CHAPTER

1

Biomarkers and Inflammatory Network in Aging: Targets for Therapies

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INTRODUCTION

Aging is recognized as a complex process, induced by intricate interactions between genetic, epigenetic, stochastic, and environmental factors. These factors contribute to a loss of molecular fidelity that results from the random accumulation of damage (particularly to nuclear and mitochondrial DNA) at the cellular, tissue, and organ levels and/or to the whole body, compatible with the "disposable soma" theory of aging [1]. This theory states that both the architecture and functioning of physiological processes and regulatory (*immune and endocrine*) systems are modified during aging, which leads to a deterioration of homeostatic capacity. In elderly people, induced homeostatic processes show increased amplitudes and take longer to return to baseline. Accordingly, elderly people are more vulnerable to internal and external stressors, frailty, disability, and disease. In addition, the decline in DNA integrity, one of the main types of random damage that reduces cellular fidelity and induces cellular senescence, is caused by altered or lack of expression of stress resistance and survival genes that are involved in the cellular and organismal defense to environmental stresses, and which maintain homeostasis [2]. However, wide variations have been observed in the occurrence, complications, speed, and age- and gender-specific manifestation of the aging process occurring at the cellular, tissue, and organ levels, and/or in the whole body both within and between individuals of the same species or of different species. In humans, there are individuals aged ≥90 years who are still in good mental and physical condition, and others who show cognitive difficulties and/or the onset of chronic inflammatory diseases, such as Alzheimer disease (AD), cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM), and cancer at age ≥60 years [3].

The large heterogeneity in the aging rate in humans has been ascribed to genetic and different environmental factors. However, the overall impression is that environmental factors are the major determinants of both aging and age-related diseases [3,4]. No genetic program has emerged to explain the aging process [4]. This conclusion is based on studies on the heritability of age-related diseases and aging. In particular, they suggest that the heritability of age-related disease is similar to current estimates of the heritability of life expectancy [5,6]. Population-based and twin studies on late-onset disorders such as AD, cancer, CVD, and T2DM indicate that heritability is less than 40% [6]. Lifespan studies in worms and mice suggest heritability to be 10–35% [5]. Most literature reviews on human life expectancy are based on Scandinavian twin studies that estimate heritability at about 25–33% [7,8]. However, this relatively small genetic contribution does not imply that genes are irrelevant. On the contrary, modern genetic techniques identified mutations in familial forms of AD that have helped to unravel the molecular mechanisms of disease, such as the toxicity of amyloid beta peptide and potential therapeutic targets in more common sporadic late-onset AD [9]. Thus, genetic contributions to aging and diseases of later life are probably complex and the effects of individual genes are probably weak [4]. Furthermore, there is a distinction between the genetics of aging and exceptional longevity. Human genome-wide genetic analyses have revealed only a few age-related loci and polymorphic longevity genes [10–12]. Among these,

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current promising candidates are sirtuins and forkhead box O proteins (FOXOs), and the field of epigenetics. Functional genomics has revealed a group of genes that are differentially expressed in aging, such as immune/inflammatory genes [13].

Another critical point emerging from the above observations is that in humans biological age rather than chronological age is a better determinant of both the aging rate and onset of the common diseases of later life [14]. This concept opened an important area of research focused on addressing the complex question of whether aging should be considered the "cause or effect of disease," and, consequently, eliminating the confusing influence of disease from research into aging. With the aim of resolving this dilemma, over the last few decades gerontologists have focused their efforts on measuring biological aging by identifying potential molecular targets as *biomarkers* of human aging [15]. On the one hand, this might finally cast light on the paradigm "aging: a cause or effect of disease" and, on the other hand, it could identify potential treatment strategies. The hypothetical treatment of aging could retard or prevent age-associated diseases, resulting in widespread health and social and economic benefits. Such treatments could include genetic engineering, such as gene therapy or endogenous gene repair, pharmacological therapies, or changes in lifestyle.

Many of these aspects are summarized in this chapter. Particular emphasis is given to describing the cellular and serum biomarkers of inflammation. In particular, the data discussed in this chapter are based on expert opinion derived from the author's findings derived from studies on age-related diseases and inflammation.

AGING BIOMARKERS: DEFINITION AND SELECTION CRITERIA

To date, when one talks about a biomarker, one refers, as established officially by the National Institutes of Health, to a "feature objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [16].

In the case of the aging process, this definition might concern measures related to physical changes, such as gray hair, reduced skin elasticity, wrinkles, reduced muscle strength, or changes affecting near vision, which are thought to result from molecular mechanisms occurring in old age [17]. However, these changes reflect chronological age rather than biological age. Biological age represents the most important indicator of health and potential lifespan [14]. Consequently, in considering these changes as aging biomarkers, the problem of measuring the real age of an individual remains. A biomarker of real aging should preferably reflect a process associated with aging, be easily reproducible in cross-species comparisons, and be easily obtainable. In addition, more than one biomarker of aging should be considered, since aging is assumed to be the consequence of deterioration of more than one system or process. This assumption leads to the decision to preferentially use "panels" of biomarkers associated with conditions, alterations, or changes to a set of critical systems to assess the biological age of any organism [15,17].

Gerontologists began to face this problem in the early 1980s, with the development of a large number of aging biomarkers [15,17]. Despite numerous efforts and the support of this research by the National Institute of Aging, to date most biomarkers, including inflammatory markers, hormones, markers of oxidative stress, and telomere shortening are still under discussion [15,17]. In addition, most (perhaps all) markers have not been supported by longitudinal studies in humans. Moreover, they have been developed for a variety of purposes, which are not sufficiently defined. Most investigators have used biomarkers as tools for comparing rates of aging between different populations or between subgroups of a single population. In contrast, others have sought biomarkers for identifying individual predisposition to aging. The latter is much more challenging, principally because aging, as a biological process, is not well defined at the individual level. Furthermore, searching for comparative or predictive biomarkers has resulted in the attempted use of panels of measures associated with survival, healthy old age, frailty, and age-related (multi)morbidity and mortality [15,17]. Classic examples of these panels are indicators of physical function, body mass and composition, inflammation, endocrine function, and micronutrient status.

