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Biomarkes of aging

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1. ABSTRACT

Aging is a complex process that negatively impacts the development of the different systems and its ability to function. Moreover, the Aging rate in humans is not the same, principally due to genetic heterogeneity and environmental factors. The aging rate is measured as the decline of functional capacity and stress resistance. Therefore, several attempts have been made to analyse the individual age, (so-called biological age) compared to chronological age. The biomarkers of aging are age-related body function or composition, these markers aim to assess the biological age and predict the onset of age-related diseases and/or residual lifetime. Such biomarkers should help in one hand to characterise the biological age and on the other hand to identify individuals at high risk of developing age-associated diseases or disabilities. Unfortunately, most of the markers under discussion are related to age-related diseases rather than to age, so none of these markers discussed in literature is a true biomarker of aging. Hence, we discuss some disease-related biomarkers useful for a better understanding of aging and the development of new strategies to counteract it, essential for improving the quality of life of the elderly population.

2. INTRODUCTION

Aging results from a breakdown of self organizing system and reduced ability to adapt to environment. In addition, it has been suggested that normal human Aging is associated with a loss of complexity in a variety of fractal-like anatomic structures and physiological processes (1). Furthermore, using a variety of measures that employ fractal analysis, Aging has been shown to be associated with a loss of complexity in blood pressure (2), respiratory cycle (3), stride interval (4), and postural sway dynamics (5).

However, there is a judge difference among people that age: there are people at the age of 90 years old still in good mental and physical condition and other that at 60 years old have extensive cognitive difficulties and chronic diseases. Overall, understanding Aging means being able to quantify physical inability, mental functional capacity, organs and apparatus deregulation (6).

Aging is considered a process that changes the performances of most physiological systems and increases susceptibility to diseases and death. The Aging phenotype

is a complex interaction of stochastic, environmental, genetic and epigenetic variables. However, these variables do not create the Aging phenotype but generate the lost of molecular fidelity and therefore as the random accumulation of damage in the human organism's cells, tissues, or whole organism during life increases, the probability of disease and death also augments in proportion (7).

In the society, the public perception of advanced Aging involves the inability to survive due to chronic diseases and the combined loss of mobility, sensory functions, and cognition (8) with an exponential growth of health costs linked to increased size of elderly in the Western World. So, biomarkers of human Aging are urgently needed to assess health state of elderly and the possible therapeutic interventions.

What a biomarker for Aging should be or predict is quite broadly defined. At the minimum, a biomarker should not only (i) reflect some basic property of Aging, but also (ii) be reproducible in cross-species comparison, (iii) change independently of the passage of chronological time (so that the biomarker indicates biological rather than chronological age), (iv) be obtainable by non invasive means, and (v) be measurable during a short interval of life span. A biomarker should reflect the underlying Aging process rather than disease (9). It should vary as an individual ages, but not strictly chronologically; instead, its quantity should correlate with remaining life span and with the likelihood of acquiring multiple age-related conditions. Furthermore, scientists will likely need a set of numerous biomarkers, perhaps some that provide a window on particular tissues and others that give a glimpse of an entire organism, to replace life span as the best measure of Aging.

Another problem, which is even more challenging, is that unless we understand how Aging "works," we might not be able to define ideal biomarkers at all. A biomarker would have only limited utility without understanding of the biological complexity of the system and above all how we can influence the complexity of structure. Moreover, which tissues or organs are preferable to evaluate as a predictable marker? Maybe it can be taken from blood, urinary tract or central nervous system; biochemical markers are better suitable than histology markers that are still to find out.

Unfortunately, most of the markers under discussion are related not only to age, but also to diseases, and thus none of the markers discussed in literature is a pure biomarker of Aging. A recent report has stated that biomarkers collected in physical exams, such as markers of cardiovascular diseases (CVD) and diabetes are useful predictors of healthy Aging (10). In the present review, we will discuss markers based on immunosenescence, inflammatory responses and oxidative stress. The review is based on data from author laboratories rather than on an extensive review of the literature.

