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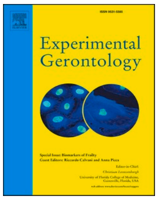

Review

Whole organism aging: Parabiosis, inflammaging, epigenetics, and peripheral and central aging clocks. The ARS of aging

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ABSTRACT

The strong interest shown in the study of the causes of aging in recent decades has uncovered many mechanisms that could contribute to the rate of aging. These include mitochondrial ROS production, DNA modification and repair, lipid peroxidation-induced membrane fatty acid unsaturation, autophagy, telomere shortening rate, apoptosis, proteostasis, senescent cells, and most likely there are many others waiting to be discovered. However, all these well-known mechanisms work only or mainly at the cellular level. Although it is known that organs within a single individual do not age at exactly the same rate, there is a well-defined species longevity. Therefore, loose coordination of aging rate among the different cells and tissues is needed to ensure species lifespan. In this article we focus on less known extracellular, systemic, and whole organism level mechanisms that could loosely coordinate aging of the whole individual to keep it within the margins of its species longevity. We discuss heterochronic parabiosis experiments, systemic factors distributed through the vascular system like DAMPs, mitochondrial DNA and its fragments, TF-like vascular proteins, and inflammaging, as well as epigenetic and proposed aging clocks situated at different levels of organization from individual cells to the brain. These interorgan systems can help to determine species longevity as a further adaptation to the ecosystem.

1. Introduction

Two opposing views are that aging rate is programmed (PA) or non-programmed (non-PA). Giacinto Libertini (1988) and Vladimir Skulachev (1997) were the first proponents of PA, focusing on telomere shortening, or mitochondria and ROS, as mechanisms of aging, respectively. In our opinion, the available evidence strongly supports the PA view. Many compelling observations support the PA position. Non-PA wear-and-tear based theories of damage generation, like that responsible for progressive damage of inanimate objects, cannot generate the huge differences in longevity between animal species (longevity will be used as surrogate of maximum longevity from here on). Animal cells and extracellular materials are roughly composed of the same kinds of biological macromolecules in species with large differences in longevity. Therefore, we should expect wear and tear to erode short- and long-lived species at rather similar rates instead of at rates differing by almost 1million-fold.

Different species have lifespans that differ by at least 10^5, meaning that longevity is a genetically programmed species-specific trait. This constitutes the strongest evidence against non-PA wear-and-tear theories of aging proposing that animals age due to entropy-driven processes like inanimate objects. These theories ignore the fact that animals are energetically open systems that can locally decrease their own entropy at the expense of the environment, and they include the prediction that pro-aging genes cannot exist (Kirkwood, 1999). But >40 such genes are already known (Mitteldorf, 2016a; Folgueras et al., 2018).The genetically programmed character of aging agrees with one of the four Bernard Strehler rules of aging—the endogenous origin of aging

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Abbreviations: AP, aging program; ARS, (whole organism) aging regulation system; cf-mtDNA, cell free mtDNA; CARS, cell aging regulation system; DAMPs, damage-associated molecular patterns; DR, dietary calorie restriction; DRs, dietary restrictions in general (referring to dietary, protein, and methionine restriction); EARS, extracellular aging regulating system; MetR, methionine restriction; miRNA, micro RNA; mitBER, mitochondrial base excision repair; mitROSp, mitochondrial reactive oxygen species production; nDNA, nuclear DNA; mtDNA, mitochondrial DNA; PA, programmed aging; SASP, senescence-associated secretory phenotype; TF, transcription factor.

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(Strehler, 1962)—and constitutes the "big effect": the huge inter-species differences in longevity. Furthermore, the longevity of individual animals of a given species has been successfully increased up to 1.4-fold in mammals, both via dietary restriction (DR) and single gene mutations. This constitutes the "small effect". Many other aging-related facts discovered during the last decades, arising from both laboratory and field studies in the wild, support the notion that aging is a genetically programmed biological function that is adaptive at the group level (Libertini, 1988; Barja et al., 1994a, 1994b; Skulachev, 1997; Bowles, 1998; Guarente and Kenyon, 2000; Kenyon, 2001; Bredesen, 2004; Longo et al., 2005; Goldsmith, 2014; de Cabo et al., 2014; Jones et al., 2014; Mitteldorf, 2016a). Aging can be seen as a continuation of development and adult maturity leading finally to death, in most cases through degenerative diseases.

Intermediate positions between proponents of programmed and non-programmed aging have also been articulated (de Magalhães, 2012; Lenart and Bienertová-Vašků, 2017; Flatt and Partridge, 2018). We believe that their viability is limited due to contradiction between various PA and non-PA mechanisms and many results obtained from both laboratory and field studies.

Many findings are inconsistent with non-PA wear-and-tear evolutionary theories of aging. The main one concerns the oft-quoted wrong prediction of a strong decline in the force of natural selection with age because old animals would rarely, if ever, be observed in the wild (Medawar, 1952; Williams, 1957). This problem for programmed aging was solved by the finding that old animals do exist in the wild in very substantial numbers (Promislow, 1991; Ricklefs, 1998; Bonduriansky and Brassil, 2002; Moorad and Promislow, 2010, Nussey et al., 2013). Additionally, although mainstream evolutionary theories of aging predict that evolution should inevitably lead to increasing mortality and declining fertility with age after maturity, a study in 46 different species found that there is great variation in the wild, including increasing, constant, decreasing, humped, and bowed trajectories for both long- and short-lived species (Jones et al., 2014). This is very difficult to explain with wear-and-tear evolutionary theory, and it fits well with the concept that aging is differently programmed in different species. Programmed aging theory considers aging to be an adaptation produced by the action of natural selection at the group level, whereas non-programmed wear-and-tear-based evolutionary theories of aging are against a role for natural selection in the evolution of aging.

Concerning disposable soma theory (Kirkwood, 1977), the strong increase in weight-specific metabolic rate as both body size and longevity decrease across species is, rather significantly, exactly opposite to what the theory predicts. Animals of small body size process huge amounts of energy per gram but nevertheless have short rather than long lives. In addition, many mammalian females expend huge amounts of energy in reproduction compared to males (men expend <0.2 % of their24-hour metabolic rate during sexual intercourse) mostly due to the long periods of pregnancy, lactation, and offspring care. However, they do not live for less time than their sexual companions, as predicted by the theory. On the contrary, they even outlive them by around 7 %. Moreover, DR increases longevity while disposable soma theory predicts the contrary due to the strong decrease in energy intake. These problems, plus several other objections (Mitteldorf, 2016a), render it unlikely that aging is mainly due to a lack of energy for maintenance (defence plus repair) as predicted by the theory. Disposable soma theory is based on a theoretical argument without a detailed physiological mechanism that could mediate it. Moreover, it is contradicted by data obtained from animals both in the laboratory and in the wild.

