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Biomarcadores do envelhecimento - Uma visão global

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Resumo

A entropia vence sempre. Um organismo multicelular é capaz de desenvolver e manter a sua identidade apenas por algum tempo até que a deterioração prevaleça sobre a síntese e o organismo envelheça.

O envelhecimento tem sido definido como o declínio, em função do tempo da capacidade funcional e da resistência ao stress, associada ao risco aumentado de morbilidade e mortalidade. Estas são várias alterações no corpo humano que podem levar à perda de função e à manifestação de doenças crónicas.

No entanto, enquanto as doenças geralmente possam ser diagnosticadas de forma fidedigna, o processo de envelhecimento requer abordagens mais sofisticadas para avaliar a sua progressão.

Numerosas foram as tentativas feitas para estabelecer biomarcadores para quantificar o envelhecimento humano a nível celular, tecidular e do organismo. Novas técnicas experimentais permitiram a geração e acumulação de uma grande quantidade de dados relacionados com o envelhecimento, contudo a análise e uso prático deste tipo de dados requer abordagens computacionais adaptadas.

Desenvolvimentos promissores consideram múltiplas combinações de vários tipos de biomarcadores preditivos e podem lançar luz sobre o processo de envelhecimento, proporcionando uma maior compreensão sobre o que contribui para um envelhecimento saudável.

Esta revisão resume as mais recentes e inovadoras descobertas, considerando três categorias principais de biomarcadores: não-moleculares, moleculares e baseados na Ômica.

Palavras-chave: Entropia, envelhecimento, biomarcadores, doença crónica

Abstract

Entropy always wins. A multicellular organism is able to develop and maintain its identity for only so long before deterioration prevails over synthesis and the organism ages.

Aging has been defined as the time-dependent decline of functional capacity and stress resistance, associated with increased risk of morbidity and mortality. These are various changes in the human body that may lead to the loss of function and the manifestation of chronic diseases.

While diseases can generally be reliably diagnosed, the aging process itself requires more sophisticated approaches to evaluate its progression.

Numerous attempts have been made to establish biomarkers to quantify human aging at the cellular, tissue and organismal level. New experimental techniques have allowed the generation and accumulation of a huge amount of aging-related data; however the analysis and practical use of this data also requires adapted computational approaches.

Promising developments consider multiple combinations of various types of biomarkers predictors and may shed light on the aging process providing further understanding of what contributes to healthy aging.

This review summarizes the current state-of-the-art findings considering three main biomarkers categories: non-molecular, discrete molecular and Omics-based biomarkers.

Keywords: Entropy, aging, biomarkers, chronic disease

1. Introduction

*"*To die of old age is a death rare, extraordinary, and singular and therefore so much less natural than the others: it is the last and most extreme sort of dying […]"(1)

On August 4, 1997, a French woman from Arles named Jeanne Louise Calment died at the age of 122 years and 164 days. She was widely reported to have been the oldest human and there were documents to confirm this.(2)

Aging is one of the major causes of death in the developed world, even though it is rarely recognized as such. It is usually some other cause that is written down on death certificates, e.g., heart failure, stroke, cancer, pneumonia, respiratory failure. However, there is no doubt that these pathologies are brought on largely by underlying age-related biological changes.(1)

While chronological age is arguably the strongest risk factor for aging-related death and disease, it is important to distinguish chronological time from biological aging, because individuals of the same chronological age may exhibit greatly different susceptibilities to age related diseases and death, which is likely reflective of differences in their underlying biological aging processes.(3)

It has been widely acknowledged that life expectancy has increased over the past centuries as a specific result of improved medical care, vaccination and hygiene. The process of aging is a dynamic, chronological process characterized by the gradual accumulation of damage to cells, progressive functional decline and increased susceptibility and vulnerability to diseases. In addition, aging is closely connected to the onset and progression of multiple age-related diseases, such as cancer, type 2 diabetes mellitus and cardiovascular and neurodegenerative diseases.(4) According to the World Health Organization such diseases affect mainly adults and elderly individuals and this imposes the greatest burden on global health with staggering costs to healthcare services.(2)

Aging research has reached an unprecedented advance over recent years, particularly with the discovery that the rate of aging is controlled, at least to some extent by genetic pathways and biochemical processes conserved in evolution.(5)

One of the major goals of geroscience research is to define 'biomarkers of aging', which can be thought of as individual-level measures of aging that capture inter-individual differences in the timing of disease onset, functional decline and death over the life course.(3) Hypothetically, a biomarker of aging should reflect the underlying biology and a change in biomarker levels should have parallel changes that occur in the susceptibility to disease and loss of function.(6)

Historically, biomarkers of aging were selected on the basis of their strong correlation with age, regardless of the disease burden and do not report on the specific mechanisms of aging that affect each individual.(7)

Later on, the American Federation for Aging Research (AFAR) formulated the criteria for aging biomarkers as follows:

1. It must predict the rate of aging. In other words, it would tell exactly where a person is in their total life span. It must be a better predictor of life span than chronological age.

2. It must monitor a basic process that underlies the aging process, not the effects of disease.

3. It must be able to be tested repeatedly without harming the person. For example, a blood test or an imaging technique.

4. It must be something that works in humans and in laboratory animals, such as mice.

However, to date no such marker or marker combination has been developed.(8)

Aging research has long been considered one of the most costly types of biological studies, because it is time-consuming and expensive to use mammals as research subjects.(2)

Some researchers focus their work on specific potential biomarkers or aspects related to them, but trying to understand the big picture seems to be an even more challenging task, with different researchers trying different approaches. Otín et all 2017, clustered the basic process that underlies aging at the cellular and molecular level in three main categories according to their function: primary, antagonistic and integrative aging-related processes. The primary processes are the ones related to cellular damage. Antagonistic processes have opposite effects depending on their intensity. At low levels, they mediate beneficial effects but at high levels, they become deleterious. A third category comprises the integrative hallmarks which directly affect tissue homeostasis and function (Figure 1).(5)

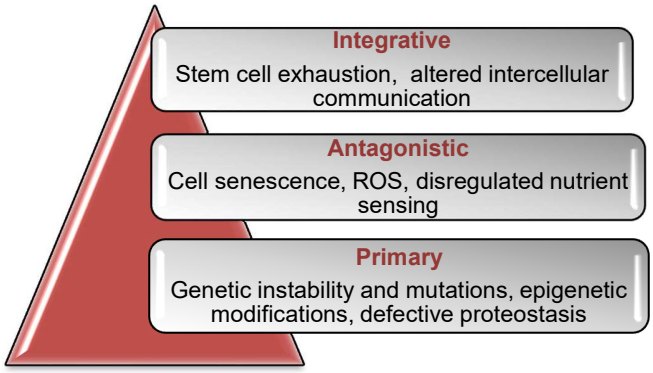

Figure 1: Main categories of biomarkers.

In the bottom, primary processes are strictly related to cellular damage and cumulative negative effects. In the middle, those are 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. At the top, there are integrative processes which are the end result of the previous two groups and are ultimately responsible for the functional decline associated with aging.(5)

Biomarkers that can track the initial steps of aging progression at the molecular and cellular level seem promising, particularly in terms of a rising trend to counteract aging at early-stages by direct molecular and cellular interventions, before the degenerative processes become profoundly deleterious and/or spread throughout the body.(7) Common molecular features of aging, including decreased telomere length and changes in DNA methylation (DNAm), among others have been identified as possible based-biomarkers at cellular/molecular levels.(9)

Physiological measures of aging including blood pressure, heart rate, grip strength and walking speed have shown relatively moderate correlations with chronological age and can be assessed through functional performance testing. Multiple system-related ages, such as brain, cardiovascular, renal, endocrine and musculoskeletal can also be assessed and have demonstrated their usefulness in predicting the rate of aging. Taken together, these measures are beneficial in predicting disease and mortality. However, examining each feature separately fails to comprehensively explain and capture the variability of the aging process.(9) There is evidence that aging differs noticeably across individuals and between various organs in the same individual(9). The functional decline in specific tissues and organs due to the accumulation of aged cells, complicated by intrasystem/ intersystem interactions and by environmental factors (e.g.,

diet, lifestyle) may be best measured by systemic molecular biomarkers.(7) Although these biomarkers reflect deleterious consequences of aging rather than primary causes, such as molecular damage, they provide essential input to develop prediction models for biological age, comprising information from multiple systems throughout the body.(7)

The systemic and multifactorial nature of aging (Figure 2) explains why understanding its biology and mechanisms are so complex and why, as a consequence, aging research is continuously in need of multidisciplinary and global approaches. Novel experimental techniques have allowed the generation and accumulation of a huge amount of aging-related data, including genomic, transcriptomic, microRNA, proteomic, antigen, methylation, imaging, metagenomic, mitochondrial, metabolic and physiological. This data provides an unprecedented detailed overview of the aging process.(10)