3. BIOMARKERS OF IMMUNOSENESCENCE

The modifications of the immune system in the elderly are evaluated as a deterioration of the immune system, the so-called immunosenescence, which is thought to be mostly the result of the declining effectiveness of T cells. It contributes to higher morbidity and mortality caused by the increased susceptibility to infectious diseases or their reactivation as well as to autoimmune phenomena and cancer (11-13).

It is well established that the percentage and the number of naïve T cells is lower in the elderly than in the young. Age-associated thymic involution is thought to materially contribute to this phenomenon. Reciprocally, the percentage and numbers of memory and effector-memory cells are higher in the elderly. In fact, lifelong and chronic antigenic load results the major driving force of immunosenescence, which impacts on lifespan by reducing the number of virgin antigen-non experienced T cells, and filling the immunological space with expanded clones of memory and effector, antigen-experienced T cells. Thus, the repertoire of cells available to respond to antigenic challenge from previously unencountered pathogens is shrinking (11,14,15).

Several studies have underlined the importance of ubiquitous viruses causing chronic latent infections, such as Herpes Viruses, in determining characteristic aspects of T-cell branch senescence such as the progressive exhaustion of naïve lymphocytes, the increase in memory cells and the T repertoire shrinkage. Particularly, the Herpes virus Cytomegalovirus (CMV) seropositivity has been associated with many of the same phenotypic and functional alterations of T-cell immunity previously considered as Aging biomarkers. CMV-specific lymphocytes represent, even in immunocompetent subjects, a sizable proportion of both the CD8+ and the CD4+ memory compartment and they increase with age, with a significant increase in the proportions of highly differentiated effector memory and effector $CD45RA^+$ CD8 T cells in comparison to younger subjects. Furthermore, the increase of these cells is due to the expansion of terminally differentiated exhausted lymphocytes and expanded clones restricted towards specific epitopes, with the accumulation of large oligoclonal expansions of $CD8^+$ T cells (16-20).

Thus, the analysis of T cells in longitudinal studies in octogenarians and nonagenarians has defined an “immune risk phenotype” (IRP), originally characterized by an inverted CD4/CD8 ratio and low lymphoproliferative response, that constitutes a major predictor of no survival. In addition, very old subjects with a health status impaired by the most common age-related diseases exhibit an increase in CMV-specific effectors T cells, mostly CD4+, with a parallel increase in anti-CMV IgG antibodies. As previously stated, persistent CMV infection induces chronic stimulation of specific T cells that leads to terminal differentiation to senescent cells with an altered functional capability. Thus, elderly have high expansions of CMV specific CD8 T cells that display an effector memory phenotype characterized by the low expression of CD28 and increased expression of NK-associated receptors. Therefore, a critical indicator of incipient mortality is T cell repertoire attrition. Furthermore, several studies have suggested a positive association between *in vitro* T cell function and individual longevity (17, 21-29).

Also in the B compartment age-dependent changes indicate that advanced age is characterized by lack of B instructive immune response to new extracellular pathogens. B cell number is decreased and the B-cell repertoire is influenced by Aging through the quality of the antibody response. Changes in B cell repertoire have been described, and decreased B cell diversity in old age is correlated with poor health status. In addition, decreased IgM and IgD levels in elderly suggest a shift from the naïve (CD27-) compartment of the B cell branch towards the memory (CD27+) compartment. However, data are controversial since not all studies have shown, in elderly, a significant decrease of naïve CD27- B cells and an increase of CD27+ memory B cells (30-35).

Circulating B cells can be divided on the basis of the expression of IgD and CD27 into different functional subsets. In aged, a double-negative (DN) IgD-CD27- B cell subset is significantly increased. The origin of DN cells is not well understood as they might derive from activated CD27+ memory cells that have lost CD27 expression (32, 36-38). Hence, they might be similar to the effector T exhausted T cells, previously stated.