To defeat aging the "small effect" is not enough, and the causes underlying the "big effect" must also be uncovered. The "small effect" is likely controlled by the same aging regulation system (ARS) controlling the "big effect", although in the former case the global ARS pro-aging output should be less intense and/or include a different number or kind (pro- or anti-aging) of aging effectors. The cellular aging regulation system (CARS) is composed of three main parts (Fig. 1) as described in detail in Barja (2019):

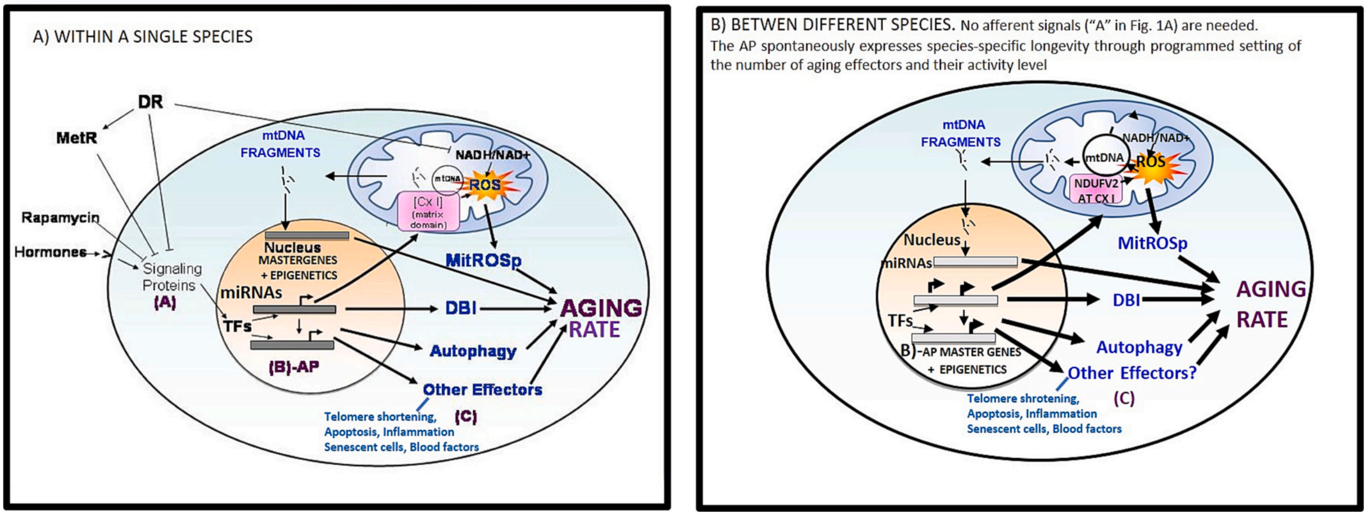

Fig. 1. Cell aging regulation system (CARS). The CARS broadly depicts known integrated mechanisms of cellular longevity control. A (left): Different kinds of dietary restrictions (DR, MetR, and protein restriction) and rapamycin, and other signals coming from the environment, alter humoral, endocrine, and finally cytosolic signalling proteins like mTOR, AMPK, PI3K, AKT, and many others, whose effects are mediated in many cases through TFs like FOXOs, TEFB, and other substances that regulate expression of nuclear AP genes (part B center). The AP output (solid arrows leaving the nucleus on the right side of the figure), in turn, modifies the activity of the multiple *Aging Effectors* (Part C, right) including: (a) ROSp in mitochondria (mitROSp), b) fatty acid double bonds (measured as double bond index, DBI) that stimulate lipid peroxidation, c) autophagy, and d) other likely processes such as apoptosis, inflammaging, proteostasis, telomere attrition, mitBER, etc. There is emerging evidence that epigenetics is also involved in CARS. The integrated response of the CARS to environmental signals modulates **the intra-specific** aging rate by up to 1.4-fold ("small effect"). B (right): An overlapping AP with additional components and wider output activity, composed only of parts B + C in the scheme, could also determine differences in longevity **between different species**. The aging program (AP) spontaneously generates the species-specific rate of aging effector activities, determining the species aging rate and longevity without the need for any afferent stimulus from outside (no part "A" of A), resulting in up to 10^5 fold differences in longevity between species (the "big effect"). TFs = transcription factors. mitBER = mitochondrial DNA base excision repair.
A is reproduced with permission from Barja (2019).

A) Cytoplasmic pre-nuclear signalling (mostly signalling proteins)
B) The nuclear genetic aging program (AP)
C) Post-nuclear aging effectors (executors of aging).

Within single species, extracellular and environmental stimuli including dietary restrictions, longevity extending drugs like rapamycin and or single gene mutants affecting hormonal afferences to the AP can modify the expression of its many target genes (Fig. 2), changing longevity by up to 1.4-fold ("small effect"; Fig. 1A), whereas between species parts B+ C of the CARS are enough to determine longevity because the species-specific level of AP activity sets the aging rate of the species (Fig. 1B). The ARS operates at whole organism level and thus includes both the CARS and the EARS (extracellular aging regulation system). This review complements a previous review dedicated to CARS, and is thus limited to the control of aging at the extracellular and whole organism levels (Barja, 2019).

2. Are epigenetic changes and "clocks" part of the aging program?

In addition to varying their expression in response to cytoplasmic, hormonal, and environmental signals, AP genes can also be modified by epigenetic factors. Epigenetic changes like methylation and hydroxymethylation of DNA, as well as acetylation, methylation, phosphorylation, ubiquitination, or sumoylation of histones can be important factors in aging. Epigenetic marks are responsible for changes in gene expression during cellular differentiation, and in response to environmental stimuli like different kinds of DR and rapamycin (D'Aquila et al., 2013; Yong-Quan et al., 2019). Some authors even support the existence of an "epigenetic clock" (Jones et al., 2015; Mitteldorf, 2016b; Horvath and Raj, 2018; Levine et al., 2020). This would be essentially different from the "epigenetic drift" during aging which corresponds to stochastic changes (Jones et al., 2015). Selecting 353 CpG sites applied to different human tissues and cell types and to the entire human lifespan, it was observed that the "DNAm" (DNA methylation) age strongly correlated ($r^2 = 0.92$) with chronological age (Horvath and Raj, 2018), outperforming telomere length ($r^2 < 0.25$). This "epigenetic" clock emerged as a potential biomarker of aging (Horvath and Raj, 2018).