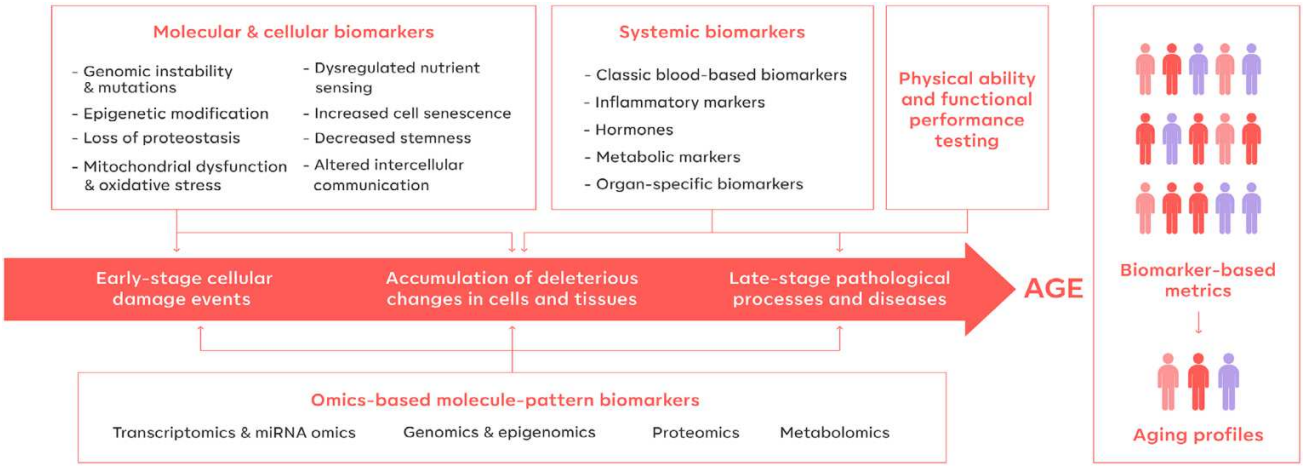

Figure 2. The diversity of aging biomarkers in humans. Image presented by Kudryashova et all.(7)

Existing groups of biomarkers show distinct preferences for different stages of aging progression. As people age very differently, the most accurate way to measure degeneration and to predict future outcomes seems to be the use of integrative biomarkers-based metrics, able to define personalized trajectories of aging or aging profiles.(7) However, the analysis and practical use of the information contained within this huge amount of data also requires adapted computational approaches, such as machine learning and, more recently, the development of deep learning techniques which are the cornerstones of modern artificial intelligence technologies.(10) The personalized system-oriented trajectories of aging should be considered or,

in other words, criteria for defining a person's aging profile needs to be formulated. Simple accumulation of biomarkers from all types of data (non-molecular, discrete molecular and omics-pattern) would, if revealing aging profiles is the aim, be rather informative at the young adulthood and midlife stages.(7)

The attractive feature of artificial intelligence is its ability to identify relevant patterns within complex, nonlinear data, without the need for any a priori mechanistic understanding of the biological processes. AI unveils the mechanistic relationships taking place within the body. Today, deep learning and artificial intelligence algorithms have been successfully developed and applied in many pharmaceutical areas.(10)

2. Methodology

PubMed was used as the search engine with Medical Subject Headings (MeSH) terms "aging", "physiology", "telomere" and "biomarkers" used combined in advanced PubMed search using the following search formulas:

i. ("Biomarkers"[Mesh]) AND "Aging"[Mesh] = 7635 articles.
ii. ("Biomarkers"[Majr]) AND "Aging"[Majr] = 975 articles + filtered for the last 5 years = 255 articles.
iii. ("Biomarkers"[Majr]) AND "Aging"[Majr] AND (Telomere[MeSH Terms]) = 26 articles.
iv. (Aging[MeSH Major Topic]) AND (Telomere[MeSH Major Topic]) = 736 articles + filtered for the last 5 years.
v. ("Aging/physiology"[Mesh]) AND "Telomere/physiology"[Mesh] = 975 articles.
vi. (Aging/physiology[MeSH Major Topic]) AND (Telomere/physiology[MeSH Major Topic]) = 485 articles.

All English-language data obtained was included. Systematic reviews, clinical trials, meta-analyses, etc. were included. Cited papers in the selected publications were also considered.

Trip medical database using the regular search with the terms "histone modification aging", "Omics-based biomarkers aging" was used and a selection from articles published within the last

5 years was made. The searches were performed between 22nd of September 2020 and 11th of January 2021.

3. Cellular and molecular biomarkers

The accumulation of molecular damage within a cell due to genomic instability and mutations, epigenetic modification, loss of proteostasis, mitochondrial dysfunction and oxidative stress lead to the loss of normal cell function and altered proliferation.(7) As a result, cells can undergo a transition into an irreversible non-proliferative senescence state, that if the cells that have become senescent are stem cells, it contributes to decreased stemness. Besides a senescence state, cells can go into a reversible non-proliferative quiescent state or die by various cell death mechanisms. Altered paracrine and endocrine communication, as well as intercellular matrix remodelling, also affect cell functioning.(7)

3.1 Genetic instability and mutations

The genetic lesions arising from extrinsic or intrinsic damage are highly diverse and include point mutations, translocations, chromosomal gains and losses, telomere shortening and gene disruption caused by the integration of viruses or transposons.(5)

Detailed measures of DNA damage can be performed by DNA sequencing; however, this is not as easy as it sounds since each cell in a tissue acquires different mutations that get lost in bulk sequencing efforts. Therefore, single cell DNA sequencing is required and recent studies have been performed to assess mutation rate with age.(7)

Mitochondrial DNA has been considered a major target for aging-associated somatic mutations due to the oxidative microenvironment of the mitochondria, the lack of protective histones in the mtDNA and the limited efficiency of the mtDNA repair mechanisms compared to those of nuclear DNA. However studies have shown that most mtDNA mutations in adult or aged cells appear to be caused by replication errors early in life, rather than by oxidative damage caused by respiratory chain dysfunction in different tissues.(5)

In addition to these direct lesions in the DNA (point mutations, translocations,…), defects in the nuclear architecture, known as laminopathies, can cause genome instability and result in premature aging syndromes.(5)

An important type of DNA damage is the shortening of telomeric repeat sequences at the end of chromosomes during DNA replication in most somatic cells or as a result of cumulative oxidative stress.(7) Telomeres are terminal nucleoprotein complexes of chromosomes in eukaryotes, play an important role in chromosomal DNA protection, gene expression and regulation of stress-related signalling pathways by controlling cell senescence and organismal aging. Telomere length reflects a delicate balance between shortening and elongation of telomeres, is mainly controlled by telomerase and telomeric repeat-containing RNA.(2) Telomere shortening is viewed as a mitotic clock that counts down the number of cell divisions. The cellular enzyme telomerase counteracts telomere shortening by RNA-templated addition of DNA nucleotides onto telomeres, thereby lengthening the telomeric DNA.(11)

Centenarians and their offspring have been found to have longer telomeres relative to controls, (7) also the average telomere length varies and there are gender differences with women having a longer telomere length and life expectancy compared to men, perhaps owed to the hormonal differences, e.g., in estrogen levels, as well as the effect of the X chromosome. Decreasing immune surveillance and increasing inflammation are related to age and both are linked to telomere shortening and lower telomerase activity.(2) The use of telomere length (TL) as a biomarker of overall aging is still a debated issue, because TL inconsistently predicts clinical and functional outcomes, life expectancy and death.(7)

Telomeres can be regarded as DNA breaks that are made invisible to the DNA repair machinery through the formation of specialized nucleoprotein complex known as shelterin, the infliction of exogenous DNA damage to telomeres becomes invisible.(5)

Mammalian somatic cells do not express telomerase and this leads to the progressive and cumulative loss of telomere-protective sequences from chromosome ends.(5) Indeed, ectopic expression of telomerase is sufficient to confer immortality to otherwise mortal cells.(5)

While telomere length reflects the cumulative effects of genetic and environmental factors, telomerase activity is dynamic and is likely an essential modifiable factor in mediating environmental and lifestyle factors and telomere length changes.(11) Disruptions to the normal function of telomeres and telomerase are associated with human disease. One of the most closely associated of these diseases is perhaps, cancer. In around 90% of cancers, the expression of telomerase is increased, while similar, yet benign tumours do not display this increase in telomerase.(12)

A growing body of literature links shorter telomere length and lower telomerase activity with various age related diseases and earlier mortality.(11)

Southern blotting, polymerase chain reaction (PCR)-based methods, single telomere length analysis and fluorescence in situ hybridization (FISH) are relatively new technologies for measuring telomere length. Quantitative PCR and FISH are frequently applied in clinical and epidemiological studies.(2)

In the last four years however, there has been a huge step forward. The advent of the clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) system has led to a wide array of targeted genetic studies that are already being employed to modify telomeres and telomerase, as well as the genes that affect them.(12) CRISPR-Cas technology has provided a powerful tool that can be used to fashion rapid and specific genomic changes in living organisms.(12)

3.2 Epigenetic alterations

Epigenetics is defined as the collection of mechanisms by which environmental stressors affect gene expression, but not the underlying genetic sequence. These mechanisms primarily involve DNA methylation, histone modifications and gene regulation by noncoding RNA.(13) DNA methylation as well as histone methylation and acetylation are highly dynamic processes that occur over the human life span and are influenced by environmental and genetic factors. These modifications also change during aging, making them a potential biomarker.(7)

The ability of epigenetic systems to affect all other hallmarks of aging puts them into a key position to affect the basic mechanisms (driving forces) of aging. On the other hand, active components of the epigenetic systems are encoded in the genome and thus, are themselves under epigenetic control.(2) The genome is comprised of ≈3 billion inherited and largely stable deoxyribonucleic acid (DNA) base pairs that serve as the building blocks for more than 20,000 genes. The exposome takes shape throughout the life course, defined as the complete set of exposures an individual encounters.(14) These modifications, termed epigenetic modifications leave the DNA sequence untouched, but can alter the likelihood of a gene being transcribed and translated into a protein. Thus, the exposome adds variation to the genome, creating unique epigenomic signatures and patterns of gene expression that continue to morph throughout life. This point is well-illustrated by the rapidly expanding literature on variation in epigenetic aging.(14)