Besides, none of the identified biomarkers is a "true" biomarker of aging: most biomarkers are related not only to aging but also to diseases. Several biomarkers have indeed been developed and tested for conditions for which biological age is the single biggest risk factor, such as peripheral blood cellular telomere length, which is an indicator of immunosenescence and does not correlate with disease-specific diagnoses. In addition, biomarkers of age-related diseases and aging have been documented only in young-old populations (typically aged 60–85), and not in the oldest old (aged 85 and above) [15,17]. For example, it has been demonstrated that blood pressure, indicators of metabolic syndrome, and telomere length do not associate significantly with age-related morbidity or mortality in population-based studies of the oldest old [18–20]. Thus, in general, the utility of biomarkers of aging and age-related diseases for understanding the health trajectories of the oldest old is unexplored territory. It is important that this gap is filled, given the rapid growth in the number of very old people in many contemporary populations.

TABLE 1.1 Selection Criteria for an Aging Biomarker

1	It must predict the rate of aging. Operationally, it must be a better predictor of lifespan than chronological age alone.
2	It must monitor a basic process that underlies the aging process, not the effects of diseases.
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 both humans and laboratory animals. So, it must be tested in laboratory animals before being validated in humans.

In order to clarify, it is important to know not only how such a biomarker is defined, but also the criteria for its selection. Accordingly, the American Federation for Aging Research has proposed detailed criteria, which have been recently reviewed by Johnson [17] and Sprott [15] (see Table 1.1). Based on these criteria, a true biomarker of aging, in order to be both accurate and useful, should predict a person's physiological, cognitive, and physical function in an age-related way. At the same time, it should be easily testable and not harmful to test individuals. For example, it could be a blood test or an imaging technique that can be performed accurately and reproducibly without the need for specialized equipment or techniques. Preliminary testing should be done in laboratory animals, such as mice, and then in humans. Thus, a biomarker needs to be simple and inexpensive to use. It should cause little or no pain or stress [15,17].

Furthermore, current clinical and basic research into aging biomarkers is designed to exchange knowledge and resolve differences between these fields by making comparisons between clinical and basic research data. On the other hand, biomarkers represent a hot topic and have the ability to change our lives, if real predictions about individuals are made possible in the future.

INFLAMMATORY NETWORK: A DESCRIPTION AND ITS BIOLOGICAL EFFECTS ON AGING

The immune system has evolved to defend the host against microbial invasion, and to counteract tissue damage elicited by chemical or physical agents or trauma, thus maintaining tissue homeostasis and repair [21]. In both conditions, it is able to respond appropriately by inducing appropriate reactions, *the inflammatory responses* [21,22]. These can be induced under different stimuli and can be initially evoked as localized tissue reactions and subsequently as systemic cytokine-induced reactions [i.e. leukocytosis, fever, somnolence, anorexia, activation of hypothalamic-pituitary-adrenal axis, increased level of glucocorticoids, and acute-phase synthesis, e.g. of C-reactive protein (CRP) in the liver]. These characterize the so-called "*acute-phase reaction*." Inflammatory responses are orchestrated by a complex network of molecules (the *mediators*) and cells, which work together to mediate the activation of different signaling pathways and the expression and transcriptional regulation of hub genes. Such hub genes receive and direct the activity of many other genes [21,22].

Recent studies on the topology of the inflammatory network suggest a key role for some mediators in driving the different cellular interactions and regulating the type of inflammatory reaction. Several pro- and anti-inflammatory mediator molecules are involved [23]. Their release is modulated by different factors, such as the type and load of the inflammatory agent, and the activation of different receptor sensors and signaling pathways, such as the well-known nuclear factor (NF)-κB pathway [24,25]. In addition, the magnitude of their production has been demonstrated to exhibit inter-individual variation resulting from genetic heterogeneity. Single nucleotide polymorphisms (SNPs) in several genes and epigenetic factors are involved in regulating the pattern of inflammatory mediator activation [26]. Among the inflammatory mediators, tumor necrosis factor (TNF-α), interleukin-1 (IL-1), and IL-6 have important roles. These are classic proinflammatory cytokines involved in inducing both local and systemic effects. Locally, they contribute to both activation and local recruitment of inflammatory cells by working together with chemokines, which induce expression of adhesion molecules [27]. When inflammatory reactions are caused by high-intensity stimuli, the production of cytokines is increased and they are released into the circulation, thus provoking the acute-phase response. In contrast, anti-inflammatory cytokines, such as IL-10, damp down the activity of inflammatory cells by inhibiting the release of proinflammatory cytokines and therefore turning off inflammatory processes [28].

If tissue health is not restored in response to stable low-grade irritation, inflammation can become a chronic condition that causes continuous damage to the surrounding tissues. In fact, during chronic inflammatory immune responses, tissue injury and healing proceed simultaneously. The collateral damage caused by this type of inflammation usually accumulates slowly, sometimes asymptomatically for years, but can eventually lead to severe tissue deterioration [29].

The inflammatory response is not a negative phenomenon per se. It has evolved to neutralize infectious agents by playing a beneficial role until the time of reproduction and parental care. In contrast, in old age, a period largely unforeseen by evolution, it can lead to a detrimental effect through chronic inflammatory responses ("antagonistic pleiotropy") in several/all tissues and organs; these are the cause of both the aging phenotype and chronic diseases [23,29]. Low-level, chronic

inflammation, or "*inflammaging*," is characterized by a two- to fourfold increase in serum levels of inflammatory mediators and accompanies the aging process [30]. It can be used as a predictor of mortality and, as mentioned above, it is recognized as a critical risk factor in the pathogenesis of several age-related chronic diseases such as AD, CVD, frailty, functional disability, T2DM, and sarcopenia [31–33].

Several contributory factors have been associated with low-grade inflammatory activity in elderly populations, including augmentation of age-related body fat and a consequent increase in visceral adiposity, age-related decline of sex hormones, oxidative and genotoxic stress, cellular and tissue damage, changes in nutrition, alterations in the physical condition of gut microbiota, and changes to other organs (brain and liver) and systems (immune and endocrine) [31–33]. In addition, factors that promote physiological stress, such as long-term smoking and depression, also seem to contribute to elevated inflammation [31–33]. However, the most important factor affecting age-related inflammation is the life-long pathogen burden [29]. Accordingly, some recent studies have linked an individual's exposure to past infection to levels of chronic inflammation and increased risk of heart attack, cancer, and stroke [31,33]. In contrast, some evidence suggests persistent peripheral multibacterial infection, such as periodontitis, associated with gram-negative anaerobic bacteria capable of exhibiting localized and systemic infections in the host, as a possible aggravating cofactor in subjects with vascular diseases and as a risk factor for the onset of other age-related diseases, such as AD [29].