During aging there is a decrease in global DNA methylation and an increase in local methylation at CpG islands and specific promoters (Jones et al., 2015; Johnson et al., 2012, Sun and Yi, 2015; Pérez et al., 2022). Hypomethylated CpG sites are strongly enriched in the active histone mark H3K4me1, and hypermethylated sequences enriched in the repressive marks H3K27me3/H3K9me3 (Fernández et al., 2015) while expression of the strong repressors miRNAs, which seem to regulate up to 60 % of human protein coding genes (Friedman et al., 2009), also changes during aging (Sierra et al., 2015; Lai et al., 2019). MiRNA-driven epigenetic changes during aging include those in DNA methylation, histone loss or modifications, chromatin remodelling, and loss of constitutive heterochromatin at telomers, centromeres, and pericentromers, and leading to changes in gene transcription (Sen et al., 2016). Analysis of the human epigenome has found widespread tissue-specific differential CG methylation, allele-specific methylation and

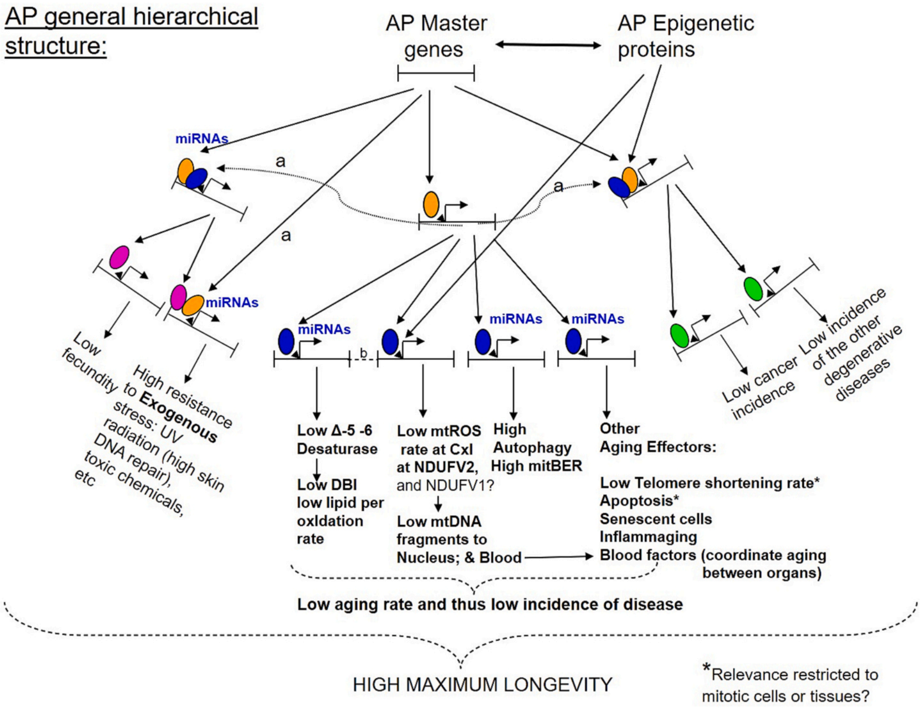

Fig. 2. General proposed structure of the AP. AP target genes producing proteins affecting the endogenous aging rate, degenerative diseases, resistance to external stress, fecundity, and other traits can be organized into clusters working through TF transcriptional cascades and complex interactions. The controller genes (in superior hierarchical levels in space or time) produce regulatory proteins (ovals) that control the graded expression of other genes. Regulatory proteins that contain similar DNA binding sequences are depicted with the same colour. The grouping of genes controlled by similar regulatory proteins shown in the figure is only one of the many possible combinations, and it is arbitrarily shown only as an example. Gene expression is also influenced by other actors (e.g., upstream promoters, enhancers) not shown in the figure, as well as by miRNAs and epigenetic proteins. The graded activation/repression of the target structural genes finally affects aging rate as well as other traits needed for final expression of high longevity (e.g., low incidence of degenerative diseases and high resistance to external sources of stress). Interrelations among genes in the cluster are expected to be much more complex than depicted, and would include crossed regulations both at the horizontal level and vertical levels spanning more than one level per relationship. The real number and kinds of final target genes must be much greater than shown in the figure, and the master genes at the upper control level can be multiple, although their number should be much smaller than the number of target genes. This will strongly facilitate future possible manipulations aimed at greatly increasing maximum longevity. The figure should be considered a highly simplified example of a much more complex network of genetic interrelationships, and not as a precise scheme. According to our present knowledge, the target genes included in the figure should be present in the real cluster, although not necessarily in the sub-clustering tandem positions shown, which were arbitrarily selected from among many possible combinations. The longevity gene cluster, like homeotic genes controlling development, is expected to be highly conserved among animal species, which will strongly facilitate its discovery. For further explanation see text. "a": horizontal or multilevel (in addition to single level) hierarchical interactions, and overlapping of regulatory elements; "b": hypothetical example of two genes clustered in tandem in the same region; "m": master genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Modified from Barja (2008).

transcription, and the presence of non-CG methylation in almost all human tissues, which correlates with tissue-specific functions (Schultz et al., 2015).

Changes in specific histones have also been described during aging, including global increases in H4K20 trimethylation and H3S10 phosphorylation (Thomson et al., 1999; Bártová et al., 2008), which contribute to modulate the rate of aging. Such DNA modifications modulate gene expression through regulation of chromatin structure, which is known to change during aging. Many age-dependent histone methylations are reversed by both DR and rapamycin treatments in mouse brain (Gong et al., 2015; Lim et al., 2022; Ogura et al., 2021). Histone deacetylases like SIRT1, and the mitochondria-specific SIRT3 influence the level of acetylation of corresponding DNA methyltransferases, which, in turn, affects level of DNA methylation and are involved in the decrease in oxidative damage and antiaging effects induced by DR in mice (Someya et al., 2010; Zhou et al., 2022). SIRT3 deacetylates various Complex I subunits (Huang et al., 2010) which could modulate its mitROSp rate. SIRT3 attenuates palmitate-induced mitROSp and inflammation in proximal tubular cells (Koyama et al., 2011). SIRT1 is activated by DR decreasing NADH/NAD+, and specifically targets acetylated histones like H4K16ac and H3K9ac as well as non-histone proteins such as PPARα, and it deacetylates DNA methyltransferase 1 (Feil and Fraga, 2012). Epigenetics seems to influence at least two main effectors of the AP, the mitochondria (Wallace and Fan, 2010; D'Aquila et al., 2015; van der Wijst and Rots, 2015) and the autophagy system (Lapierre et al., 2015). ROS-induced decreases in DNA methylation, up-regulation of DNA methyltransferases, and hypermethylation in promoters have been described (Rang and Boonstra, 2014).

In spite of these advances, the significance of epigenetic changes with age is not well understood, and it is not known which changes in CpG methylation are cause and which are consequence of aging. However, unlike Horvath's original DNAm clock, the "PhenoAge" of Levine and Horvath (Levine et al., 2018) exhibits strong correlations with gene expression that are reasonably assumed to cause damage (Horvath and Raj, 2018). DNA PhenoAge acceleration was found to be associated with increased activation of pro-inflammation and decreased activation of the DNA damage response and nuclear mitochondrial signatures (Horvath and Raj, 2018; Levine et al., 2018). In addition, longer-lived DR, rapamycin-treated, and growth hormone gene KO mice all showed slowed epigenetic hepatic aging (Wang et al., 2017). There is evidence that a single short reprogramming early in life has anti-aging effects (Alle et al., 2022), and human trials using various interventions (DR, exercise, a plant-based diet, metformin, and vitamin D3) showed capacity to slow down or reverse an aging clock suggesting that aging clocks are malleable in humans, although more rigorous clinical trials are needed to validate these initial findings (Johnson et al., 2022). A mammalian methylation array measuring up to 36 k highly conserved CpGs per species has been recently developed that applied to the 200 species studied (Arneson et al., 2022).