Epigenetic age-related changes include nuclear lamina breakdown, leading to alteration of heterochromatin that is normally anchored to the nuclear lamina and changes in chromosome structure and gene regulation which lead to increases in transcriptional noise with age.(13)

Unlike DNA mutations, epigenetic alterations are – at least theoretically – reversible, hence offering opportunities for the design of novel anti-aging treatments.(5)

3.2.1 Histone modifications and chromatin remodelling

Epigenetic events regulate gene expression at both transcription (histone modification and DNA methylation) and translation (small non-coding RNA) levels.(15) Post-translational modifications of the histone amino tails affect their affinity to DNA and interacting proteins.(1) Generally, acetylation and phosphorylation of histone tails are considered to be euchromatin marks and methylation of histones is enriched in heterochromatin regions. However, some exceptions to this theory exist.(1) In addition to histone tail modifications, the nucleosome density affects the level of DNA packaging as well. Therefore, DNA regions with a high density of nucleosomes are likely transcriptionally inactive while transcriptionally active DNA regions are characterized by looser packaging and a low density of nucleosomes.(1)

DNA- and histone-modifying enzymes act in concert with key chromosomal proteins, such as the heterochromatin protein 1a (HP1a) and chromatin remodelling factors, whose levels are diminished in both normally and pathologically aged cells. Epigenetic modifications in histones and DNA-methylation determine changes in chromatin architecture, such as global heterochromatin loss and redistribution, which constitute characteristic features of aging.(5)

In some cases, changes in histone modification or DNA methylation (hypermethylation or hypomethylation) can cause neurodegenerative disorders, such as Parkinson's disease (PD), AD and Huntington's disease.(15)

It is not clear yet whether manipulations of histone-modifying enzymes can influence aging through purely epigenetic mechanisms, by impinging on DNA repair and genome stability or through transcriptional alterations affecting metabolic or signalling pathways outside of the nucleus.(5)

3.2.2 DNA methylation

Chronological time has been shown to elicit predictable hypo- and hyper-methylation changes at many regions across the genome and, as a result the first generation of DNAm based biomarkers of aging were developed to predict chronological age,(16) while the total DNA methylation level slowly decreases with age, cytosine methylation (Figure 3) at specific loci containing CpG dinucleotides (referred to as CpG sites) changes differently with age, becoming both hyper- or hypo-methylated in different genomic locations.(7)

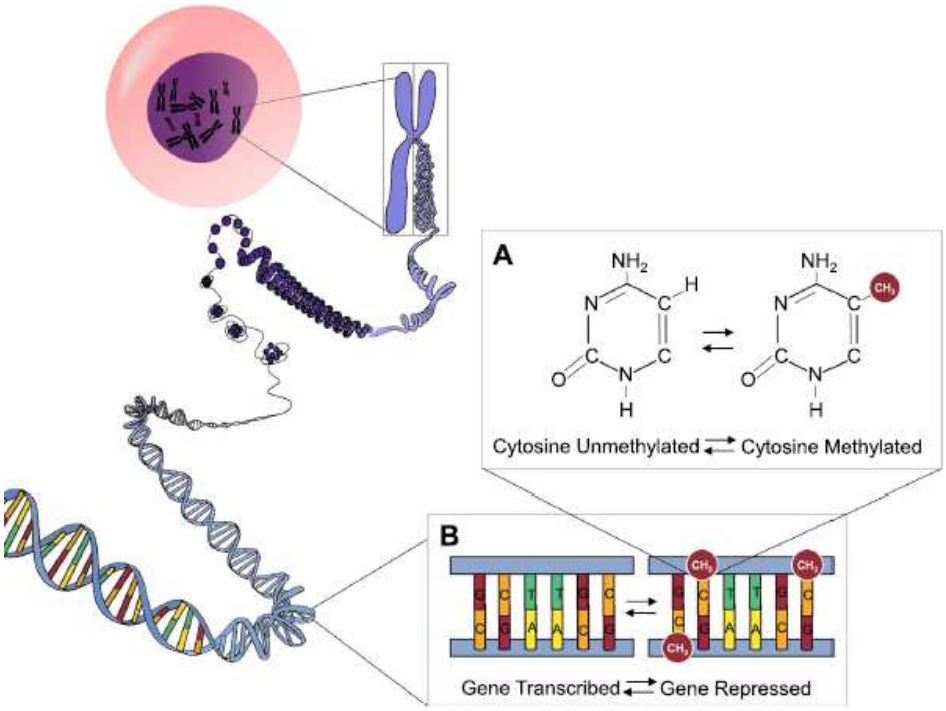

Figure 3. Cytosine methylation specific loci containing CpG dinucleotides.

Image presented by Gillespie et all.(14)

DNA methylation occurs when a methyl group (CH3) is added to the cytosine of a phosphate-linked cytosine-guanine (CpG) base pair. Removal of methyl groups can be accomplished by the conversion of 5-methylcytosine to 5-hydroxymethylcytosine.(14)

DNA methylation is a well-studied epigenetic mechanism that regulates gene expression by adding or removing a methyl group at the fifth carbon of the pyrimidine ring of cytosine to form 5mC.(15) When CpGs are methylated, gene expression can be repressed. When CpGs are demethylated, gene expression can be enhanced.(14) Moreover, DNA methylation was found to occur predominantly on cytosines followed by guanine residues (CpG).(15) Transfer of methyl groups is mediated by DNA methyl transferase enzymes including DNMT1, DNMT3A and DNMT3B, promoting the establishment and maintenance of DNAm patterns.(14)

Until recently, DNAmAge and telomere length were associated with age and mortality independently of each other,(8) however recent studies using the CRISPR/Cas system have shown that when cells were deprived of the DNA methyltransferase 2 (DNMT2), which catalyses the addition of methyl groups to tRNA, they suffered both a decrease in telomere lengths and

telomerase activity. Interestingly, the loss of DNMT2 resulted in the compensatory upregulation of other DNA methyltransferases, including DNMT1, DNMt3A and DNMt3B. These methyltransferases primarily methylate DNA, which led to global DNA hypermethylation. In turn, this hypermethylation induced cellular senescence apoptotic pathways.(12) The age-dependent gene methylation patterns were partially conserved between humans and mice and the conserved genes were mainly among the hypermethylated ones.(2) There is evidence that indicates that DNA CpG methylation predictably changes with age, with these CpGs localized near genes related to cell death, cellular proliferation and tissue development.(13)

Other new studies in line with those of the DNMT2, show that the lack of compensatory global methylation likely prevented telomere shortening and cell senescence. Understanding how changes in methylation state are induced at the telomerase reverse transcriptase (TERT) locus is vital, as they may provide insight as to why the expression levels differ in disease states with no obvious mutation.(12)

About 350 CpG loci were found to be differentially methylated with age (aCpGs) in human peripheral blood leukocytes (PBLs), with approximately 200 hypermethylated (hyper-aCpGs) and 150 hypomethylated (hypo-aCpGs). More than 95% of aCpGs were located within 500 bp of transcriptional start sites (TSS).(2) Of 476,366 CpGs covered by the Illumina Human Methylation450 BeadChip, about 29% were found to be differentially methylated across ages 14–94 years in PBLs. Of these aCpGs, 60.5% were hypomethylated and 39.5% hypermethylated with age. For most sites, DNA methylation changed approximately linearly with age. The strongest positive correlation of the methylation level with age was seen for a CpG site located in the ELOVL2 gene promoter CGI (methylation levels were from 0.35 in the youngest to 0.89 in the oldest individuals). Among the sites that become hypermethylated with age, 81.4% were located in CGIs, compared to only 2.8% for the hypomethylated sites.(2)

Methylated cytosines are found primarily at CpG dinucleotides, but are also found at non-CpG sites (CpA, CpT, and CpC). The general functions of CpG and non-CpG methylation include gene silencing or activation depending on the methylated regions. CpG and non-CpG methylation are found throughout the whole genome, including repetitive sequences, enhancers, promoters and gene bodies. Interestingly however, non-CpG methylation is restricted to specific cell types, such as pluripotent stem cells, oocytes, neurons and glial cells.(15)

A number of recent studies have identified a measure of DNA methylation age (DNAmAge), also referred to as the epigenetic clock, as a viable biological age predictor. Two of these clock

measures, (Horvath,2013) and (Hannum et al., 2013) calculators, are currently perhaps the most robust predictors of chronological age.(8)

The blood-based algorithm by Hannum and the multi-tissue algorithm by Horvath produce age estimates (DNAm age) that correlate with chronological age well above r=0.90 for full age range samples(3) more exactly (r= 0.96 for Horvath and r = 0.91 for Hannum) and small mean deviations from calendar age (3.6 and 4.9 years, respectively) in their corresponding validation cohorts.(8)

The Hannum et al. algorithm was derived and validated using whole blood from two independent cohorts (N1 = 482; N2 = 174) of Caucasian and Hispanic men and women aged 19 to 101. The model was built by regressing chronologic age onto percent DNAm at 485,577 CpGs quantified, using the HumanMethylation450 BeadChip for each individual's population of whole blood cells. Clinical parameters were controlled. Seventy-one CpGs were identified as highly associated with chronological age among the training and validation sets, generating the quantitative definition of the apparent methylomic aging rate as the ratio of predicted epigenetic age to chronological age. More than 90% of the predictive CpGs were associated with known genes, which were over-enriched for biological pathways of cell communication, locomotion, cell proliferation and growth and under-enriched for Gprotein coupled receptor activity, ribosome, RNA splicing and M phase, providing a link with putative sites for aging and age-related disease.(14)