Of interest, B naïve lymphocytes are increased in offspring of healthy centenarians. It is well known that centenarian offspring, who are in their 70s and 80s, have a survival advantage when compared with control subject of the same age range whose parents died at an average life expectancy (39). The main lymphocyte differences observed between the two groups concern B cells. Indeed naïve B cells are more abundant as well as double negative B cells are less abundant in centenarian offspring. These data are similar to that found in previously experiment on young subjects. So, B cell compartment of the offspring of centenarians seems to be more similar to that of young respect to the old one (40).

However, the age-dependent B cell changes here briefly discussed indicate that the loss of naive B cells could represent a hallmark of immunosenescence and could provide a biomarker possibly related to the life span of humans (32).

The importance of a well-preserved NK cell function in elderly is underscored by data showing that low NK cell activity is associated with development of infections and death due to infection in immunologically normal elderly subjects with an impaired performance status. The relative risk for the development of infection increased in accordance with the decrease in the NK cell activity and a low NK cell activity was associated with short survival due to infection. Furthermore, people aged > 85 year with low numbers of NK cells were reported to have three times the mortality risk in the first 2 years of follow-up than those with high NK cell numbers. Other aspects of NK cell function, such as the secretion of chemokines or interferon-γ in response to interleukin (IL)-2 are also decreased in the aged. Hence, high NK cytotoxicity associates with healthy Aging and longevity, whereas low NK cytotoxicity associates with increased morbidity and mortality due to infections, atherosclerosis, and poor response to influenza vaccination. Together, these results support the notion that preserved NK cytotoxicity should be considered a biomarker of healthy Aging and longevity, whereas low NK cytotoxicity is a predictor of morbidity and mortality due to infections (29, 41-43).

4. BIOMARKERS OF INFLAMMATION

Aging is accompanied by chronic low-grade inflammation state, showed by a 2 to 4-fold increase in serum levels of inflammatory mediators which acts as predictors of mortality independent on pre-existing morbidity. This pro-inflammatory status of the elderly underlies biological mechanisms responsible for physical function decline, and inflammatory age-related diseases are initiated or worsened by systemic inflammation (44-46). In fact, an inflammatory response appears to be the prevalent triggering mechanism driving tissue damage associated with different age-related diseases and the term "inflamm-Aging" has been coined to explain the underlining inflammatory changes common to most age-associated diseases (44,45,47-49). It is mostly the consequence of the body ability to counteract and modulate the effects of a variety of stressors, which cause the accumulation of molecular and cellular scars. However, a wide range of different aetiological factors contributes to increased low-grade inflammatory activity in elderly including a decreased production of sex steroids, smoking, subclinical disorders such as atherosclerosis, asymptomatic bacteruria, a higher amount of fat as well as cellular senescence (49,50).

However, there is a link among an individual exposure to past infection, levels of chronic inflammation and increased risk of heart attack, stroke, Alzheimer's disease (AD), Parkinson's disease, cancer, type-2 diabetes, sarcopenia, functional disability and high mortality risk. In addition, within individuals, C-reactive protein (CRP) levels are also correlated with the number of seropositivities to common pathogens, suggestive of infection history (44, 51-62).

Tumor necrosis factor(TNF)-alfa is an independent prognostic marker for mortality in persons aged 100 years and in elderly nursing home patients detectable serum level of TNF- alfa were associated with death within 13 months. Plasma levels of TNF-α are correlated linearly with Interleukin (IL)-6 and CRP reactive protein in centenarians, indicating an interrelated activation of the entire inflammatory cascade in the oldest old. High circulating levels of TNF- alfa and IL-6 as well as CRP have been related to CVD and frailty. Moreover, TNF- alfa has been linked to AD and type 2 diabetes, and IL-6 has been demonstrated to be a strong predictor of mortality itself. Increased levels of CRP, IL-6 and TNF- alfa are associated with insulin resistance in elderly non-diabetic subjects and highly sensitive CRP levels are significant predictors of subsequent diabetes and metabolic syndrome as well as AD. The prospective InCHIANTI study demonstrated that high levels of IL-6, CRP and IL-1, are significantly associated with poor physical performance and muscle strength (60,63-78).