Of special note is *inflammaging* in centenarians, whose data seem paradoxical. Centenarians have increased levels of both inflammatory and anti-inflammatory mediators and significantly different frequencies of protective genotypes from old subjects [29,34]. Therefore, it is necessary to identify appropriate molecular targets as biomarkers likely to be present in long-lived subjects and capable of simultaneously influencing different organs of the body with pleiotropic characteristics. This might permit a preferential and selected development of pleiotropic therapeutic interventions capable of acting concomitantly on different targets and at different levels.

CELLULAR INFLAMMATORY BIOMARKERS

As above mentioned, aging is not a genetically programmed process [4]. On the contrary, it is considered to be an entropic process, involving the loss of molecular fidelity and subsequent accumulation of waste products [2,35]. In addition, it is now thought that during evolution the host defense and the aging processes have become linked [2]. As a consequence, host defense mechanisms seem to be involved in the aging process and activate the inflammatory network to induce the so-called *senescence-associated secretory phenotype* (SASP), which is represented by a myriad of factors, including proinflammatory mediators [36]. A large array of defense factors and mechanisms characterize the inflammatory network and all (or most) are linked to the NF-κB system, an ancient specialized signaling pathway involved in host defense [24,25]. In particular, the NF-κB system is a cytoplasmic sensor that can be activated by both immune attack and a plethora of external and internal danger signals, such as a oxidative and genotoxic stress and tissue injuries [24]. Thus, the NF-κB system is at the hub of the aging inflammatory network; NF-κB activators are pro-aging factors. Sustained NF-κB activation seems to be associated with advancing age. As a result of harmful responses (e.g. chronic inflammatory responses, increased apoptotic resistance, decline in autophagic cleansing and tissue atrophy), sustained NF-κB activation can elicit a host defense "catastrophe" (by activating several inflammatory and entropy pathways) that improves both the aging process and the risk of developing age-related degenerative diseases (Fig. 1.1) [2,36,37]. In light of this evidence, research into aging has focused on identifying pro-aging factors, the "NF-κB activators," as hypothetical cellular inflammatory biomarkers. Some of these will be described next.

Mitochondrial Dysfunction, Oxidative Stress, Activation of Inflammasomes, and Decline of Autophagic Cleansing

Upon aging, mitochondrial alterations are observed, including increased production of oxidation molecules and diminished functional activity; this condition is termed *dysfunctional mitochondria* [38,39]. An increase in mitochondrial oxidation seems, indeed, to accompany aging, with protein carbonyls, thiobarbituric acid reactive substances, ROOH, and 8-hydroxy-2′-deoxyguanosine being the major markers. Mitochondrial dysfunction and oxidative stress are, hence, associated with the aging process, but also with the pathogenesis of several diseases, i.e. metabolic and neurodegenerative diseases. These effects are commonly attributed to disturbances in energy metabolism, increased reactive oxygen species (ROS) production, and the crucial role of mitochondria in apoptotic cell death. In addition, mitochondria dysfunction and oxidative stress seem to provoke and potentiate inflammatory responses, even if the mechanisms remain elusive [38,39]. However, recent findings provide evidence for a crucial role for mitochondria in the regulation of innate immunity/inflammatory responses through different mechanisms [40,41]. The first is mediated by ROS, which can induce

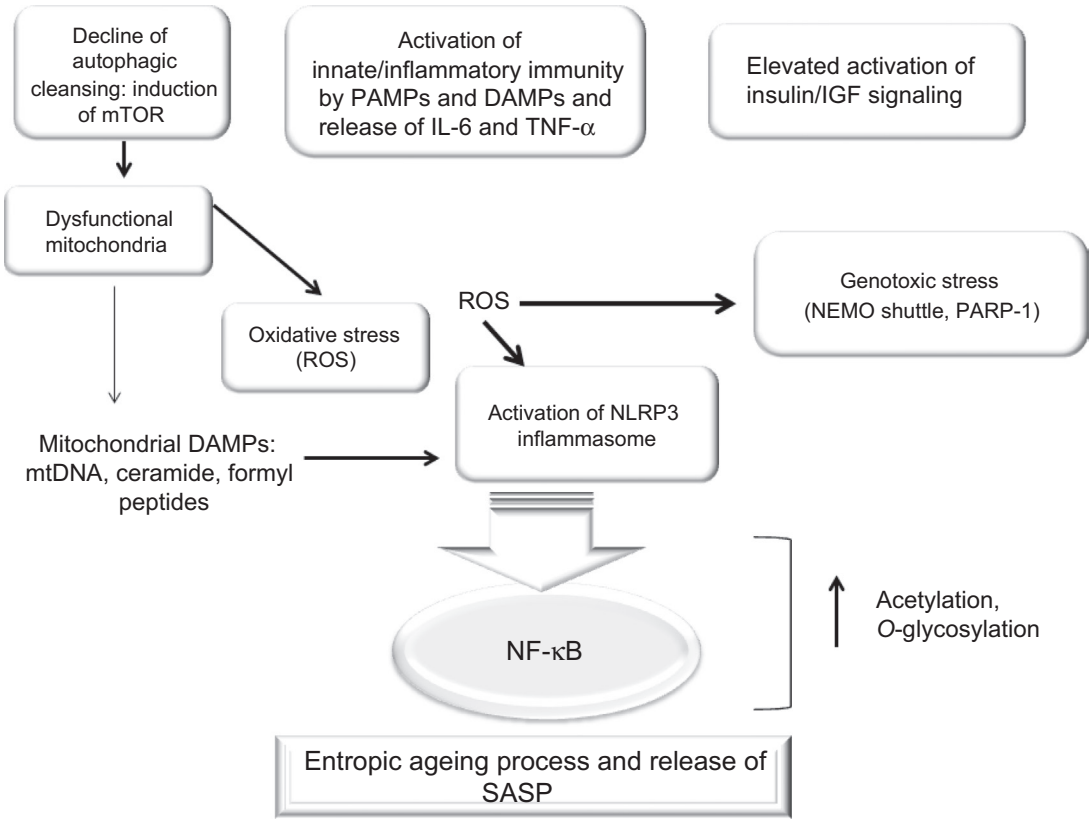

FIGURE 1.1 **NF-κB system activation is at the hub of the aging inflammatory network.** NF-κB system activation is induced by different factors, such as mitochondria dysfunction, oxidative stress, activation of inflammasomes, decline of autophagic cleansing, activation of innate/inflammatory responses by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), elevated induction of insulin/insulin-like growth factor I (IGF-I) pathway, acetylation, *O*-glycosylation of components of the NF-κB pathway, and genotoxic stress. The NF-κB system induces the aging process and release of the senescence-associated secretory phenotype (SASP). IL-6, interleukin-6; NEMO, NF-κB essential modulator; NLRP3, Nod-like receptor protein 3; PARP-1, poly (ADP-ribose)-polymerase 1; ROS, reactive oxygen species; TNF-α, tumor necrosis factor.