The Horvath (2013) algorithm was derived and validated using 82 datasets (7,844 non-cancer samples) reflecting percent DNAm at 21,369 CpGs available across both the platforms. Participant ages varied widely and 51 tissue and cell types were assessed.(14) The Horvath clock is a multi-tissue predictor based on methylation levels of 353 CpG sites on the Illumina 27 k array.(8) The selection of the CpG sites for both predictors (Hannum and Horvath) was done using a similar penalized regression model, yet they only have six CpG sites in common. Nevertheless, the correlations between the clocks appear to vary from fairly strong (r=0.76) to moderate (r= 0.37) in independent studies.(6)

The variations in epigenetic age between different tissues of the same person were small. The notable exceptions were breast tissue in women (epigenetically older compared with other tissues) and sperm in men (epigenetically younger compared with other tissues).(2) As could be expected, the epigenetic age of embryonic stem cells (ESCs) is close to zero. Interestingly, induced pluripotent stem cells (iPSCs) do not differ from ESCs by their epigenetic age. Therefore, epigenetic age is reset when iPSCs are produced from differentiated somatic cells.(2)

Another interesting feature of the epigenetic clock is that the offspring of semi-supercentenarians exhibit lower epigenetic age than age-matched controls (Horvath et al., 2015). As centenarians are an excellent example of successful, healthy agers, who managed to escape or postpone the onset of major aging diseases, their offspring's youthful DNAmAge could indicate that common (genetic or shared environmental) factors matter for protection from aging diseases and DNAmAge maintenance.(8)

Epigenetic clocks are independent of mitotic divisions and senescence and change most rapidly during development, suggesting that the clock's rate of change represents the work done to maintain epigenetic stability.(13)

The most recently presented "GrimAge" clock further elaborates the "phenotypic age" concept, combining clinical biomarkers with DNA methylation levels.(7) This clock was built using 1030 unique CpGs and a selection of plasma proteins that have previously been associated with mortality or morbidity (adrenomedullin, CRP, PAI1, and GDF15).(2)

More recently, TRIIM (Thymus Regeneration, Immunorestoration and Insulin Mitigation) treatment with a cocktail of substances, comprising human growth hormone, proto-hormone DHEA and metformin, demonstrated epigenetic rejuvenation, reversing epigenetic age by 2.5 years in a one-year treatment window.(7) Nevertheless, thus far there is no direct experimental demonstration that organismal lifespan can be extended by altering patterns of DNA methylation.(2)

3.3 Defective proteostasis

Proteostasis involves mechanisms for the stabilization of correctly folded proteins, most prominently the heat-shock family of proteins and mechanisms for the degradation of proteins by the proteasome or the lysosome.(5) Proteostasis efficiency decreases with time, especially in non- or minimally-dividing cells, such as skeletal muscle and neurons, which are forced to deal with accumulating protein waste and contribute to many aging associated diseases.(7)

Impaired proteostasis can be detected directly by the formation of aggregates, either ordered (amyloid β and hyper-phosphorylated tau in Alzheimer's disease) or amorphous (-α-crystallin in cataracts; α-synuclein of Lewy bodies in Parkinson's disease), or indirectly by increased production of proteostasis network machinery (lysosomal β-galactosidase in senescence; chaperone Hsp70 as a marker of oxidative stress). Moreover, the integrity of the proteome can be efficiently examined in proteomics studies.(7) There are regulators of age-related proteotoxicity, such as MOAG-4 that act through an alternative pathway distinct from molecular chaperones and

proteases. All these systems function in a coordinated fashion to restore the structure of misfolded polypeptides or to remove and degrade them completely, thus preventing the accumulation of damaged components and assuring the continuous renewal of intracellular proteins.(5)

3.4 Mitochondrial dysfunction and oxidative stress

The mitochondria are now known to be important in the regulation of the aging process. The primary function of these organelles is to generate energy for the organism in the form of adenosine triphosphate (ATP), but they are also involved in other physiological processes which have links to aging, such as apoptosis, autophagy and production of reactive oxygen species (ROS).(2)

Mitochondrial function has a profound impact on the aging process. Mitochondrial dysfunction can accelerate aging in mammals, but it is less clear whether improving mitochondrial function can extend lifespan in mammals, although suggestive evidence in this sense already exists.(5)

The observed decline in ATP production with age (≈8% per decade) causes energetic deficiency and likely contributes to lower physical performance in elders.(7) Recent studies have suggested a complex relationship between oxidative stress and aging, as some low level of ROS may provoke signalling pathways associated with longevity; moreover, most strategies to mitigate ROS have not led to lifespan extension in animal models.(7) In others words according to this concept, mild toxic treatments trigger beneficial compensatory responses that surpass the repair of the triggering damage and actually produce an improvement in cellular fitness when compared to the starting pre-damage conditions.(5)

Preclinical models and data from human studies suggest that insulin/IGF signalling and the efficiency of mitochondrial energy production are key regulators of the aging process. In addition, studies of these pathways have provided both rationales and potential drug targets for therapeutic interventions. A number of investigations along these lines have already been completed in animal models with the aim of finding a way of slowing the aging process and extending the human health span. Interventions such as caloric restriction and exercise and administration of nutritional compounds and drugs, such as antioxidants, omega-3 fatty acids, metformin and aspirin, target the mitochondria to delay or counteract the effects of aging.(2)

3.5 Deregulated nutrient-sensing

Altered energy metabolism is a major feature of aging. At the cellular level, it occurs predominantly due to the disruption in nutrients-sensing pathways highly involved in aging progression, such as the mechanistic target of rapamycin (mTOR) (nutrient sensor), the adenosine monophosphate (AMP)-activated protein kinase (AMP sensor), the protein deacetylase sirtuins (regulated by NAD+) and insulin/insulin-like growth factor 1 (IGF-1) signalling (IIS) pathways.(7) These signalling pathways make a network with positive and negative feedback control.(2)

Literature suggests that nutrient sensing and signalling (caloric restriction and mutations in related signalling pathways), lead to increased lifespans in several model organisms.(17)

Aging research has lately paid attention to the intracellular mechanistic target of rapamycin (mTor) signalling pathway, mediated by the growth hormone (GH) and insulin growth factor1 (IGF1), sensitive to insulin and caloric restriction. (18) Paradoxically, GH and IGF-1 levels decline during normal aging, as well as in mouse models of premature aging. Thus, a decreased IIS is a common characteristic of both physiological and accelerated aging, while a constitutively decreased IIS extends longevity. These apparently contradictory observations could be accommodated under a unifying model by which IIS down-modulation reflects a defensive response aimed at minimizing cell growth and metabolism in the context of systemic damage. According to this view, organisms with a constitutively decreased IIS can survive longer, because they have lower rates of cell growth and metabolism and hence, lower rates of cellular damage. Along the same lines, physiologically or pathologically aged organisms decrease IIS in an attempt to extend their lifespan.(5)

IIS pathway is related and interconnected with nutrient-sensing system mTOR - (as mentioned before), mTOR pathway acts by sensing high amino acid concentrations; AMPK however senses low energy states by detecting high AMP levels; and sirtuins sense low energy states by detecting high NAD+.(5)

In mammals, the target of rapamycin (mTOR) pathway integrates multiple signals from nutrients, growth factors and oxygen to regulate cell growth, proliferation and survival.(2) Inhibition of mTOR has been found to extend lifespan in multiple species,(2) mTOR activity increases with age, whereas treatment of old mice with the mTOR inhibitor rapamycin significantly increased their life span.(2)

The other two nutrient sensors, AMPK and sirtuins, act in the opposite direction to IIS and mTOR, meaning that they signal nutrient scarcity and catabolism instead of nutrient abundance and anabolism.(5) AMPK activation has multiple effects on metabolism and, remarkably shuts off

mTORC1(5), sirtuins (SIRT 1–7), localized in different cell compartments are another group of mediators that are associated with promoted longevity; by deacetylating histones or non-histone substrates in a nicotinamide adenine dinucleotide (NAD+) dependent manner, sirtuins and especially SIRT1, located in the nucleus, can regulate gene-expression. Because NAD+ levels are sensitive to diet and exercise, sirtuins are involved in metabolism and sense life-style changes, thus impacting health status.(18) Also, Sirt1 (the mammalian orthologue of Sir2) has been associated with neuroprotection, reduction of fat storage and insulin secretion from pancreatic-β cells. Sirt1 increases expression of genes involved in fatty acid oxidation in response to low glucose, thereby providing a switch from glucose to a fatty acid oxidation metabolism under low caloric conditions. Some dietary activators of Sirt1 have been identified such as resveratrol and melatonin. Another sirtuin family member (SIRT3) is thought to be involved in increasing the mitochondrial glutathione antioxidant defence system under caloric restriction conditions,(2) but perhaps the most conclusive studies are related with SIRT6, which regulates genomic stability, NF-.B signalling and glucose homeostasis through histone H3K9 deacetylation,(5) mice overexpressing Sirt6 have a longer lifespan than control animals, associated with reduced serum IGF-1 and other indicators of IGF-1 signalling.(5)

Caloric restriction appears to work by lowering mitochondrial O2 consumption, leading to reduced generation of damaging ROS. This has been linked to changes in the mammalian target of rapamycin (mTOR) and sirtuin pathways.(2)

