyk K., Płoski R., Branicki W. Development of a forensically useful age prediction method based on DNA methylation analysis. Forensic Sci. Int. Genet. 2015;17:173–179. doi: 10.1016/j.fsigen.2015.05.001.
34. Zhou, A., Wu, Z., Zaw Phyo, A. Z., Torres, D., Vishwanath, S., & Ryan, J. (2022). Epigenetic aging as a biomarker of dementia and related outcomes: A systematic review. *Epigenomics*, **14**, 1125–1138.