On the other hand, white blood cell count (WBC) is an important predictor of all-causes mortality in aged, mostly CVD and a high leukocyte count may identify high-risk individuals who are not currently identified by traditional CVD factors (79,80).

During normal Aging, the gradual loss of telomeric DNA in dividing somatic cells can contribute to replicative senescence, apoptosis, or neoplastic transformation. Hence, an association between telomere length and mortality in 143 normal unrelated individuals over the age of 60 years. Those with shorter telomeres in blood DNA had poorer survival, attributable in part to a higher mortality rate from heart disease and a mortality rate from infectious disease. Telomere shortening of blood cells, likely due to increased rounds of replication depending on life-long immune-inflammatory stimuli, contributes to mortality in many age-related diseases. Hence, these results suggest their possible role as biomarkers. However, unfortunately they have not been yet extensively confirmed (81).

The majority of above described immune-inflammatory aspects, that characterize the immunosenescence, are also detectable in extreme longevity, where a higher frequency of genetic markers associated with a reduced pro-inflammatory ability seems to counteract the onset of the main age-related disorders. Centenarians are quite capable of mounting effective inflammatory responses; however, inflammatory status, correlated to increasing risk of developing frailty and diseases, is compensated by the concomitant development of strong and effective anti-inflammatory responses (39, 82,83).

4.1.Oxidative stress

The "free radical theory of Aging" has captivated the scientific attention as a possible biological explanation of the entire Aging process (84, 85). Oxidative stress has been linked to a variety of medical problems related to Aging, such as CVD, cancer, diabetes and AD (86, 87). Many *in vitro* markers of oxidative stress are available, but most are of limited value *in vivo* because they lack sensitivity and/or specificity or require invasive methods. Thus the results of studies investigating oxidative stress in human Aging are still controversial, and there are still limited and conflicting results available in the literature (88-91).

There is considerable literature supporting a key role of oxidative stress in the clinical phenotype, with direct evidence of significant increases in oxidative DNA damage, protein oxidation and lipid peroxidation (86,87,92). Moreover, numerous studies have demonstrated that oxidative stress is increased in frail, institutionalised elderly people, and may lead to an accelerated Aging, while in free living elderly this increase is not always significant.

An example of biomarkers of oxidative stress, *in vivo*, is the measure of lipid peroxidation, although producing contradictory results. Lipid oxidation not only causes membrane disruption, it also produces aldehydic species, such as malondialdehyde (MDA), able to perpetrate further damage by binding to and modifying proteins (93). MDA, though certainly not perfect, has widely performed as a biomarker to ascertain whether lipoperoxidation has taken place (93, 94) and it has been often utilized to evaluate human Aging. *In vivo* results in elderly are quite ambiguous. In numerous studies plasma MDA, evaluated by means of the thiobarbituric acid test, was significantly higher in healthy elderly, confirming the presence of increased lipoperoxidation in old age. Nevertheless, other studies in healthy older subjects, reported a biological antioxidant status similar to those of younger elderly subjects (95).

Recently, isoprostanes (IsoPs), compounds that are produced *in vivo* by free radical-induced peroxidation of arachidonic acid, have been also proposed to assess oxidative stress status *in vivo*, but there are still few evidences of their consistency (96). The data from literature suggest that lipid peroxidation is a less sensitive marker of oxidative stress than protein oxidation (90). The most widely studied oxidative stress-induced modification to proteins is the formation of carbonyl derivatives. Carbonyl formation can occur through a variety of mechanisms including direct oxidation of certain amino-acid side chains and oxidation-induced peptide cleavage. Protein carbonyl level is a stable and generic signal of protein oxidative damage (97, 98). The importance of protein oxidation in Aging is supported by the observation that levels of oxidized proteins increase with animal age, but again in humans *in vivo* studies results are poor and uncertain. Although all organs and all proteins can potentially be modified by oxidative stress, certain tissues and specific protein targets may be especially sensitive. For example, recent studies have applied redox proteomic, a branch of proteomics that identifies oxidatively-modified proteins, to characterize specific proteins in brain Aging, and a number of proteins that are oxidatively modified in AD have been identified (99).