the assembly of multiprotein inflammatory complexes called "*inflammasomes*" [38,39,42]. Nod-like receptor protein 3 (NLRP3) is a component of these complexes and is a major sensor of cellular stress signals, e.g. ROS. Its activation triggers the caspase-1-mediated maturation of precursors of the IL-1β and IL-18 cytokines [42]. Thus, an endogenous stress-related inflammatory response is induced, termed "*para-inflammation*" by Medzhitov [43]. However, the exact mechanism involved in ROS-induced NLRP3 activation is still unclear. Zhou and colleagues demonstrated that oxidative stress can activate NLRP3 inflammasomes via redox regulation of the thioredoxin/thioredoxin-interacting protein balance [44]. It is also possible that ROS directly oxidizes thiol groups in the leucine-rich repeat domain of NLRP3 to activate the inflammasomal pathways. In addition, mitochondria (1) are involved in the control of antiviral RIG-like receptor signaling pathways; (2) contain NLRX1 receptors, which monitor ROS production; and (3) secrete several damage-associated molecular patterns (DAMPs) following loss of their integrity, such as ROS, ceramide, mitochondrial DNA, and formyl peptides, which can provoke local and systemic para-inflammatory responses by inflammasomes via NLRP3 [40,41].

All of these observations emphasize that disruption of mitochondrial integrity and a deficiency in cellular housekeeping can trigger NLRP3 and NLRP1 (another member of inflammasomes) activity in some tissues, such as brain, and by this means stimulate inflammation [38,39]. In this respect, the effective autophagic uptake and lysosomal degradation of dysfunctional mitochondria form a crucial element in maintaining tissue homeostasis [37]. Autophagy is, indeed, an ancient housekeeping mechanism that controls cellular homeostasis by facilitating the removal of misfolded proteins and dysfunctional organelles, such as mitochondria [37]. There are indications that autophagic capacity is compromised in aging and age-related diseases, e.g. AD, as proposed in the "*garbage can*" hypothesis of Brunk and Terman [45]. On the other hand, there is growing evidence that inflammasomes are activated under many pathological conditions; thus, a deficiency in autophagic housekeeping could trigger activation of an inflammatory component and promote pathogenesis [37–41].

After 10 years of experimental work, the garbage can hypothesis still seems to be valid, since different research approaches have clearly demonstrated both the decline of autophagy and increased mitochondrial dysfunction with aging [37,46,47]. Thus, the decline in autophagy during aging creates problems in cellular housekeeping functions, which stimulate NF-κB signaling and thus, directly or via inflammasomes, trigger SASP and the inflammatory phenotype [37]. Moreover, there are indications that inflammatory NF-κB signaling can repress autophagy and thus induce the destructive interplay between autophagy and inflammasomes [37,38]. For instance, TNF-α can induce or repress autophagy in an NF-κB-dependent manner. In the presence of NF-κB signaling, TNF-α activates mammalian target of rapamycin (mTOR), a major autophagy inhibitor. In contrast, in cells lacking of NF-κB activation, TNF-α stimulates the expression of Beclin-1, an enhancer of autophagy [37,38].

Immune Innate Activation by PAMPs and DAMPs

During aging, adaptive immunity clearly declines, a condition defined as *immune senescence* [48]. In contrast, innate immunity seems to be activated, thus inducing a chronic inflammatory phenotype, as mentioned above [29]. Innate immunity is activated through the linking of pattern recognition receptors (PRRs), consisting of multiligand and evolutionarily conserved receptors [e.g. Toll-like receptors (TLRs), Nod-like receptors (NLRs), and RIG-like receptors (RLRs)], with a plethora of both invading pathogen structures, called pathogen-associated molecular patterns (PAMPs), and endogenous danger molecules, the DAMPs [25]. These latter represent the debris from apoptotic cells, fragments of extracellular matrix, and abnormal molecular modifications that accumulate during aging (26). PRR-dependent innate/inflammatory responses induce the release of different inflammatory mediators by the NF-κB pathway [24]. Among the PRRs, TLRs (mainly TLR2 and TLR4) recognize not only some PAMPs but also a large number of different alarmin age-type DAMPs, including high mobility group box 1 (HMGB1), S100, heat shock protein (HSP) 60 and HSP70, and defensins [25,33]. In addition, both TLR2 and TLR4 may play a crucial role in the pathogenesis of several age-related diseases [33]. Accordingly, genes encoding these molecules seem to modify both the susceptibility to age-related diseases and survival to extreme age, as recently described in our study [26]. Their action seems, at least in part, to be mediated by pro/anti-inflammatory genotypes that are able to determine the negative or positive control of inflammation. In contrast, the +896A/G (Asp299Gly; rs4986790) and +1196C/T (Thr399Ile; rs4986791) TLR4 SNPs have been phenotypically associated with changes in the production of pro- and anti-inflammatory cytokines; principally, the Asp299Gly SNP seems to have a key role in AD, atherosclerosis, prostate cancer, and, reciprocally, in longevity [26,33].

In addition, during aging different macromolecules (DNA, lipids, and proteins) can be targeted by different age modifications; for example, the Maillard reaction, a well-known nonenzymatic glycosylation mechanism, is induced as a result of the enhancement of oxidative stress and hyperglycemia [2]. Interestingly, protein glycation products, called AGEs (advanced glycation end products), which are considered to be pro-aging factors, activate the NF-κB pathway by linking to characteristic PRR receptors, the RAGE receptors (receptor for advanced glycation end products). The AGE content increases in tissues during aging, and the AGE process increases in atherosclerosis, diabetes, neurodegeneration, and several inflammatory diseases. The major harmful effect of AGE in aging seems to be maintenance of the antiapoptotic and proinflammatory phenotype. Of special note is the glycation of collagen and elastin, which seems to have a key role in vascular pathologies [25].