Within the perspective of programmed aging, some epigenetic factors could be codified by AP genes. Continuing development after maturity, epigenetic enzymatic proteins controlled by AP genes could methylate/demethylate DNA and modify histones, changing the gene expression of hundreds of AP target genes helping it to increase biological age, e.g. from the 4th to the 5th decade of human life (Fig. 3). In this sense, the AP will be both a genetic and an epigenetic phenomenon. Many aging changes seem to be the result of changes in gene expression. Gene expression would be adjusted late in life, or different AP genes would be turned on or off, in a way that promotes self-destruction. Some will be aging genes (e.g., those that codify for aging-linked mitROSp ay complex I) and other will be pleiotropic genes with altered gene expression in old individuals. In addition, epigenetic factors will also epigenetically modify the activity of the AP master genes themselves situated at different levels in the cluster (Fig. 2), producing a different pattern of changes in target gene expression during the next stage of the lifespan. Repetition of this process will progressively lead the individual towards the end of his lifespan. In this hypothetical model, epigenetics cooperates with genomic DNA in the execution of the species AP (Fig. 3). This makes sense, since chromosomes are constituted by both proteins and DNA, and genetics and epigenetics are profoundly interrelated and cannot be separated from each other. Therefore, it would not be surprising if genetics and epigenetics plus their mutual interrelationships control species aging by *unfolding* the AP during the lifespan. Such control would be easier than development, a process in four dimensions, while aging only proceeds essentially through one, time. Both the proteins responsible for epigenetic changes (methylases, demethylases, sirtuin deacetylases, phosphorylases, etc.) miRNAs, and the AP master and target genes would be involved in programmed aging, and epigenetics would be just another part of the AP (Figs. 1 and 2). This model may help to explain why the pattern of chromatin epigenetic modifications and the biological age are different at different ages in each

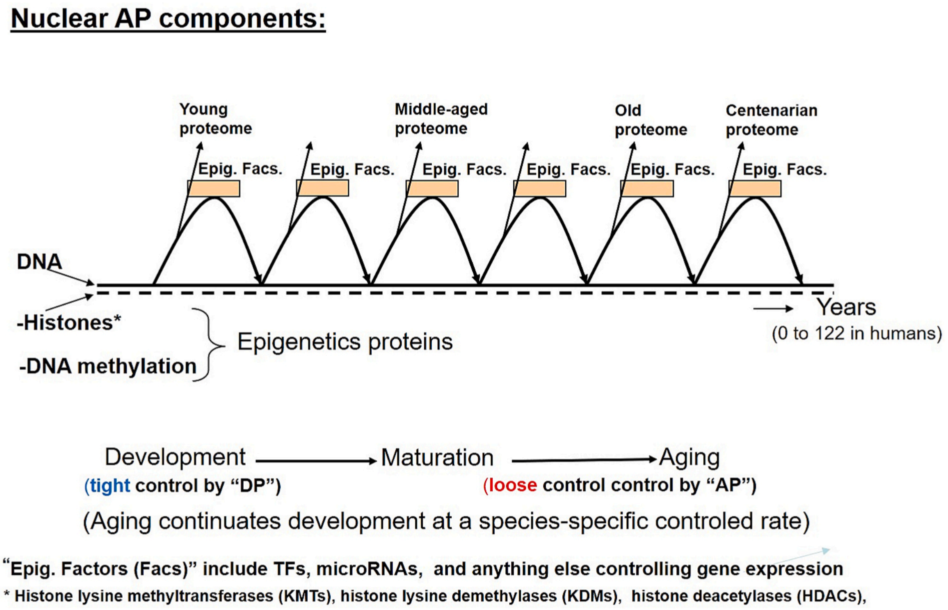

Fig. 3. Epigenetics and aging. The model depicts how the genetic information in the AP and its organization determine the activities of proteins involved in epigenetics, which in turn modify the gene expression of AP target genes, thus helping the AP to generate age changes that carry the individual, e.g., from 25 to 35 years of biological age. The epigenetic proteins also modify the AP itself through DNA methylation/demethylation, histone modification, and changes in chromatin structure. This changes the AP, which then generates a different variation in gene expression of AP target genes during the following decade. In this way, the two components of the chromosomes modify each other: the DNA changing the epigenetic proteins and these in turn modifying the DNA and histones. Repetition of the process brings biological age towards the end of the life span. One decade of life is arbitrarily chosen to exemplify the process. Solid line: DNA. Dotted line: histone proteins. DP = developmental program.

tissue and species.

3. Heterochronic parabiosis reveals systemic pro- and anti-aging factors involved in the extracellular aging regulation system (EARS)

Although a high burden of senescent cells can cause damage to a large area of surrounding tissue, it is unlikely that this local effect directly and systemically affects other organs. There is evidence however, that a putative hormone secreted by brain cells triggered by the mitochondrial unfolded protein response (UPR^{mt}) causes damage far away in the intestine in individuals with brain degenerative diseases (Berendzen et al., 2016; Shen et al., 2022). Cellular aging effectors like autophagy, telomere shortening, etc., all work intracellularly and therefore cannot directly explain damage during aging extracellularly and/or to targets situated far away in other organs. This concerns the extracellular matrix of many tissues and its components like collagen and elastic fibers, glycosaminoglycans, cell adhesion molecules, the avascular cartilage and eye lens, and curtailed irrigation of tendons. Nevertheless, the lens develops cataracts, many bones, ligaments and joints develop arthritis, and the extracellular tissue compartment is damaged during aging in a process related to low grade inflammation (Berenbaum, 2013; Mobasheri et al., 2015; Toba and Lindsey, 2018; Vidović and Ewald, 2022). Why does a rat suffer these pathologies in only two years' time whereas it takes around 60 years for humans to develop them, although the extracellular components affected are made of similar materials in both species? What causes aging in the extracellular compartment and what are the main EARS components? Few definitive answers to these important questions are available.