Oxygen free radicals can induce a variety of damage to DNA, including DNA single and double strand breaks, base modifications and abasic sites (100). 8-hydroxy-2-deoxyguanosine (8-OHdG) is by far the most studied oxidative DNA lesion and has gained much attention because of its mutagenic potential (101). The formation of 8-OHdG in leukocyte DNA and the excretion of 8- OHdG into urine have been frequently measured by high performance liquid chromatography or mass spectrometry to assess oxidative stress in humans. Compared with the determination of 8-OHdG in leukocyte DNA, the measurement of urinary 8-OHdG offers some advantages, because it is non-invasive, there is less production of artifacts during sample procedure, it better represent a marker of oxidative DNA damage and repair from all cells in the organism (102). Although some studies have identified an age-related increase of 8-OHdG in healthy volunteers (103), prospective study of oxidatively damaged DNA as a predictor of risk for age related pathologies is extremely difficult and few interesting results have been obtained to date. In addition, the accumulation of oxidation products several studies have

associated Aging with a progressive loss of antioxidant defence (104, 105).

Reactive oxygen species (ROS) production is largely counteracted by an intricate antioxidant defence system that includes the enzymatic scavenger superoxidodismutase (SOD), catalase and glutathione peroxidise (GSH-Px). SOD speeds the conversion of superoxide to hydrogen peroxide, whereas catalase and glutathione peroxidise convert hydrogen peroxide to water. The most recently discovered SOD isoenzyme is the extracellular SOD (EC-SOD) that plays a primary role as main enzymatic scavenger of superoxide in the extracellular space (106). Numerous researches evaluated the impact of Aging process on EC-SOD activity, but the results are yet disparate. There are conflicting evidences about the effect of Aging on GSH-Px activity. In the French PAQUID study, there were no changes in GSH-Px with age (107). BELFAST study has shown a decline in GSH-Px in well free-living nonagenarians (95) and other studies have demonstrated the same in institutionalized old subjects (109). In a recent population-based study, cognitive decline was associated with lower activity of the protective selenium-dependent GSH-Px and a higher activity of Cu/Zn-SOD (110).

In addition to these well characterized antioxidant enzymes a variety of other non-enzymatic, low molecular mass molecules are important in scavenging ROS. These include ascorbate, pyruvate, flavonoids, carotenoids, uric acid and perhaps most importantly, glutathione (GSH), an ubiquitous antioxidant which is present in millimolar concentrations within cells. GSH depletion can enhance oxidative stress, GSH level and the ratio between GSH and oxidized glutathione (GSSG) is decreased in models of Aging and correction of low tissue glutathione increased longevity (109,110). Thus it has been speculated that glutathione status could be an indicator of health and functional age. In the BELFAST study GSH plasma levels were increased in nonagenarians compared to septo/octogenarians (95). It has been recently shown in 204 volunteers with a broad age spectrum that blood GSH concentration declines with age (111). Another study, measuring cysteine/cystine and GSH/GSSG redox in plasma of 122 healthy individuals aged 19-85 years, showed a steady, linear increase in oxidative events throughout adult life and in particular that the capacity of the GSH antioxidant system is maintained until 45 years in healthy subjects and then declines rapidly (112).

Recently, total plasma carotenoid levels have been suggested as a possible health indicator in elderly populations. The 'Epidemiology of Vascular Aging' (EVA) study have determined the association between baseline total plasma carotenoids and mortality. Low total plasma carotenoid levels were significantly associated with all-cause mortality in men but not in women (113).