Induction of the NF-κB Signaling Pathway by Proinflammatory Cytokines

Activation of innate immunity in the aging process (see above) determines the production and release of SASP, including different inflammatory molecules. Among these, proinflammatory cytokines are mostly observed to be elevated in the elderly. These cytokines can also activate the NF-κB pathway and in this way can propagate and aggravate inflammatory changes. IL-6 and TNF-α are clearly upregulated with aging, even if their exact role in the aging process has been difficult to establish because of their complex, cell type-specific functions [24].

Insulin/Insulin-Like Growth Factor Signaling

Excessive insulin/insulin-like growth factor (IGF) signaling has been demonstrated to enhance and accompany the aging process [2]. On the other hand, suppressing insulin/IGF signaling triggers FOXO signaling, thus inducing the long-lived phenotype. Insulin/IGF signaling promotes detrimental aging effects via NF-κB pathway activation of the IκB kinase α/β complex. As a consequence, improved inflammatory responses and resistance to apoptosis are induced. Given that inhibition of the insulin/IGF signaling pathway can activate FOXO-dependent lifespan extension, the NF-κB pathway may have a role in driving the aging process via the insulin/IGF axis [49].

Protein Modification: Acetylation and O-Glycosylation

Components of the NF-κB pathway are the targets of several post-translation modifications that trigger the activation of the pathway, but also regulate the transcriptional efficiency of the NF-κB system [2,24]. Phosphorylation and ubiquitylation are the major regulatory changes in the activation step. However, acetylation and *O*-glycosylation can control the transcriptional efficiency of the NF-κB system. Interestingly, acetylation and *O*-glycosylation seem to modify the NF-κB pathway in response to stress. For example, inflammatory responses can be potentiated through the acetylation of NF-κB components. In addition, increased protein acetylation can activate cellular senescence. In contrast, sirtuin molecules (see below), such as SIRT1 and SIRT6, can deacetylate the p65 component of NF-κB and thus repress NF-κB signaling [2,24].

Chronic hyperglycemia seems to induce glucotoxicity through the formation of AGEs or via the production *O*-linked *N*-acetylglucosamine (O-GlcNac)-modified proteins; this suggests glucose as a potential pro-aging factor. On the other hand, levels of *O*-glycosylated proteins increase during aging. In particular, increased *O*-glycosylation of IKKβ protein, which can enhance NF-κB activity, has been observed during aging. *O*-glycosylation can also target the p65 NF-κB protein and potentiate the transcriptional efficiency of NF-κB components. In addition, p53 can inhibit glycolysis and thereby suppress the activation of IKKβ/NF-κB signaling [2,24].

Genotoxic Stress

One of the major stochastic aging mechanisms is genomic instability [2,50]. DNA lesions appear during aging in both nuclear and mitochondrial DNA as a result of free radicals and oxidative stress. The major pathways activated by genotoxic stress are the p53, NF-κB, and PARP-1 [poly (ADP-ribose)-polymerase 1] pathways [50]. In particular, activation of NF-κB signaling represents one of the principal cellular features induced by DNA damage [51]. The DNA damage-dependent NF-κB activation cascade is defined as the NEMO shuttle, since under genotoxic stress an NF-κB essential modulator (NEMO) forms a complex with both PIDD (p53-induced protein with a death domain) and RIP-1 (receptor-interacting serine/threonine-protein) kinase [52]. This complex accumulates in the nucleus, and a nuclear matrix-associated SUMO E3 ligase (PIASy) then sumoylates the NEMO protein. Sumoylation is a prerequisite for ataxia telangiectasia mutated (ATM) kinase-mediated phosphorylation of NEMO. Subsequently, NEMO is desumoylated and the NEMO/ATM complex is exported from the nucleus to the cytoplasm, where it activates IKK kinases by triggering NF-κB signaling. This prevents p53-induced apoptosis, since the IKK kinases phosphorylate p53 and induce its degradation by proteasomes [52].

Another hallmark of DNA damage is induction of PARP-1, a ubiquitously expressed member of the PARP family of enzymes that modify proteins by poly(ADP-ribosyl)lation. PARP-1 is a DNA damage sensor that maintains genome integrity by regulating DNA repair [53]. In addition, PARP-1 is a novel coactivator of NF-κB signaling, which potentiates NF-κB activation by genotoxic stress [24,53]. Furthermore, it is one of the proteins involved in regulating the length of telomeres, nucleoprotein structures located at the ends of chromosomes [53]. Telomeres are subject to shortening at each cycle of cell division and are highly sensitive to damage induced by oxidative stress. During aging, both chronic inflammation and oxidative stress induce increased base oxidation. In contrast to the majority of genomic DNA, there is evidence that telomeric DNA is deficient in the repair of single-strand breaks. Thus, oxidative stress causes persistent damage to telomeres and a faster rate of telomere shortening, which induces cellular senescence and a faster rate of biological aging. Since chronic oxidative stress plays a major role in the pathophysiology of several chronic inflammatory diseases, it has been hypothesized that telomere length reduces at a faster rate during oxidative stress. On the other hand, telomere shortening is assumed to be a biomarker of premature cell senescence in vascular and metabolic diseases [54,55]. Therefore, telomere length as well as the evaluation of PARP-1 function and integrity might be useful biomarkers of both biological aging and disease onset and progression [53].

SERUM INFLAMMATORY BIOMARKERS

Serum biomarkers (SBs) are commonly defined as indicators of physiological or pathological states that are detectable in serum. They are generally utilized as appropriate and rapid tools for the diagnosis of pathologies and for monitoring therapy or therapeutic efficacy, good health status, and systemic status. A wide range of SBs related to tissues, organs, and the whole body exists, even if none can be considered as sufficiently well-established and specific to be useful as an ideal aging biomarker. Among the SBs, the circulating inflammatory components of SASP (i.e. IL-6, TNF-α, proinflammatory cytokines, and CRP) are well investigated (see Fig. 1.1). These are associated with several chronic aging conditions, such as CVD, T2DM, physical disability, and cognitive decline [29–31].