It is now increasingly appreciated that aging is also systemic. It is more than conceivable that the AP of each individual cell is related to those of other cells through humoral factors. This ensures that the aging rate does not differ too much between different tissues and organs of the same individual. This could also help to explain the results of heterochronic parabiosis experiments in which young and old mice are surgically joined and develop a common vascular system. Early studies of this type, observed increased longevity of old and decreased longevity of young heterochronic parabionts compared to homochronic parabionts, both in males and females (Ludwig and Elashoff, 1972), and found increased longevity of AL-fed mice joined to DR ones (McCay et al., 1957). Resurrection of heterochronic parabiosis in the present century confirmed health benefits for the old parabiont connected to the young one including enhanced myogenesis, hepatogenesis, bone regrowth, neurogenesis, and cognition, and strong detrimental effects for the young parabiont connected to the old one (Fig. 4A). The young parabiont becomes pale, shrivelled, and anaemic, and can die of parabiotic disease (Conboy et al., 2013). Studies of this type concluded that the rejuvenating effects on the old parabiont are due to positive effects of humoral factors coming from the young parabiont acting on the microenvironment of the stem-like tissue cells of the old partner. Thus, improved muscle regeneration and satellite cell activation in muscle of aged mice and enhanced proliferation of aged liver progenitor cells were observed in the old parabiont, and the opposite changes occurred in the young one. The improvements observed in the old parabiont were reproduced by direct addition of young serum to tissue cultures of old cells (Conboy et al., 2005). Studies using young parabionts marked with azido-nor-leucine labelled proteins have identified 70 proteins transferred from the young to the old partner involved in old tissue remodelling like those promoting intercellular interactions, the GDF5 regulator of reinnervation or vascular remodelling factors, proteins with positive effects on muscle cells like IGF-1 and follastatin, and LIF, leptin and cerberus stimulating muscle repair through notch signalling or

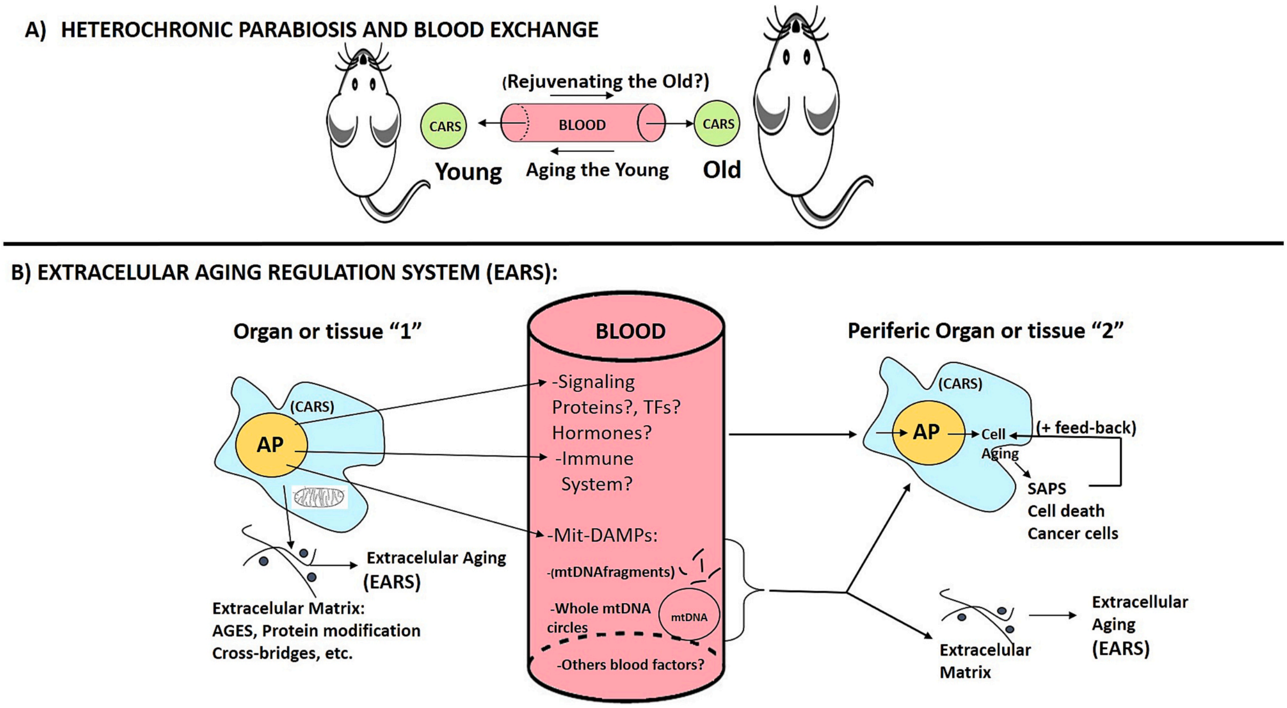

Fig. 4. The extracellular aging regulation system (EARS) hypothesis. A) Heterochronic parabiosis experiments between young and old rodents indicate that substances or cells present in the blood modulate aging of tissues and organs situated far from their sites of production. B) In intact animals, the EARS model hypothesizes that TF-like signalling substances, leukocytes, DAMPs (damage-associated molecular patterns) including cell free mtDNA fragments, hormones, and others are produced/activated in a regulated manner by the AP towards the extracellular space and also enter the bloodstream. Reaching their target, they cause damage to the extracellular space locally or in other organs, or they modulate the degree of expression (through TF-like signalling proteins) of the AP of other cells, even those situated far away in the organism. DAMPs like mtDNA fragments produce extracellular damage during aging by eliciting an immune response, generating inflammaging due to the presence of similarities between mtDNA and bacterial DNA. All the above effects, taken together, can lead to a loose synchronization of the aging rate at the whole organism level and also help explain the rejuvenating and pro-aging effects observed in heterochronic parabiosis experiments (A).

inhibition of TGF-β or Wnt, and leukocyte-specific proteins like interleukins and TNF-β that regulate inflammation (Liu et al., 2017). However it is under discussion whether there are young factors in blood that are missing in old rodents, or blood factors that affirmatively cause damage in the aged ones.

Another experimental approach, heterochronic blood exchange, allows vascular connection of young and old animals without them being surgically coupled, is less invasive, avoids several disadvantages of heterochronic parabiosis, and also demonstrates positive effects on tissues of the old partner (Mehdipour et al., 2022). Using this kind of technique, damage to the brain and muscle was found to be related to increases of B2M protein in these organs, the invariant chain of MHC class I that becomes elevated in inflammation, although interestingly, BM2 concentration did not increase in the shared blood. The authors indicated that not only proteins but also leucocytes could be involved in the negative effects exerted by the old partner on the young parabiont (Rebo et al., 2016).