3.6 Increased cellular senescence

Senescent cells are characterized by an inability to divide due to stable growth arrest, driven by telomere attrition (replicative senescence) or by external stress stimuli, such as DNA damage or abnormal activation of oncogenes (stress-induced premature senescence).(7) Since the amount of senescent cells increases with aging, it has been widely assumed that senescence contributes to aging; it is possible that senescence is a beneficial compensatory response that helps tissues to get rid of damaged and potentially oncogenic cells.(5)

In recent years, it has been recognized that senescent cells manifest dramatic alterations in their secretome, which is particularly enriched in pro-inflammatory cytokines and matrix metalloproteinases and is referred to as the ‘senescence-associated secretory phenotype’ (SASP). This pro-inflammatory secretome that may contribute to aging(5) the SASP, typically assessed by a few dozen secreted proteins, has been greatly underestimated. There are several candidate biomarkers of cellular senescence that overlap with aging markers in human plasma,

including Growth/differentiation factor 15 (GDF15), stanniocalcin 1 (STC1) and serine protease inhibitors (SERPINs).(19) Lysosomal β-galactosidase (β-gal) activity is the most frequently applied molecular biomarker of senescence in culture and tissue samples, plasma β-gal activity has been recently proposed as a potential systemic biomarker of aging.(7) There are several others biomarkers widely validated, but are found within the cell and therefore, require cell isolation (and usually destruction) for analysis. For clinical implementation, it is more useful to rely on molecules that are secreted in the adjacent extracellular area and can be detected without disturbing the cells in human subjects.(7) SASP factors therefore hold potential as plasma biomarkers for aging and age-related diseases, marked by the presence of senescent cells.(19) To understand the pleiotropic phenotypes of senescent cells, a shift from traditional reductionism to more systematic, multi-parametric approaches is needed. The development of sophisticated high-throughput methods and machine learning tools that can handle multi-omics data will help achieve this goal.(20)

3.7 Stem cell exhaustion

The decline in the regenerative potential of tissues is one of the most obvious characteristics of aging. For example, hematopoiesis declines with age, resulting in a diminished production of adaptive immune cells, a process termed immunosenescence and in an increased incidence of anemia and myeloid malignancies.(5) Counting stem cell numbers within organ tissues is fraught with obvious difficulties, but for particular groups, such as circulating hematopoietic stem cells, some important research has been done.(7)

Although deficient proliferation of stem and progenitor cells is obviously detrimental for the long-term maintenance of the organism, an excessive proliferation of stem and progenitor cells can also be deleterious by accelerating the exhaustion of stem cell niches.(5)

Mutations naturally occurring in blood cells during a lifetime and revealed by whole-genome sequencing, can be used as a bar code for tracking and quantifying adult stem cells and their offspring in the bloodstream. This technique has been applied to demonstrate that all peripheral white blood cells of a 115-year-old woman were the offspring of only two hematopoietic stem cell clones, suggesting that the diversity of active stem cells is significantly depleted at this advanced age.(7)

Data on the quantity of active stem cells in other organs and how their abundance and functionality changes with age would be very pertinent, especially in the context of novel

therapeutic approaches, such as direct reprogramming of differentiated tissue cells or rejuvenation of dysfunctional adult stem cells, designed to maintain robust stem cell pools.(7)

3.8 Altered intercellular communication

Beyond cell-autonomous alterations, aging also involves changes at the intercellular communication level, be it endocrine, neuroendocrine or neuronal. Thus, neurohormonal signalling (eg, renin-angiotensin, adrenergic, insulin-IGF1 signalling) tends to be deregulated in aging as inflammatory reactions increase, immunosurveillance against pathogens and premalignant cells declines and the composition of the peri- and extracellular environment changes, thereby affecting the mechanical and functional properties of all tissues.(5)

Inflammatory markers associated with aging exhibit significant associations with a measure of epigenetic age acceleration. However, this association was not independent of other key aspects of immune system aging.(21)

The intracellular space has efficient intracellular mechanisms of waste recycling, however it lacks such machinery and disposal of garbage outside the cell which is a serious age-related problem.(7)

A prominent aging-associated alteration in intercellular communication is ‘inflammaging’, a smoldering pro-inflammatory phenotype that accompanies aging in mammals. Inflammaging may result from multiple causes, such as the accumulation of pro-inflammatory tissue damage, the failure of an ever more dysfunctional immune system to effectively clear pathogens and dysfunctional host cells, the propensity of senescent cells to secrete pro-inflammatory cytokines, the enhanced activation of the NF-kB transcription factor or the occurrence of a defective autophagy response. These alterations result in an enhanced activation of the NLRP3 inflammasome and other pro-inflammatory pathways, finally leading to an increased production of IL-1ß, tumour necrosis factor and interferons. Inflammation is also involved in the pathogenesis of obesity and type 2 diabetes, two conditions that contribute to, and correlate with aging in the human population.(5)

Regulatory and effector molecules secreted into an immediate environment, such as various SASP components, or found in circulating body fluids, such as hormones, systemic inflammatory markers and circulating miRNAs, also contribute to altered cell communication and activity with age.(7)

4. Systemic biomarkers

What deserves attention is that the effects of an individual's aging biomarkers vary at the cellular and systemic levels. For the exploration of aging biomarkers and anti-aging targets, more attention should be paid to the overall effect.(2)

The functional decline in specific tissues and organs due to the accumulation of aged cells, complicated by intrasystem/intersystem interactions and by environmental factors (e.g., diet, lifestyle) may be best measured by systemic molecular biomarkers. Although these biomarkers reflect deleterious consequences of aging, rather than primary causes such as molecular damage.(7)

4.1 Inflammatory, metabolic, hormonal, organ-specific and blood-based biomarkers

Many markers require access to tissues or resources that are not feasible for large clinical trials or clinical practice. Relatively few markers can be directly measured from samples obtained from blood draw or have validated and reliable blood-based surrogates.(6)

A number of parameters routinely measured in a comprehensive metabolic panel (glucose, albumin, creatinine, alkaline phosphatase, bilirubin), complete blood count (red blood cell counts, hemoglobin, hematocrit) and lipid profile (total cholesterol, low- and high-density lipoprotein cholesterol, triglyceride concentration) were found to correlate with age, age-related disease and mortality.(7)

A database study conducted through the examination of 11,555 different publications, identified 20 circulating biomarkers associated with mortality risk. These most significant associations were found for brain natriuretic peptide, cholesterol fractions, C-reactive protein, erythrocyte sedimentation rate, fibrinogen, granulocytes, homocysteine, intercellular adhesion molecule-1, N terminal-pro brain natriuretic peptide, white blood cell count, granulocytes, homocysteine, intercellular adhesion molecule-1, neutrophils, osteoprotegerin, procollagen type III aminoterminal peptide, uric acid, soluble urokinase plasminogen activator receptor, tissue inhibitor of metalloproteinases 1 and tumour necrosis factor receptor II.(2)

Targeting Aging with Metformin (TAME) Biomarkers Workgroup established a framework for the selection of blood-based biomarkers for next-generation of geroscience clinical trials.(7)

Each biomarker and its role is briefly explained in the graphical table below (Figure 4).

Blood-based biomarkers for geroscience-guided trials

Biomarker	Underlying Biologic Process & Role
IL-6, CRP TNFRII	**Inflammation & Intercellular Signaling** Interleukin 6 (IL-6) is a proinflammatory cytokine and Tumor Necrosis Factor-α RII is a TNF -α receptor involved in acute-phase response. C-Reactive Protein (CRP) is an acute phase protein produced in response to inflammation. Cytokine dysregulation is a driver of pathophysiologic processes leading to disease, functional decline, frailty, and death.
GDF15	**Stress Response & Mitochondria** Growth Differentiating Factor 15 (GDF15) is a member of the TGF-ß superfamily robustly associated with mortality, cardiovascular events, cognitive decline and dementia. GDF15 is increasingly recognized in mitochondrial dysfunction, and as a biomarker of aging.
IGF-1 Insulin	**Nutrient Signaling** Disruption of the insulin/ insulin-like growth factor (IGF-1) signaling pathway is implicated in longevity in animal models. In humans, IGF-1 and fasting insulin are responsive to caloric restriction, and low IGF-1 in growth hormone receptor deficiency conveys disease protection.
Cystatin-C	**Kidney Aging** Cystatin C, an extracellular inhibitor of cysteine proteases, is a marker of renal disease and aging. It is an independent risk factor for all cause and CVD-related mortality, and multi-morbidity, and higher levels are consistently associated with poor physical function and cognition.
NT-proBNP	**Cardiovascular Health** B-type natriuretic peptides (BNP, NT-proBNP) are secreted in response to cardiomyocyte stretching to decrease vascular resistance. NT-proBNP has a greater-half life and accuracy compared with BNP and is used to diagnose and establish prognosis for heart failure.
HGBA1c	**Metabolic Aging** Glycated hemoglobin (hemoglobin A1c, HGBA1c) is formed in a non-enzymatic glycation pathway and is a marker for 3-mo average plasma glucose. High HGBA1c reflects poor glucose control, and in older nondiabetics is strongly associated with death, chronic disease, and functional decline.
Molecular Signature	**Epigenetic, Interdependent, Multi-Omic** Data intensive molecular platforms can explore global changes in epigenetic, transcriptomic, proteomic and proteostasis, and small metabolite signatures. These approaches may better capture complex and multifactorial processes underlying aging.