As mentioned above, IL-6 is a proinflammatory cytokine produced by both lymphoid and nonlymphoid cells, such as T and B cells, monocytes, fibroblasts, vascular

endothelial cells, adipocytes, skeletal muscle cells, and other kinds of cells. Its release and expression are under transcriptional regulation mediated by induction of the inflammatory response [27].

TNF-α is mainly produced by macrophages, but also by other lymphoid and nonlymphoid cells, such as adipocytes, mast cells, fibroblasts, cardiac myocytes, vascular endothelial cells, and neuronal cells. It induces IL-6 and IL-8 production and release through the activation of different pathways [56,57].

In healthy elderly people, an age-related increase in IL-6 and TNF-α levels has been observed. In particular, the prospective InCHIANTI study showed a significant association between high levels of CRP, IL-1, and IL-6 and poor physical performance and muscle strength [58]. In addition, TNF-α is considered to be an independent prognostic marker for mortality in centenarians, and in elderly nursing home residents its detection in serum may be a predictor of early mortality [59,60].

In centenarians, a linear increase in serum TNF-α levels with CRP and IL-6 has been detected, demonstrating inter-related activation of the inflammatory cascade in very old people [59]. A specific correlation between the above-mentioned cytokines and specific age-related diseases has been demonstrated. In particular, TNF-α seems to be associated with AD, insulin resistance, and T2DM [60–62]. In addition, high circulating levels of TNF-α and IL-6, as well as CRP, have been associated with CVD and frailty [63–66].

CRP is a member of the pentraxin superfamily, which includes both short and long pentraxins. It is a highly conserved plasma protein consisting of five identical 21,500-Da subunits. It was identified in 1930 as one of the first acute-phase proteins. CRP is, indeed, a stable plasma marker of systemic inflammation, with a half-life of 19 h. During the acute-phase response, its levels may rapidly rise up to 1000-fold above reference values. The main source of CRP is the liver, even if its production has recently been demonstrated in other sites, such as macrophages, kidney, and neuronal and endothelial cells. Its production is regulated during acute-phase response by the proinflammatory IL-1, IL-6, and TNF-α cytokines. Increased CRP levels might be significant predictors of T2DM and are related with insulin resistance and metabolic syndrome, as well as with AD [66–69].

MicroRNAs (miRNAs) are small, noncoding regulatory RNAs composed of 18–25 nucleotides. They act as gene expression regulators, promoting the degradation and/or translational inhibition of the mRNA target by binding to either the coding region or the 3′ untranslated region (UTR). As base pairing is often imperfect, one miRNA can regulate many targets, and several miRNAs can inhibit a single mRNA. In general, miRNAs regulate many physiological processes such as cell proliferation, development, cell death, cell response, survival, and replicative senescence, but they are also involved in cancer formation [70,71]. Moreover, some studies have demonstrated a role for miRNAs in aging processes, and the presence of different miRNA expression profiles between young and old people [71]. In addition, miRNAs have been demonstrated to have the capacity to modulate age-related inflammation [72]. In particular, some miRNAs take part in feedback loops, both regulating the expression of cytokines and being subjected to regulation by NF-κB [73]. One of these is miRNA146a, which together with miRNA146b negatively regulates the expression of IL-6 and IL-8 in fibroblast and probably also acts on IL-1 [74]. Because its expression depends on NF-κB, this provides a negative feedback loop. Also, the miRNA let-7 inhibits IL-6 expression, but in this case a positive feedback loop is established because let-7 is negatively regulated by NF-κB [75]. Moreover, NF-κB may be indirectly regulated by miRNAs. Indeed, miRNA21 negatively regulates the expression of pellino 1, a ubiquitin ligase that acts on IRAK-1 (interleukin-1 receptor-associated kinase 1), thus activating the downstream signaling cascade that leads to the nuclear translocation and activation of NF-κB [76]. This means that miRNAs could have a role in aging, regulating gene expression, and interacting with the NF-κB pathway.

MOLECULES AND MECHANISMS LINKED TO THE NF-κB SIGNALING SYSTEM ARE POTENTIAL TARGETS FOR ANTI-AGING AND ANTI-AGE-RELATED DISEASE TREATMENT STRATEGIES

Growing evidence suggests that activation of the NF-κB signaling system during aging is responsible for inflammaging [30–33,36]. This is plausible since nearly all insults that enhance the aging process are well-known activators of the NF-κB signaling system, as illustrated in Fig. 1.1. The NF-κB signaling system also represents the keystone of host defense, receiving input signaling from the PRR receptors and subsequently organizing the transcriptional output response against the acute danger [24,25]. In both cases, sustained activation of the NF-κB signaling system can trigger and enhance the aging process in many different ways, as described above [2,36]. Thus, the NF-κB system is at the hub of the aging process. This concept leads us to consider molecules and mechanisms linked to the NF-κB signaling system as potential aging biomarkers and targets for the development of new therapeutic strategies against aging and age-related diseases.

On the basis of data reported herein, some inferences can be proposed (see Table 1.2). The presence of "high-risk" levels of IL-6 and TNF-α in elderly people suggests the possibility of developing preventive measures

TABLE 1.2 Targets and Potential Therapeutic Interventions

Targets	Therapies
Elevated levels of IL-6, TNF-α	Monoclonal antibodies against these cytokines and their receptors NSAID Agonists of cytokine receptors or PRR receptors for people who do not respond to (or comply with) NSAID therapy Antibody-mediated stimulation of decoy TLRs, such as TAM receptors, or of intracellular TLR regulators for people with proinflammatory alleles of the *TLR4* and *TLR2* genes Statin therapy Physical activity Administration of prebiotics and probiotics: proinflammatory cytokine lowering, CRP reduction
Oxidative stress	Caloric restriction: increase in the level and activation of adenine nucleotide translocase and the uncoupling proteins to reduce the mitochondrial membrane potential, which results in a decrease in superoxide radical ($O_2^{\bullet -}$) production Polyphenols
Mitochondria dysfunction	Caloric restriction: improving in SIRT1 levels
Activation of NF-κB pathway	Caloric restriction: improving the levels of sirtuin proteins Terpenoids: resveratrol induces activation of sirtuins via the AMPK pathway Use of specific miRNAs Administration of prebiotics and probiotics
Decline of autophagic cleansing	CR: inhibition of mTOR Rapamycin: inhibition of mTOR
Increased insulin/IGF1 pathway	Metformin with CR mimic response