4. Inflammaging, extracellular aging effectors, and the EARS

Extracellular and systemic effects inducing aging could thus be due to proteins and other kinds of substances that reach cells and the extracellular matrix in the same tissue or even in other organs through the circulatory system (Fig. 4B). In the case of targeting cells in other organs, they could signal their AP to modify gene expression and induce aging, analogously to what happens with CARS (Barja, 2019) in environmental signalling of DRs (Fig. 1A). This could help to coordinate aging rate across organ systems of the same organism, and help to explain aging in the extracellular compartment (EARS). In addition, a provocative hypothesis that cannot be ruled out is that the immune system itself could be involved in the EARS too (Zhang et al., 2010; Picca et al., 2018, 2022; Franceschi et al., 2000; Motta et al., 2022). From the point of view of programmed aging, in order to damage or kill other cells at a distance it is difficult to imagine something more efficient than the immune system, due to its huge capacity for phagocytosis, hydrolytic digestion, lysis, and the killing of bacteria and infected or abnormal cells using highly efficient tools including free radical production enzymes like the NADPH oxidases of the neutrophil plasma membrane. The capacity of NADPH oxidase for ROS generation is large and can increase by one order of magnitude or more its rate of superoxide radical generation during the neutrophile respirator burst, with the potential to spread oxidative stress throughout the organism.

In this respect, from the year 2000 on it has become established that the old individual, although suffering from a decline in immunity, is also characterized by a low-grade chronic inflammatory state. During aging there is an overall decline in immune system efficiency, termed immunosenescence, characterized by changes like involution of the thymus, reduced naive T cells and B cells, and increased memory T cells in the acquired immune system, decreased phagocytic capacity and chemotaxis of neutrophiles and macrophages, impaired dendritic cell function, and reduced cytotoxicity of natural killer cells. Immunosenescence increases susceptibility to infections and malignancy, seems to decrease the response to vaccinations, impairs wound healing, and increases autoimmunity and production of autoantibodies (Baylis et al., 2013; Watad et al., 2017; Fulop et al., 2018; Sanada et al., 2018; Feehan et al., 2021).

Paradoxically, there is also an up-regulation of the inflammatory response with age, resulting in a low-grade chronic sterile systemic proinflammatory state described for the first time at the turn of the century and called inflammaging (Franceschi et al., 2000). Inflammaging is characterized by increased levels, with age, of proinflammatory cytokines like IL-1 and IL-6, tumour necrosis factor (TNF-α), and other substances promoting inflammation produced by both immune and senescent cells (senescence-associated secretory phenotype-SASP) under stimulation of transcription factors like NF-kB (Liang et al., 2022). Inflammaging is associated with oxidative stress and the pathogenesis of age-related diseases like Alzheimer disease, atherosclerosis and cardiovascular diseases, osteoarthritis, muscle sarcopenia, and cancer (Baylis et al., 2013; Motta et al., 2022). Phagocytes like macrophages are hypothesized as being at center stage of inflammaging. It is believed that among the stimuli driving inflammaging are lifetime exposure to antigens, gut microbiota and nutrients, and recognition of damage-associated molecular patterns (self DAMPs) including altered proteins and cell-free mtDNA (cf-mtDNA) and viral and bacterial products called pathogen-associated molecular patterns (non-self PAMPs). DAMPs and PAMPs bind to pattern recognition receptors like Toll- (TLR) and NOD-like receptors (NLR proteins), cGAS and AHR receptors, and Rig-like receptors acting through interferon response elements, present in the nucleus, cytosol, and plasma membrane (Fulop et al., 2018; Franceschi et al., 2018; Zindel and Kubes, 2020). NLR proteins form multiprotein complexes termed inflammasomes, thought to be important drivers of sterile inflammaging, and then recruit caspase-1 enabling proteolytic cleavage of IL-1 family cytokines (Latz and Duewell, 2018). DAMPs release induces chronic inflammation (Zhang et al., 2010), and cf-mtDNA especially is associated with aging, various degenerative diseases, and damage to tissues including skeletal muscle (Picca et al., 2018; De Gaetano et al., 2021). During inflammaging the initial inflammatory response produces tissue damage and ROSp that cause further tissue damage and the release of additional cytokines, resulting in a vicious cycle favouring a chronic proinflammatory state leading to pathophysiological changes (Baylis et al., 2013). Cell damage triggers the mitochondrial release of cf-mtDNA, N-formyl peptides, TFAM, cytochrome *c*, and lipids such as cardiolipin that can act as DAMPs by activating the receptors of innate immunity in various cell types like neutrophiles, adipocytes and endothelial cells.

cf-mtDNA are fragments of mtDNA within the extracellular space, mtDNA inside vesicles (Giuliani et al., 2017; De Gaetano et al., 2021), or even extruded whole mitochondria (Thurairajah et al., 2018). They trigger inflammation due to their similarity to bacterial DNA since mitochondria originated from bacteria during evolution of the eukaryotic cell through symbiogenesis. During that evolution mtDNA decreased in size but retained the circular structure and abundant unmethylated CpG motifs similar to bacterial genomes which activate receptors like TLR9 present both in lysosomes and on the neutrophile surface, leading to NF-kB signalling and increased expression of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β (Giuliani et al., 2017; Sanada et al., 2018). mtDNA also binds the cytosolic cGAS playing a prominent role in cell senescence by inducing the SASP phenotype, and binds STING (stimulating of interferon genes) protein at the endoplasmic reticulum, activating cGMP synthase, resulting in downstream pro-inflammatory interferon secretion and also contributing to NLRP3 inflammasome generation (Picca et al., 2017; Thurairajah et al., 2018; Xu and Núñez, 2022). Active and passive mtDNA release is closely linked to mitROSp and subsequent mtDNA oxidation (8-oxodG) and fragmentation during aging (Picca et al., 2017; Aucamp et al., 2018; Storci et al., 2018). This is consistent with the concept that mitochondrial dysfunction, increased mitROSp, and deficient autophagy can lead to inflammation, apoptosis, necrosis, and cell death (Green et al., 2011; Picca et al., 2022).

Mitochondrial signalling seems essential for induction of innate and adaptive immunity through the stimulatory effects of mitROSp and mitochondrial DAMPs on cytokine release, inflammasome activation, and sterile inflammation, as well as regulation of both T and B cells (Weinberg et al., 2015; Picca et al., 2017), and mitROSp can also activate the inflammasome (Giuliani et al., 2017). Importantly, mtDNA fragments are released in the extracellular fluid as a product of mitochondrial damage, and its plasma levels increase during aging (Pinti et al., 2014; Franceschi et al., 2018), lending support to the idea that circulating mtDNA is part of a systemic signalling constellation that initiates and propagates inflammaging (Picca et al., 2022). A great amount of cf-mtDNA is found in the blood in various diseases as well as in normally aged individuals; it has a high pro-inflammatory capability

(Franceschi et al., 2018), and is an independent predictor of mortality in the elderly (Storci et al., 2018).