Figure 4: Blood-based biomarkers remained as candidate markers to be used as an exploratory outcome. Image presented with athor permission available in Justice et all.(6)

Authors like Franceschi et all, (2018), also consider IL-1 and IL-8 as inflammatory biomarkers of chronic inflammation(22), due to that fact that many of these mediators are classical SASP components. Cellular senescence is likely to participate in inflammaging, although the true extent to which senescence contributes to systemic markers, as compared to other inflammatory drivers remains unknown.(7)

Metaflammation (the metabolic inflammation accompanying metabolic diseases) is thought to be the form of chronic inflammation that is driven by nutrient excess or overnutrition; metaflammation is characterized by the same mechanisms underpinning inflammaging.(22) This unifying hypothesis indicates that an individual's metabolic history probably affects their immunobiography and eventually the individual's inflammaging phenotype. (22) Inflammatory markers associated with aging exhibit significant associations with a measure of epigenetic age acceleration. However, this association was not independent of other key aspects of immune

system aging (such as estimated naïve T cell type percentages). (21) Information regarding age-related changes in immune function can be obtained from immune-phenotype profiling (lymphocyte subset panel, CD4:CD8 T cell ratio).(7)

Serum IgG-G0 digalactosylated or agalactosylated N-linked glycan structures is another potential biomarker, based on the fact that Vanhooren and colleagues demonstrated that the log ratio of the relative abundance of two N-linked glycan species increases progressively with age and is associated with features of healthy and unhealthy aging.(22) In terms of microRNAs, miR-17-5p has been identified as potentially being involved in longevity and cancer through its role in cell cycle regulation, proliferation and apoptosis.(2)

Metabolic aging and aging of the endocrine system can be evaluated by changes in hormone production such as GH and IGF-1. Moreover, declines in sex hormones like estrogen and testosterone (menopause and andropause, respectively) trigger downstream fading effects, such as muscle mass loss and physical frailty. Dehydroepiandrosterone (DHEA) and its ester metabolite dehydroepiandrosterone sulphate (DHEA-S) are essential steroid precursors for sex hormones that profoundly decrease from the third decade of life and serve as prospective candidate biomarkers of human aging.(7) These are too potential systemic aging biomarkers that were not included in the TAME Biomarkers Workgroup framework, but are mentioned in the referred biography.

5. Omics-based biomarkers

An era is now approaching where progressively maturating Omics-based technologies are prepared to meet long-promised goals for a better understanding of the complex mechanistic basis of disease. Arguably, the paradigm shift from a traditional “hypothesis-driven” research environment to one that is primarily “discovery-based” will fail to sit comfortably with many researchers.(23) The rise of technologies that simultaneously measure thousands of data points represents the heart of systems biology. These technologies have had a huge impact on the discovery of next-generation diagnostics, biomarkers and drugs in the precision medicine era.(24)

Genes, gene expression products (i.e., transcripts and proteins) and metabolites are the main biomarker families. Given this molecular diversity of biomarkers, the increase in high-throughput omics technologies offers an amazing opportunity to capture the whole picture of biological systems in a hypothesis-free and unbiased mode.(24)

5.1 Genomics

Next-generation sequencing (NGS) techniques using a massive parallel sequencing strategy have profoundly changed the clinical genomic landscape. High-throughput sequencing (HTS) techniques can be classified according to their applications for investigating genomes, epigenomes or transcriptome.(24)

Recent advances in various 'omics' technologies enable quantitative monitoring of a myriad of biological molecules in a high-throughput manner and allow the determination of their variation between different biological states on a genomic scale.(2)

One approach has been to study population groups sometimes called the oldest-old, such as centenarians who lived long lives essentially free of disease. With this in mind, a number of genome-wide association studies (GWAS) of various human populations have been performed to identify genes associated with living a long time.(2)

A relatively large human cohort (*n* = 300 447) found 12 candidate loci associated with health span. Among them, three single nucleotide polymorphism located at or near CDKN2B (cyclin-dependent kinase inhibitor), LPA (apolipoprotein A) and HLA-DQA1 (leucocyte antigen DQA1) loci were identified and reported to be associated with the longevity phenotype.(7)

Other GWAS studies have shown associations of *APOE* and *FOXO3A* variants with longevity and these findings have been confirmed in other population studies. Not surprisingly, maintenance and repair of the genome have also been identified as important factors for supporting a long life. This has been shown by studies on centenarian populations, which have found that such individuals appear to have an enhanced DNA repair capacity with significantly lower genomic and cellular damage.(2)

5.2 Epigenomics

General alteration of the chromatin structure and the epigenetic signature of the genome is a major characteristic of the aging process, which can be assessed by the newly available techniques that can scan the whole epigenome.(22) Chemical modifications of DNA, histones, non-histone chromatin proteins and nuclear RNA define the epigenome.(24)

Different strategies have been developed to assess the epigenome. Epigenomics methods generally focus on chromatin structure and include histone modification ChIP-seq (chromatin immunoprecipitation sequencing), thus allowing the identification of DNA-associated protein-

binding sites. DNase-seq combines DNase I digestion of chromatin with HTS to identify regulatory regions of the genome. DNA methylation and ATAC-seq (assay for transposase-accessible chromatin sequencing) allow the mapping of chromatin accessibility genome-wide. Recently, an epigenome-wide study suggested that inter-individual variations in high-density lipoprotein (HDL) particle metabolism rely on epigenome modifications.(24)

5.3 Transcriptomics

The gene expression pattern in a cell/tissue can broadly reflect its functional state. The transcriptome is the complete set of RNA transcripts, including ribosomal RNA (rRNA), messenger RNA (mRNA) that represents only 1.5 to 2 percent of the transcriptome, transfer RNA (tRNA), miRNA and other non-coding RNA (ncRNA). Quantitative analyses of the transcriptome can be performed with either microarrays (Chips) or RNA sequencing (RNAseq). Microarrays are based on specific hybridization of RNA transcripts to DNA probes and High-throughput sequencing-based (HTS-based) expression profiling by RNA-seq allows comprehensive qualitative and quantitative mapping of all transcripts.(24)

Comparisons of young and old tissues from several species have identified age-related transcriptional changes in genes encoding key components of inflammatory, mitochondrial and lysosomal degradation pathways.(5)

A main transcriptomic age predictor was established based on 11 908 significantly expressed genes with the average absolute difference between predicted age and chronological age of 7.8 years.(7)

Blood-based sets of gene expression profiles have been developed to fulfil criteria for a transcriptomic age predictors. Holly et al., (2013) demonstrated that 6 (IL-6) and blood urea, as well as higher levels of serum albumin and muscle strength, were to be considered transcriptomic age predictors able to differentiate biologically young group from the rest.(8)

Several studies have been performed with different tissues and a wide range of transcripts have been associated with aging, such as Q10 enzyme in the epidermal tissue, however these studies have limited value because it is clear now that each tissue of our body has its own age-dependent gene expression pattern.(7)

The transcriptomic age and the epigenetic clock describe different aspects of biological aging. When simultaneously examining multiple cohorts that have their transcriptomic profiles produced using different array platforms, it is critical to control technical variables and probe design to

ascertain whether the signatures are truly platform-independent. Nevertheless, the transcriptomic age predictors still await broader validation in independent cohort studies.(8)

5.4 Proteomics

The proteome consists of all the proteins expressed by a biological system. Posttranslational modifications rely on a highly specialized enzymatic arsenal, specific to each cellular type, which leads to the generation of different proteomes from the same genome. These modifications add layers in proteome complexity and thus, broaden their functionalities. Hence, proteins exhibit different conformation, localization and interactions, depending on space and time factors. The development of proteomics assays is triggered by these complexity challenges.(24) Mass spectrometry-based proteomics remains the gold standard for protein biomarker screening, yet it lacks throughput in the validation phase.(7)

Over the last two decades, several studies have shown effects of aging on protein glycosylation as measured from human serum or plasma.(8) Glycan structures are known to modulate the half-life of plasma glycoproteins. Removal of sialic acid from glycans with terminal Gal and GalNAc residues, which can interact with hepatic asialoglycoprotein receptors shortens the half-life of these glycoproteins. Conversely, the half-life of glycoproteins possessing glycans covered with sialic acid should be prolonged, thus the half-life of haptoglobin with highly sialylated N-glycans may be longer. It is known that haptoglobin binds free hemoglobin released from erythrocytes and thereby inhibits its oxidative activity. The high levels of sialylated haptoglobin in SSCs may be simply a sign of extreme aging, it may contribute to extreme longevity and healthy aging through antioxidant activity.(25)

Recently, Kristić and colleagues tried to construct a prediction model for age based on three individual glycans. The GlycanAge was built in one cohort and replicated well in the others (among which TwinsUK was included). The GlycanAge index was associated with health variables such as fibrinogen, HbA1c, BMI, triglycerides and uric acid after correction for age and gender.(8)

Menni and co-workers developed an age predictor who calculated a protein-derived age variable from four age-associated proteins found in plasma (PTN, CHRDL1, MMP12, and IGFP6). The predictor (trained in TwinsUK data) was validated in independent cohorts.(8) The strongest association with age was shown for chordin-like protein 1 (CHRDL1), which is involved in bone morphogenic protein signalling, retinal angiogenesis and brain plasticity. Levels of CHRDL1 increase in older individuals, correlating with lower cardiovascular risk and higher birth weight (a

well-known determinant of aging trajectories), suggesting its potential as a biomarker of healthy aging.(7)

Basity Nathan et all (2020) presented the first “SASP” Atlas, a comprehensive, curated and expanding online database of the soluble senescence-associated secretory phenotype, based on proteomic related techniques. They identified several candidate biomarkers of cellular senescence that overlap with aging markers in human plasma, including Growth/differentiation factor 15 (GDF15), stanniocalcin 1 (STC1) and serine protease inhibitors (SERPINs), which significantly correlated with age in plasma from a human cohort, the Baltimore Longitudinal Study of Aging (BLSA).(19)