CR, caloric restriction; CRP, C-reactive protein; IL-6, interleukin-6; mTOR, mammalian target of rapamycin; NSAID, nonsteroidal anti-inflammatory drug; PRR, pattern recognition receptor; SIRT1, NAD-dependent protein deacetylase sirtuin-1; TAM, Tyro3/Axl/Mer; TLR, Toll-like receptor; TNF-α, tumor necrosis factor.

using specific inhibitors, such as monoclonal antibodies, against these cytokines and their receptors. A reduction in inflammatory mediators may be also induced through nonsteroidal anti-inflammatory drug (NSAID) therapy. For people who do not respond to (or comply with) NSAID therapy, other more sophisticated preventive approaches may be possible, including the use of agonists of cytokine receptors or PRR receptors, e.g. TLR2 and TLR4, particularly in carriers of high inflammatory response alleles [26,33]. On the other hand, activation of PRR receptors, such as TLR2 and TLR4 by PAMPs or DAMPs, particularly upon aging, induce the release of a large number of SASP components, such as proinflammatory IL-6 and TNF-α cytokines, via the NF-κB signaling system [25,33]. In addition, the magnitude of cytokine production and of all proinflammatory mediators, in general, has been shown to vary between individuals, probably based on genetic heterogeneity. One or more functional SNPs in one or more innate immunity genes might be responsible. Accordingly, recent studies have suggested a role for the +896A/G TLR4 SNP in cytokine production. In particular, high levels of proinflammatory cytokines were observed in carriers of the +896A/G TLR4 SNP [26,33].

Another possible therapeutic intervention in subjects with proinflammatory alleles of *TLR2* and *TLR4* genes is antibody-mediated stimulation of the decoy TLR receptors, such as the tyrosine kinase TAM (Tyro3/Axl/Mer) receptors, or of the intracellular TLR regulators [i.e. suppressor of cytokine signaling (SOCS) molecules], which are involved in inhibition of the inflammatory response by mediating TLR degradation, or activation of competitive or dephosphorylating factors [77]. The sequential induction of these pathways and their integration with upstream TLR and cytokine signaling networks may limit the inflammatory response and maintain innate immune system homeostasis. A better understanding of the regulatory mechanisms of this cascade may have important implications for therapeutic intervention in human immune disorders and reduce the risk of development of several age-related diseases [26,33].

In addition, it has been demonstrated that statin therapy has beneficial effects in reducing primary and secondary CVD risk through its lipid-lowering and anti-aging actions, such as reducing levels of inflammatory molecules, especially CRP and IL-6. On the other hand, results from the Justification Trial Evaluating Rosuvastatin (JUPITER) demonstrated that statin treatment in apparently healthy subjects with elevated CRP and non-elevated cholesterol low-density lipoprotein results in a significant reduction of both these markers and CVD [78].

Another possible anti-aging strategy, which may reduce the biological effects of NF-κB signaling in aging, is caloric restriction (CR) [79]. Restricting the intake of calories has been practiced as a method for increasing both the length and quality of life for over 500 years. Experimental work confirming the success of this approach in animals has accumulated over the last 80 years. CR may extend life by up to 50% in rodents, with progressively less impact the later in life it is started. This effect is matched by profound impacts on age-related diseases, including reduced risk of cancer, autoimmune disease, CVD, neurodegenerative disorders, and T2DM [79]. The disposable soma theory of aging suggests that CR evolved as a somatic protection response to enable animals to survive periods of food shortage [3]. The shutdown of reproductive function during CR is consistent with this suggestion, but other

features of the phenomenon are less consistent with this theory. Some researchers have, indeed, proposed that in rodents it may be mostly an artifact of domestication. CR induces profound effects on animals at all levels, from the transcriptome to whole animal physiology and behavior. Animals under CR lose weight, which is disproportionately contributed to by white adipose tissue. Generally, animals on CR change their activity patterns. Thus, they are more active prior to food delivery each day, but their total activity may be unchanged or reduced [79]. There has been considerable debate over the effects of CR on the resting metabolic rate (RMR). Total RMR declines but as body mass and body composition also change it is unclear whether metabolism at the tissue level also declines, is unchanged, or even increases. Body temperature universally decreases. Hunger is increased and does not seem to decline even with very long-term restriction. Circulating adipokines are reduced, reflecting the reduction in white adipose tissue mass under CR [79]. There is also a large reduction in circulating insulin and glucose levels. There are profound tissue level changes in metabolism, with a generalized shift from carbohydrate to fat metabolism. Four pathways have been implicated in mediating the CR effects: the insulin/IGF-I signaling pathway, the sirtuin pathway, the 5′-adenosine monophosphate (AMP)-activated protein kinase (AMPK) pathway, and the mTOR pathway [79]. These different pathways may interact and may all play important roles in mediating different aspects of the response. Exactly how they generate the health benefits remains open to debate. However, one of the major impacts of CR is to reduce oxidative stress [80]. As described above, the main cellular source of ROS is mitochondria. Isolated mitochondria from animals under CR show reductions in ROS production. In particular, CR results in increased levels and activation of adenine nucleotide translocase and the uncoupling proteins, which reduce the mitochondrial membrane potential, resulting in a decrease in superoxide radical ($O_2^{\bullet -}$) production. This results in reduced damage to the lipids in the mitochondrial membrane, which is further reduced by increased membrane lipid saturation [80]. Increased levels of superoxide dismutase convert superoxide into hydrogen peroxide and increased levels of Se-dependent glutathione peroxidase and catalase convert this to water, thus reducing the production of the toxic hydroxyl radical ($HO^{\bullet}$). Lowered levels of $HO^{\bullet}$ reduce oxidative damage to proteins and DNA, which is further ameliorated by enhanced levels of protein degradation and base-excision repair, respectively [80]. Furthermore, CR induces mitochondrial biogenesis, as measured by changes in mtDNA levels and protein levels [81]. Such effects on mitochondrial biogenesis are consistent with the idea that there may be a tissue level increase in oxygen consumption under CR, which is accommodated by the reduced overall energy budget of the reduced amount of metabolizing tissue.