In recent years, a role for miRNAs in inflammaging has also been emerging. These are small noncoding RNAs that modulate gene expression by posttranscriptional modification of mRNAs through inhibitory binding to their 3′-untranslated region, although in some cases they can also activate gene transcription or stabilize the mRNA. A single mRNA can be targeted by several miRNAs, while a single miRNA can regulate multiple target mRNAs. miRNAs are involved in the regulation of almost all kinds of cellular functions. There are various miRNAs with a recognized role in development, aging, and inflammaging, like miRNAs 146a-5p, 21, 570, and126a-3p (Olivieri et al., 2021). Inhibition of miRNA-570 reverses oxidative stress-induced cellular senescence of lung airway epithelial cells by restoring the antiaging molecule sirtuin-1 (Baker et al., 2019), as demonstrated by suppression of the $p16^{INK4a}$, $p21^{Waf1}$, and $p27^{Kip1}$ markers of cellular senescence and suppression of SASP phenotype (matrix metalloproteinases-2/9, C-X-C motif chemokine ligand 8, IL-1β, and IL-6). miRNAs can also affect inflammaging through interaction with Toll-like receptors and their downstream signalling to NFkB activation (Olivieri et al., 2013).

Interestingly, various miRNAs have been found inside mitochondria or indirectly affecting them. Thus, miRNA-181c-5p targets mitochondrial COX I, promoting mitROSp, while miRNA-1 localizes inside mitochondria, promoting translation of ND1 and COX1 mtDNA-encoded transcripts and enhancing ATP production. Many other mito-miRNAs target the mitochondrial genome, promoting inflammation, aging, and many aging-related diseases (Giuliani et al., 2017). miRNA-146a-5p is one of the best described miRNAs involved in the SASP phenotype and inflammaging that targets ND1-ND6 mitochondrial complex I polypeptides, ATP8, SOD, and Bcl-2. Thus, many miRNAs seem to be implicated in the development of SASP by epigenetically modifying expression of mitochondrial proteins due to interaction with nDNA- or mtDNA-coded mRNAs in the cytosol or inside the mitochondria themselves.

In response to the apparent paradox that inflammaging occurs during aging together with immunosenescence, evolutionary explanations have been proposed. Thus, inflammaging has been hypothesized to result from antagonistic pleiotropy, although the basis for this was not detailed (Goto, 2008). Also in this line, it has been proposed that adaptive coevolution of immune responses to pathogens, nutrients, gut microbiota, and DAMPs throughout life could lead to inflammaging in the elderly (Franceschi et al., 2018). But these possibilities cannot explain the huge difference in the number of years needed to damage extracellular components in species with similar extracellular composition but widely different longevities. This can, however, be understood by hypothesizing that inflammaging and extracellular damage are also under the control of programmed aging, analogously to intracellular aging-related damage (CARS).

The AP promotes damage not only inside but also outside cells. The results of parabiosis experiments may also be due to TF-like signalling substances traveling in the bloodstream, promoting aging or rejuvenation through their effects on the AP of cells situated in other tissues and organs (Fig. 4B). In addition to TFs, the immune system could also be used to promoting aging of the organism through the damaging effects of the different kinds of leukocytes infiltrated in the tissues and/or their secreted cytokines, chemokines, and other substances promoting inflammaging and activating resident cells like macrophages. Mitochondrial and tissue damage also helps to inflict this wider damage though DAMP production which includes extracellular and vascular mtDNA fragments. Thus, tissue extracellular signalling substances, TFs, leukocytes, DAMPs, and many other agents likely to be discovered as being produced or stimulated in a controlled way by the AP would constitute the EARS (Fig. 4B). In order to promote damage, nothing more powerful could be used than the immune system with all its powered armaments to promote cell and tissue damage. Looking at it in this way, inflammaging is no longer a paradox and fits well within the logic of programmed aging. If this is correct, autoimmunity should not be restricted to a few diseases, since all aged individuals would be subject to chronic low grade sterile "autoimmunity" under the control of EARS. The EARS could also explain why it takes only two years for the eye lens of a rat to develop cataracts and the extracellular matrix in joints to develop arthritis, while these processes require 60 years in humans even though these organ components are made up of similar materials in the two species. Neither the direct action of mitROSp, double bond index, autophagy, telomere shortening, or apoptosis can directly explain this fact because all these aging effectors act intracellularly. This is even more evident in regions devoid of cells and blood vessels like the eye lens, which, however, can suffer from inflammatory uveitis, meaning that the immune system can reach its protein crystallins. The AP can however reach all these extracellular regions through the EARS. The EARS hypothesis further reinforces the relevance of mitochondria and free radicals in aging, because it would cause intracellular damage through the cell damaging effects of mitROSp while the resulting mtDNA fragments would cause both intracellular damage (insertions in nDNA) and extracellular damage through the long-range action of mtDNA fragments (DAMPs) traveling in the extracellular fluid and the blood circulatory system. Analogously, ROS would induce aging both intracellularly at the inner mitochondrial membrane and extracellularly at the powerful ROS generating leukocyte NADPH oxidase of the cell plasma membrane.

The EARS hypothesis is also supported by the evidence that both caloric restriction and epigenetics are involved in controlling inflammaging. Anti-inflammaging effects have been observed in rodents subjected to caloric restriction (Masternak and Bartke, 2012; Franceschi et al., 2018; Pan et al., 2018; Kim et al., 2020). Epigenetic changes promote inflammaging through changes involving sirtuin 1 (Yao and Rahman, 2012; Hwang et al., 2013; Pan et al., 2022), sirtuin 6, and NF-kB (Chen et al., 2015;Klimczak and Śliwińska, 2022), heterochromatin loss (Nardini et al., 2018), histone modifications (Cheng et al., 2018; Evans et al., 2020) and histone-dependent upregulation of SASP genes in senescent cells (Sen et al., 2016), hypomethylation of Toll receptors and DNA methylation and histone acetylation-dependent regulation of TNF-α (Ray and Yung, 2018), and epigenetic control of trained immune memory (Fulop et al., 2018).

Other substances that could be involved in coordinating aging changes between organs are hormones. The local effects of damage to individual tissues can directly affect other organs systemically via endocrine regulation. That coordinates the functions of all organs via action of hormones that move through the bloodstream from the sites of synthesis to the target organs where they can also modulate AP gene expression.

5. Is there a master clock that controls organism aging?

Finally, some authors have proposed that circadian clocks in the brain might also be connected to timing of aging on a longer scale (Chen et al., 2021; Zhu et al., 2022). Areas of the brain that control circadian rhythms could also house aging clocks (Mitteldorf, 2015, 2016b; De Nobrega et al., 2020; Lananna and Musiek, 2020; Buijink and Michel, 2021). Circadian, seasonal, and annual biological rhythms are present in mammals and other animals. Circadian rhythms are known to occur also in isolated tissue cells cultivated in vitro, but in the case of mammals they are coordinated by a central higher-order master circadian clock situated in the hypothalamic suprachiasmatic nucleus. Seasonal and annual rhythms are under the control of the suprachiasmatic nuclei plus the pituitary *pars tuberalis*. Such higher-order coordination ensures that the different clocks of the different cells in the body are synchronized and do not run at different speeds which would render the system useless. Since the three kinds of biological rhythms are controlled at the hypothalamic-pituitary level it would make sense that control of the hypothetical missing fourth biological "rhythm", the aging rate—the rhythm of life having a single cycle—would also be located in that area.