A recent proteome-wide study using mass spectrometry-based quantification in a discovery (n = 104) and replication cohort (n = 39) of initially cognitively unimpaired, longitudinally assessed older-adult post-mortem brain donors, revealing 579 proteins associated with cognitive decline, among which 38 replicated proteins were associated independently of traditional neuropathologies, such as β-amyloid plaques and neurofibrillary tangles. Notably, individuals with cognitive stability demonstrated increased neuronal mitochondrial activity and decreased inflammation and apoptosis, suggesting that proteomics could reveal not only new protein targets, but even new age-related pathways.(7)

5.5 Metabolomics

The metabolome is defined as the set of metabolites present in a given biological system, fluid, cell or tissue at a given time. Metabolomics is an omics approach based on biochemical characterizations of the metabolites and their fluctuations related to internal (genetic) and external factors (environment).(24)

Metabolomics and lipidomics could provide biomarkers at the interface between metabolism, inflammation (including age- related changes in the composition of the gut microbiota) and disease risk.(22)

Several studies had analysed several hundred plasma compounds and a large number of them showed statistically significant changes in concentration with age.(7) Yu and colleagues for example, used a targeted mass-spectrometry method to identifying 131 metabolites in fasting serum, where 11 were independently associated with age in females.(8) Hertel and colleagues (2016), used a proton nuclear magnetic resonance (H^1NMR) spectroscopy to perform an investigation in human urine samples and quantified 59 metabolites; this data was used to construct a Metabolic Age Score including all metabolites as predictors and age as the outcome.

The metabolic age score was validated and replicated in two independent cohorts and found to be associated with clinical outcomes independent of age, e.g., kidney malfunction, high HbA1c levels and hyperglyceridemia. Importantly, survival analysis showed that individuals in the first tertile of the score (lower biological age) had higher all-cause survival rates and that the prediction added value over commonly known risk factors.(8)

A particular area of interest is the metabolome of long-lived individuals. The metabolomic profile of centenarians showed peculiar mechanisms of cellular detoxification, which occurred through the specific modulation of the arachidonic acid metabolic cascade and increased cytochrome P450 enzyme activity. In particular, the longevity phenotype of arachidonic acid synthesis displayed both pro-inflammatory and anti-inflammatory characteristics, such as a high concentration of leukotriene E4 (a molecule with vasodilatation properties) or a high concentration of 15-hydroxyeicosatetraenoic acid (a molecule with anti-inflammatory properties). This metabolomic profile is in line with the hypothesis that longevity results in complex remodelling of lipids, amino acid metabolism and gut microbiota.(22)

Cross-sectional metabolome profiling was carried out on several cohorts of centenarians and offspring of nonagenarians. Metabolite profiles captured earlier in life and associated with the future ability to achieve longevity were also studied. Centenarians were revealed to have metabolic phenotypes distinct from that of elderly individuals, with alterations in lipid and fatty acid, nucleic acid and citric acid cycle metabolism; however, metabolic patterns on different cohorts had only limited overlap.(7) Researchers also showed that centenarians had a peculiar lipid profile, with unique changes in 41 of 161 measured lipid species. The lipid profile emphasized that long-living individuals have marked features of anti- inflammatory molecules, such as increased levels of phenylalanine, which inhibits the nuclear factor- κB (NF- κB) pathway and decreased levels of glycerophosphocholine (a circulating marker of cellular senescence).(22) The study suggests that the longevity plasma lipidome reflects antioxidant capacity and lower lipid peroxidation inflammatory state and β- oxidation function, likely contributing to healthy aging of studied individuals.(22)

6. Discussion

Entropy always wins. A multicellular organism is able to develop and maintain its identity for only so long before deterioration prevails over synthesis and the organism ages.(26) Cellular health is regulated across a wide range of scales from molecular to cellular and across every spatial division of the cell.(27)

The prediction of normal and pathological states in patients should be based on a dynamic understanding of gene–environment interactions on individual and population scales.(24) Pathogenetic processes of aging are highly interconnected and therefore, aging can be defined as a complex of several mutually-influencing pathological processes.(28) There are discussions on whether aging should be classified as a disease or not. A number of researchers argued that aging should be considered and treated as a disease.(28)

The new concept of medicine relies on a global and integrative approach for patient care.(24) The phenotype has three main drivers: the first one is the molecular phenome, which is defined by the underlying molecular supports of biological information. Omics strategies should enable to interrogate these supports for information retrieval; the second driver are environmental effects spanning from exposures to toxic substances or drugs to diet, together they define the exposome; and the third is the different clinical metrics used to define the clinical phenome.(24) These different biological and clinical metrics should be approached in a multi-dimensional fashion and should consider the inherent spatial and temporal scales.(24) The following picture (Figure 5) gives an overview of this multi-scale perspective.

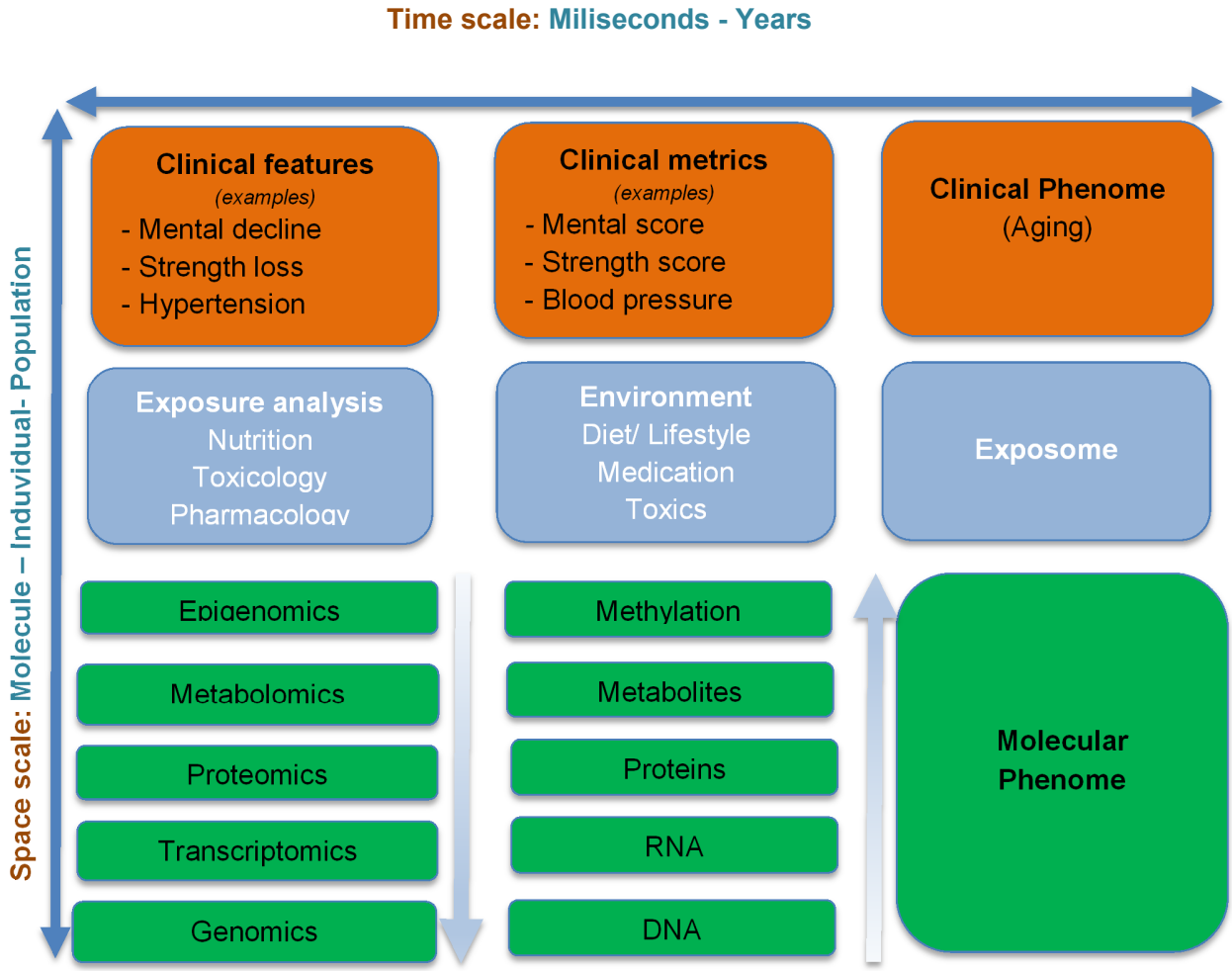

Figure 5: The holistic approach to the aging process. Adapted from Tebani A. et all.(24)

Many candidate biomarkers of human aging have been proposed in scientific literature, but in all cases their variability in cross-sectional studies is considerable and therefore no single measurement has proven to serve a useful marker to determine, on its own, biological age. A plausible reason for this is the intrinsic multi-causal and multi-system nature of the aging process.(16)

It has been proved that interconnectedness between candidates exists and a degree of hierarchical relation between them was already proposed between molecular and cellular biomarkers. The primary hallmarks are initiating triggers, whose damaging events progressively accumulate with time. The antagonistic hallmarks, being initially beneficial, become progressively negative in a process that is partly promoted or accelerated by the primary hallmarks. The

integrative hallmarks arise when the accumulated damage caused by the primary and antagonistic hallmarks cannot be compensated by tissue homeostatic mechanisms.(5)

It is reasonable to assume that a combination of several biomarkers will provide a much better tool to measure biological age than any single biomarker in isolation. It has to be taken into consideration though, that not all biomarkers are of equal weight. Therefore averaging all possible candidate biomarkers may not be appropriate,(16) moreover different biomarkers might have different weights during different lifetime periods. This and the inter-individual singular biology, significantly increases the equation complexity, making it a mathematical problem almost impossible to solve. However the exact causal network between biomarkers is an exciting challenge for future work(5) and may shed light on this process.