In addition, CR increases the levels of members of the sirtuin family (SIRT1-7), NAD^+-dependent deacetylases involved in the regulation of the activity of many proteins, energy metabolism, cell survival, and longevity [82,83]. In particular, CR increases the expression of SIRT1 in multiple tissues, even if this effect does not appear to be uniform in all tissues or across different studies [84]. It has been demonstrated that SIRT1 interacts with p65/RelA protein and specifically cleaves the acetyl group from lysine-310 of p65, which reduces the transactivation efficiency of the NF-κB system [85,86]. Thus, SIRT1 is a potent inhibitor of the NF-κB system. Autophagy is also enhanced by CR via inhibition of mTOR or activation of the AMPK pathway. This last is an evolutionary conserved sensor for disturbances in cellular energy balance and a major inducer of autophagy. Thus, CR acts directly or indirectly to inhibit the NF-κB system [79,87].

Considerable effort has been directed in recent years to finding drugs that mimic the CR response. Promising candidates are those that intersect with the critical signaling pathways identified above and include biguanides such as metformin, capable of targeting the insulin signaling pathway, stilbenes (e.g. resveratrol), which affect sirtuin activity, and drugs such as rapamycin that interact with mTOR signaling. Whether it will ever be possible to find drugs that capture the health benefits of CR without the negative side effects remains unclear [79,88,89].

As mentioned above, several plant-derived, folk medicine compounds and extracts have been claimed to have anti-aging effects [79]. However, only a few of these traditional remedies have been subjected to clinical trials. Recently, many promising compounds have been identified and scrutinized. Among these are polyphenols (i.e. flavonoids and terpenoids), the major ingredients of fruits, vegetables, and different spices [79,90]. Many polyphenols are inhibitors of the NF-κB signaling system, since they are potent antioxidants; as a consequence, they inhibit ROS production and activation of the NF-κB signaling system [91,92]. Some of them (i.e. terpenoids) can also directly inhibit IKK/NF-κB signaling [91]. Accordingly, low-doses of terpenoids have been found to trigger cellular stress response and subsequently induce adaptive stress resistance, a condition defined as *hormesis* [93,94]. Stress resistance involves several molecular adaptations via activation of the AMPK pathway and the subsequent increased expression of survival genes, such as those encoding FOXOs, p53, and sirtuins, [93,94]. Of special note is the effect of resveratrol, a stilbene phytochemical. This induces activation of SIRT1 via the AMPK pathway and indirectly inhibits the NF-κB signaling system via activation of survival genes [91].

In addition, there is promising evidence that physical activity has a role in reducing the levels of inflammatory

markers. Several theories have been advanced to explain this; however, the mechanisms underlying its anti-inflammatory effects seem complex and have not been fully elucidated. It has been recently considered that the decreased production of proinflammatory cytokines may originate from a reduction in adiposity or the release of muscle-derived IL-6 [95–98]. This last seems to induce several metabolic adaptations, i.e. hepatic glycogenolysis and lipolysis, and the release of cytokine inhibitors [i.e. IL-1ra, soluble TNF receptor (sTNFR), and IL-10] and cytokines with potent anabolic effects, such as IL-15 [95–98].

Another good target for anti-aging therapy could be miRNAs, which can be detected in serum and plasma using new advanced technologies. A good strategy could be the use of specific miRNAs to target genes encoding molecules of the NF-κB signaling system.

In addition, the administration of probiotics and/or prebiotics to the elderly seems to induce changes in several inflammatory parameters (i.e. lowers proinflammatory cytokines and reduces CRP), demonstrating that the manipulation of gut microbiota may result in modification of the aged immune system function. On the other hand, intestinal microbiota seem to play a fundamental role in maintaining human health. Their supposed importance in human physiology has recently led human subjects being labeled as "metaorganisms" because of their close symbiotic relationship with indigenous gut microbiota. The "metaorganisms" hypothesis promotes the use of dietary supplementation with probiotics and prebiotics as a therapeutic strategy to preserve human health, particularly during the life period not foreseen by evolution—"aging"—that inexorably alters gut microbiota composition, stability, and functionality [99].

CONCLUSIONS

Putting together the observations described above, chronic inflammation is emerging as a major biological mechanism underpinning the aging process and age-related diseases [30–33,36]. Thus, the large array of defense factors and mechanisms linked to the NF-κB system now seem to be involved in the aging process [2,24]. This concept leads us to propose inducers of the NF-κB signaling system as potential aging biomarkers and as promising targets for the development of new therapeutic strategies against aging and age-related diseases. Some cellular inflammatory mechanisms and plasma molecules are described in this report as potential aging biomarkers. In addition, some suggestions on their roles as promising targets for the development of new therapeutic strategies have been discussed. Our attention has been particularly focused on possible interventions in molecular survival and resistance stress pathways that may be capable of reducing or inhibiting the NF-κB signaling system. However, it is impossible to predict whether reducing or retarding the onset of the aging biological phenotype is possible by modifying lifestyle or through the use of CR-mimetic drugs and other preventive interventions. There are several reasons for being cautious. First, the major data on anti-aging effects have been obtained from studies on animals. Thus, potential therapeutic interventions based on pathways identified in model organisms may be illusory because gains in longevity achieved in these organisms seem to decline with organismal complexity or depend on their idiosyncratic physiology. Furthermore, lifespan in some organisms may be less plastic than in others. In addition, there are still enormous gaps in our knowledge about how metabolic pathways operate and interact. Serious side effects may constrain the effectiveness of pharmacological interventions.

The best treatment might be that which promotes the repair of macromolecular damage. However, it is unclear whether all toxic lesions associated with the aging process have been identified, or whether practical and appropriate strategies exist to eliminate them, such as those mentioned above.

Thus, other studies are needed to confirm and extend these current data. For example, genomic, transcriptomic, and epigenetic investigations may eventually lead to a better understanding of the molecular and cellular inflammatory mechanisms associated with biological aging. In addition, for the development of human anti-aging therapies, it would be more appropriate to identify cellular and serum aging biomarkers and potential targets using an appropriate model, such as the offspring of centenarians, i.e. healthy elderly people with a family history of longevity, as was recently suggested [100]. On the other hand, research into biomarkers of aging and age-related diseases for understanding the health trajectories of the oldest old is unexplored territory. It is important that this knowledge gap is filled, given the rapid growth in the number of very old people in many contemporary populations. The goal of this research is to guarantee improving the quality of life rather than searching for the elixir of long life.

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