If this is the case, a hierarchy of aging clocks can be conceived, with myriads of cellular aging clocks (APs) commanded by a single hypothetical master clock situated in the hypothalamus. This brain clock would tend to synchronize the pace of aging in the different organ systems, by coordinating the multitude of APs of all the individual cells (Fig. 5). This higher-order aging clock would thus be an upstream regulator of the ARS.

However, various facts argue against this possibility. On the one hand, in many ectothermic animals including vertebrates, circadian rhythmicity is not so centralized under superior tight control of a single central clock, like the hypothalamic suprachiasmatic nucleus of mammals. Instead, the control of biological rhythms is more diffusely distributed among various clocks in peripheral tissues, the hypothalamus-pituitary axis in the case of ectothermic vertebrates playing a less prominent role. Aging occurs in most animals, both endothermic and ectothermic, at well-defined species-specific rates. If a single centralized master aging clock exists exclusively in mammals and birds and not in many other animals, different organisms would regulate species longevity in different ways. In ectothermic animals longevity also varies with environmental temperature, high temperatures being usually associated with faster aging rates. In addition, in mammals there is evidence that different organs can age at substantially different rates within the same individual. This argues again that coordination of aging rates across the body is under *loose* control at the whole organism level. Furthermore, a single master aging clock would be more easily eliminated by individual selection. This could be circumvented by the existence of multiple middle hierarchy aging clocks (Fig. 5) instead of a single one (Mitteldorf, 2015).

The EARS can help to loosely coordinate the APs of the different cells in the body due to its long-distance action through substances and cells traveling in the blood circulatory system, which could help to eliminate the need for a single master clock in the brain. These aging-related blood factors can avoid excessive differences in aging rate among the different cells in the body while some variation in aging rate in the different organs systems is still allowed. These differences could even be adaptive, by promoting the death of organisms at species longevity-related ages but not at a too-tightly controlled similar age in all individuals. Variability in the age at death could be adaptive in various ways including helping individuals with advantageous traits to leave more offspring, or helping to stabilize populations in the ecosystem.

6. Conclusions

1. Programmed aging and the CARS model can reconcile the previously considered independent mechanistic “theories of aging” into a single unified theory. The so-called “hallmarks” of aging would then be effectors (executors) of the nuclear aging program, the main core of the CARS.
2. The CARS model can explain relatively slight modulation of longevity within species induced by environmental signals (DRs) or single gene mutations changing AP gene expression (part A in Fig. 1). A partially overlapping but larger AP could control the huge variation in species longevity. To determine such longevity the CARS needs only the AP and its aging effectors (parts B and C in Fig. 1), since their level of gene expression and spontaneous output activity is constitutive and species-specific and does not need any afferent signal stimulus to the AP to express itself.
3. Further research is sorely needed to clarify whether epigenetics is part of the AP and what the main aging effectors acting at extracellular and systemic levels are.
4. Results of experiments in heterochronic parabiosis and blood exchange attest to the existence of signal molecules in blood plasma that regulate (or coordinate) aging.
5. The existence of inflammaging even as other aspects of immune protection are declining maybe explained as a controlled extracellular effect of the aging program modulating particular aspects of immunity as a function of age and the pro-aging and anti-aging systemic factors flowing in the blood and extracellular fluids.

CRediT authorship contribution statement

G.B. conceptualized and wrote the outline of the manuscript; J.G., M. J., R.P., and G.B. did the investigation; R.P. and G.B. wrote, reviewed and edited the manuscript. All authors have participated in, read, and

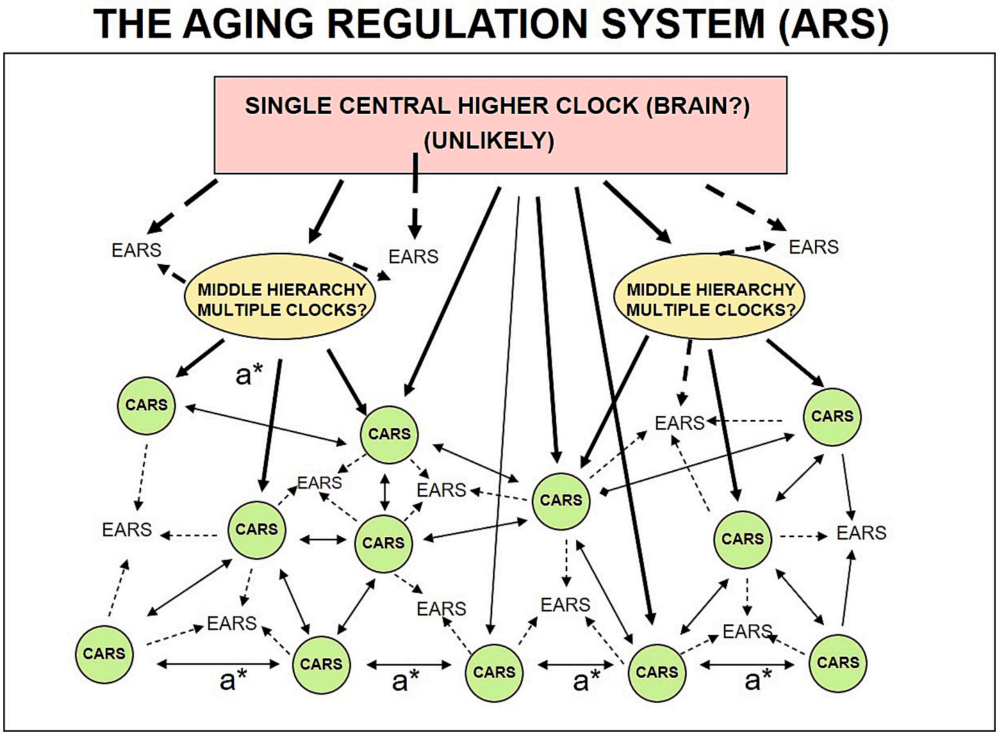

Fig. 5. Hypothetical scheme controlling whole organism aging: the ARS (aging regulation system). Although it is known that organs within a single individual do not age at exactly the same rate, there is a well-defined species longevity. Therefore, *loose* coordination of aging rate among the different cells and tissues is needed to ensure such longevity. The ARS includes both the CARS and the EARS, interrelating the two at the tissue and systemic levels. In addition, higher order master aging clocks, hypothesized as being situated in the brain and/or other organs, ensure further coordination of the aging rate and species longevity at the whole organism level. a* = blood or interstitial fluid factors affecting CARS of other cells in the same tissue or in other far away situated organs.

approved the final manuscript.

Declaration of competing interest

The authors declare no competing interests.

Data availability

No data were used for the research described in the article.

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Consent for publication

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