7. Conclusion

One of the original hypotheses of organismal longevity posits that aging is the natural result of entropy on the cells, tissues and organs of the animal slow, inexorable slide into nonfunctionality caused by stochastic degradation of its parts. We now have evidence that aging is instead at least in part genetically regulated.(27)

Genetic contribution to human variation in aging is still unclear, but most from the existing literature estimates genetic contribution in a range between 15% and 30% and across gender comparisons sometimes dropping below 15%.(29)

Human aging can be described in terms of the WHO criteria for disease, including symptomatology, etiology, course and outcomes of the disease, possible and potential interventions and linkage to genetic and environmental factors. Pathogenetic processes of aging are highly interconnected and therefore, aging can be defined as a complex of several mutually-influencing pathological processes.(28)

Taking into account the divergence in human aging scenarios, the modelling of personalized trajectories of aging or aging profiles by the integral application of all types of biomarkers, will likely be the most accurate way to predict future outcomes and facilitate early stage cellular-based intervention aiming to slow down aging.(7)

Artificial Inteligence can be used in different ways for designing personalized treatments. Designed platforms can be used as a diagnostic tool to reduce error rate and be useful to stratify patients according to their specific health condition by combining more accurate diagnostics and a better knowledge of the health conditions of the patients, these platforms can be applied to design more effective treatments.(10) However much more research is needed to identify how precisely “delaying” or “reversing” aging will impact on the metabolic disease burden.(30)

Studies on the pathways known to be involved in the aging process, have provided both rationales and potential drug targets for therapeutic interventions. A number of investigations along these lines have already been completed in animal models with the aim of finding a way of slowing the aging process and extending the human health span, such as “the effect of caloric restriction in aging delaying”.(2)

The integrative analysis of the large data sets churned out continues to prove a challenge, with advances in analytical methodology failing to keep abreast of technical advances. Nevertheless, for the first time ever an integrated approach to modelling and defining the immense complexities of health and disease is coming to light. Its implications are likely to transcend far beyond the improvement of our mechanistic understanding of health and disease or drug and biomarker discovery.(23)

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9. References

1. Song S, Johnson FB. Epigenetic mechanisms impacting aging: A focus on histone levels and telomeres. Genes (Basel). 2018;9(4).
2. Guest PC. Reviews on Biomarker Studies in Aging and Anti-Aging Research [Internet]. Doi.Org. 2017. 1–279 p. Available from: https://doi.org/10.1007/978-3-030-25650-0
3. Levine ME, Lu AT, Quach A, Chen BH, Assimes TL, Bandinelli S, et al. An epigenetic biomarker of aging for lifespan and healthspan. Aging (Albany NY). 2018;10(4):573–91.
4. Luo J, Mills K, le Cessie S, Noordam R, van Heemst D. Ageing, age-related diseases and oxidative stress: What to do next? Ageing Res Rev [Internet]. 2020;57(June 2019):100982. Available from: https://doi.org/10.1016/j.arr.2019.100982
5. López-otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The Hallmarks of Aging Longevity. Cell. 2013;153(6):1194–217.
6. Justice JN, Ferrucci L, Newman AB, Aroda VR, Bahnson JL, Divers J, et al. A framework for selection of blood-based biomarkers for geroscience-guided clinical trials: report from the TAME Biomarkers Workgroup. GeroScience. 2018;40(5–6):419–36.
7. Kudryashova KS, Burka K, Kulaga AY, Vorobyeva NS, Kennedy BK. Aging Biomarkers: From Functional Tests to Multi-Omics Approaches. Proteomics. 2020.
8. Jylhävä J, Pedersen NL, Hägg S. Biological Age Predictors. EBioMedicine [Internet]. 2017;21:29–36. Available from: http://dx.doi.org/10.1016/j.ebiom.2017.03.046
9. Foo H, Mather KA, Thalamuthu A, Sachdev PS. The many ages of man: Diverse approaches to assessing ageing-related biological and psychological measures and their relationship to chronological age. Curr Opin Psychiatry. 2019;32(2):130–7.
10. Zhavoronkov A, Mamoshina P, Vanhaelen Q, Scheibye-Knudsen M, Moskalev A, Aliper A. Artificial intelligence for aging and longevity research: Recent advances and perspectives. Ageing Res Rev. 2019;49(November 2018):49–66.
11. Lin J, Epel E, Cheon J, Kroenke C, Sinclair E, Bigos M, et al. Analyses and comparisons of telomerase activity and telomere length in human T and B cells: Insights for epidemiology of telomere maintenance. J Immunol Methods [Internet]. 2010;352(1–2):71–80. Available from: http://dx.doi.org/10.1016/j.jim.2009.09.012
12. Brane A, Tollefsbol T. Targeting Telomeres and Telomerase: Studies in Aging and

Disease Utilizing CRISPR/Cas9 Technology. Cells. 2019;8(2):186.

13. Barrere-Cain R, Allard P. An Understudied Dimension: Why Age Needs to Be Considered When Studying Epigenetic-Environment Interactions. Epigenetics Insights. 2020;13.

14. Gillespie SL, Hardy LR, Anderson CM. Patterns of DNA methylation as an indicator of biological aging: State of the science and future directions in precision health promotion. Nurs Outlook [Internet]. 2019;67(4):337–44. Available from: https://doi.org/10.1016/j.outlook.2019.05.006

15. Hyun Sik Jang, Woo Jung Shin JEL and JT Do. CpG and Non-CpG Methylation in Epigenetic Gene Regulation and Brain Function. Genes (Basel). 2017;

16. Bürkle A, Moreno-Villanueva M, Bernhard J, Blasco M, Zondag G, Hoeijmakers JHJ, et al. MARK-AGE biomarkers of ageing. Mech Ageing Dev [Internet]. 2015;151:2–12. Available from: http://dx.doi.org/10.1016/j.mad.2015.03.006

17. Sidler C, Kovalchuk O, Kovalchuk I. Epigenetic regulation of cellular senescence and aging. Front Genet. 2017;8(SEP).

18. Opstad TB, Kalstad AA, Pettersen AÅ, Arnesen H, Seljeflot I. Novel biomolecules of ageing, sex differences and potential underlying mechanisms of telomere shortening in coronary artery disease. Exp Gerontol. 2019;119(January):53–60.

19. Basisty N, Kale A, Jeon OH, Kuehnemann C, Payne T, Rao C, et al. A proteomic atlas of senescence-associated secretomes for aging biomarker development. PLoS Biol. 2020;18(1):e3000599.

20. Gorgoulis V, Adams PD, Alimonti A, Bennett DC, Bischof O, Bishop C, et al. Cellular Senescence: Defining a Path Forward. Cell. 2019;179(4):813–27.

21. Irvin MR, Aslibekyan S, Do A, Zhi D, Hidalgo B, Claas SA, et al. Metabolic and inflammatory biomarkers are associated with epigenetic aging acceleration estimates in the GOLDN study. Clin Epigenetics. 2018;10(1):1–9.

22. Franceschi C, Garagnani P, Parini P, Giuliani C, Santoro A. Inflammaging: a new immune–metabolic viewpoint for age-related diseases. Nat Rev Endocrinol [Internet]. 2018;14(10):576–90. Available from: http://dx.doi.org/10.1038/s41574-018-0059-4

23. Matthews H, Hanison J, Nirmalan N. “Omics”-informed drug and biomarker discovery: Opportunities, challenges and future perspectives. Proteomes. 2016;4(3):1–12.

24. Tebani A, Afonso C, Marret S, Bekri S. Omics-based strategies in precision medicine: Toward a paradigm shift in inborn errors of metabolism investigations. Int J Mol Sci.

2016;17(9).

25. Miura Y, Hashii N, Ohta Y, Itakura Y, Tsumoto H, Suzuki J, et al. Characteristic glycopeptides associated with extreme human longevity identified through plasma glycoproteomics. Biochim Biophys Acta - Gen Subj [Internet]. 2018;1862(6):1462–71. Available from: https://doi.org/10.1016/j.bbagen.2018.03.025

26. Michael J. F. Barresi and Scott F. Gilbert. Student Resources for Developmental Biology [Internet]. Twelfth Ed. Oxford: Oxord University Press; 2020. Available from: https://learninglink.oup.com/access/content/barresi-12e-student-resources/barresi-12e-further-development-23-7-longevity-and-senescence

27. DiLoreto R, Murphy CT. The cell biology of aging. Mol Biol Cell. 2015;26(25):4524–31.

28. Khaltourina D, Matveyev Y, Alekseev A, Cortese F, Ioviţă A. Aging Fits the Disease Criteria of the International Classification of Diseases. Mech Ageing Dev. 2020;189(February 2019).

29. Graham Ruby J, Wright KM, Rand KA, Kermany A, Noto K, Curtis D, et al. Estimates of the heritability of human longevity are substantially inflated due to assortative mating. Genetics. 2018;210(3):1109–24.

30. Martins MJ, Constância M, Neves D, Simm A. Biomarkers of Aging: From Cellular Senescence to Age-Associated Diseases. Oxid Med Cell Longev. 2017;2017:2–4.