In the context of stress response, eukaryotic cells are able to induce an evolutionarily highly conserved class of proteins known as heat shock proteins (HSPs) or stress proteins. A large body of evidence support a critical role for HSPs in cellular protection against ROS and a variety of other insults, including heat, hypoxia, ischemia, excitotoxicity, glucose deprivation, cancer, and Aging (114). The cellular protection of HSPs is attributed to their molecular chaperone function by facilitating nascent protein folding and refolding or degradation of abnormally folded proteins. Recently, the role of extracellular Hsp70 (also referred to as serum Hsp70) begun to be addressed and it has been proposed as a potential biomarker of healthy Aging (115,116), easily measurable in the blood by the classical sandwich enzyme-linked immunosorbent assay (ELISA) (117). Rea *et al* (118) examined serum Hsp70 in 60 individuals with ages ranging from 20 to 96 years. They demonstrated a progressive decline in serum Hsp70 levels in older age groups. Similarly, Jin *et al* (119), in their study of 327 healthy male donors aged between 15 and 50 years, demonstrated a decline in serum Hsp70 at older ages (between 30 and 50) although at younger ages, they noted a positive correlation with age. Terry *et al.*, (120) in their cross-sectional study, have assessed serum Hsp70 levels from participants enrolled in either the New England Centenarian Study (93 centenarian offspring plus 43 controls) or the Longevity Genes Project (87 centenarians plus 83 controls), showing that serum Hsp70 levels are lower in those individuals that reach an advanced age. In addition, they have suggested that low serum Hsp70 levels are associated with longevity independently on other covariates such as age, gender, race, income, alcohol, CVD, and a variety of other age-related diseases. It should be noted, however, that none of these studies, examined the changes in serum Hsp70 in the same individuals over time.

5. CONCLUSIONS

Aging is a complex process that negatively impacts the development of the different systems and their ability to function. A long life in a healthy, vigorous, youthful body has always been one of humanity greatest dreams. During the last years, an increasing number of scientific meetings, articles, and books have been devoted to anti-Aging therapies. This subject, full of misleading, simplistic, or wrong ideas, is very popular among the general public, whose imagery has been fascinated by all possible tools to delay Aging and to get immortality. Hence, the search for Aging biomarkers continues because such biomarkers would have tremendous relevance to the current push to identify drugs that ameliorate the Aging processes.

As discussed by Johnson (121), The American Federation for Aging Research has proposed the following criteria for a Biomarker of Aging:

1. It must predict the rate of Aging, exactly telling where a person is in his/her total life span.
2. It must monitor a basic process that underlies the Aging process rather than the effects of disease.
3. It must be able to be tested repeatedly without harming the person, as a blood test.
4. It must be something that works in humans and in laboratory animals. Hence, it should be tested in laboratory animals before being validated in humans.

Table 1. Biomarkers of unsuccessful aging

Unsuccessful Aging		Ref
Memory T cells	↓	14-16
Effector T cells	↑	
T cell repertoire	↓	
IgG anti CMV	↑	17,21-29
DN B cells	↑	40
CRP	↑	44,51-62
TNF	↑	60,63-78
IL-6	↑	
IL-1	↑	
WBC	↑	79,80
Telomere length	Short	81
MDA	↑	93-95
8-OHdG	↓	101-103
Plasma carotenoids	↓	113

For the explanation see the text

Table 2. Biomarkers of successful aging

Successful Aging		Ref
Naive T cells	↑	16-20
T cells function	↑	
NK function and number	↑	29,41-43
IgM	↑	31-33,40
Naive B cell	↑	
GSH-Px	↑	106,110
GSH	↑	
Serum HSP70	↓	115,118

For the explanation see the text

For over 15 years, the National Institute on Aging has supported research on biomarkers of Aging. While many important findings have developed, no biomarker has yet been identified" (http://www.infoaging.org). The general feeling of the Aging community is that biomarkers fulfilling all of the above criteria are unlikely to exist. Hence, we discussed some disease-related biomarkers (summarized in Table 1) as well as markers found in successful Aging (summarized in Table 2) useful for a better understanding of Aging and for the development of new strategies to counteract it essential for improving the quality of life of the elderly population. In fact, this kind of knowledge is useful to anti-Aging strategies aimed to slow Aging and to postpone death by preventing infectious diseases and delaying the onset of age-related diseases (55, 11, 122,123).

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