HEALTHY AGING

Constantin Munteanu[1,2], Diana Munteanu[1,2]

[1]*Romanian Association of Balneology, Romania*
[2]*National Institute of Rehabilitation, Physical Medicine and Balneoclimatology, Romania*
[c]***Email:*** *secretar@bioclima.ro*

ABSTRACT Demographic imbalance pyramids and strong growth of the population aged over 65 years are a serious challenge for the humankind [1,2,5,9] and the scientific community is called to respond to these challenges by maintaining the health of the elderly population to benefit from the positive role and experience that these people may have as emphasized also in the Healthy People 2020 program [4]. In this context, the development of bio-medical social programs for the elderly should be a priority of the government sector. In scientific terms is impetuous necessary to identify the relevant biomarkers of aging, to reveal biological targets upon which the medical act to be possible, resulting in a favorable adjustment of these mechanisms for decreasing aging rate, preventing the development of pathological processes or aging complications and giving individuals a positive social role [2].

Currently, health is understood as the removal of diseases in a defensive manner to the pathological process and with higher costs. Would be more effective the maintenance of health through prevention mechanisms identified by modern science [2,6].

The study of the mechanisms by which various natural or health factors can influence positively or negatively the aging process opens the path to design and obtain new products for the benefit of elderly people to maintain health for a long time and so to have socially active and positive role for others. Modern biotechnology allows today obtaining effective geroprotectors with medical use by which we can achieve the searched healthy-aging effect [2.12].

Analysis of numerous theories of aging [2,3] highlights several paradigm shifts of the researchers concerned with this area as a consequence of gradually scientific and technological development of the process [2]. Scientific research proposed for evaluation of the new therapeutic products will help to increase the knowledge in the field of geriatrics and gerontology, areas where Romania has played an essential role in the discovery of Gerovital by Professor Ana Aslan.

If early theories of aging were centered at the level of organ dysfunction or systemic level of the body, the current theories see dysfunction of the cellular and molecular mechanisms [10]. Such modern approach reduced the differences between the existing theories on the mechanism of aging, emphasizing their complementary elements, but at the moment there is no integrative theory for post-reproductive ontogenetic development that can explain all the processes involved in the multiplicity aspects of aging [10]

Post-reproductive ontogenetic concept based on synergistic and antagonistic interactions of genetic and phylogenetic processes involved in reproduction and development involves a reorganization of the regulatory developed evolutionary processes and ensures the body adaptation to internal and external factors, after reproductive function consumption. Thus, correction and rescheduling particular functions comes as a compensation to loss or damage of other

processes with impact in the aging mechanism [2,3].

Aging is a degenerative process [11], extensively studied for which many theories have been formulated [10] which has as a target scheduled or non-scheduled nature [2,3,10,13, 44].

Programmed aging theories are subdivided into three conceptual subcategories [2,13,14] genetically programmed longevity theory which assumes that aging is the result of starting or stopping certain genes being included the role of genetic instability (shortening of telomeres) in the dynamics of aging processes [14, 17]; endocrine theory - according to which aging is governed by a biological clock whose function is regulated by endocrine mechanisms, including insulin-like growth hormone IGF-1 plays an essential role [2,3,14]; and immunity theory which states that the immune system can be programmed by a decrease in its functionality, immunosenescence, which will lead to increased susceptibility to infectious diseases, respectively aging [15,16,25,26].

Theories which put the aging process due to internal and external environmental factors [10] are *Wear and Tear* Aging Theory according to which cells and tissues become aged over time [2,14]; vital level activity theory - which states that a high level basal metabolism leads to a shortening of the life of the organism [2,14]; cross-linking theory presented by Bjorksten (1942) according to which the cumulative chemical interchange of important macromolecules, including collagen, causes aging [2,14]; somatic DNA damage theory [47] which considers that aging results from genetic integrity degradation of somatic cell as a result of mutations registered both in the nucleus and in the mitochondria [2,13,14,44]; and free radicals theory which stipulates that the superoxide and other free radicals destroy the molecular components of cells and thus alter their normal function [11].

Reactive oxygen species (ROS) are probably the most significant free radicals with major implications in damage and cells and body aging [19,20]. Free radical theory is currently considered the most acceptable explanation of aging [19], although recent data obtained on a set of transgenic mutant SOD2 $^{+/-}$ or Mclk1 $^{+/-}$ mice disprove the central dogma of the theory [11]. In 1957 Harman proposed the idea that a general process of accumulation of oxygen free radicals negatively affects internal environmental factors and changes genetic factors, mechanism responsible for aging and death of all living things. The theory was revised in 1972 when was showed that mitochondria is the main center of some chemical reactions that generate free radicals.

Considering the aging being a gradual loss of functional adjustment of some complex multifactorial biological processes, the individual genotype sure has an impact on the rate of aging. However, there are not identified genetic markers of the aging process, although in the last 20 years, sustained efforts have been made [11,12,18].

Characterization of the rate of aging of the body employs the concept of biomarker of the aging [21,24,27], defined as quantitative or qualitative biological parameter that allows the prediction of functional status of the organism to a particular moment of its existence [22,24]. In this context, experts in the field suggest parameters that aim the age of nervous, cardiovascular, respiratory or excretory system and even advancing proposals for estimating the biological age of the cell. In humans, the biological age is surpassed by the psychological age, the intellectual age and the social age.

Specific combination of some parameters considered biomarkers of the aging process and the use of refractive

index as optical parameter to assess the biological status of the body, mathematical interpretation of the data by using a patented analysis method for bioclinical informations previously developed by members of the research team, lead us to consider that the efforts to identify the optimal set of biomarkers will represent a landmark for scientific research in the gerontology area.

Several authors have discussed the criteria for selection of some biomarkers of aging, emphasizing the following critical points that they should fulfill [23]: aging and longevity rate prediction, quantitative monitoring the biological quality of the body by parameters by which the determination will not harm the integrity of the organism, the data obtained must be reproducible and comparable for different species of laboratory animals and with relevance for humans [23,25,27,28].

Among the main biological markers used to predict the biological age and longevity, the American Federation of Aging Research suggests as the set of biomarkers of aging [27]: systolic and diastolic blood pressure, the heart rate at rest, total cholesterol, HDL and LDL cholesterol, lipid levels, blood glucose, body mass index [26], abdominal obesity index, T lymphocytes level, cortisol level and electrocardiogram.

Alostatic overstressing index [23] proposed by McEwen and Stellar 1993 denotes the body's dysfunctional response to chronic stress and reveal increased levels of adrenaline, noradrenaline and dopamine hormones [2]. This index was used by Karlamangla and collaborators in a recent study of mortality in people aged over 70 years, pursued over a period of 4.5 years. Patients with an alostatic overstressing increased index present a much higher risk of mortality. Thus, the study concludes that a uniform increase of alostatic overstressing index (normally located between -1.7 and 1.4) leads to an increase of 3.3 times in mortality measured in the range of 4.5 years.

The so-called **matrix protocol** [2] of aging biomarkers used by the International Institute of Longevity Montclair (New Jersey) determines the rate of aging on four levels:

I. the overall functionality of the body measured by the ratio of weight and level of fat, flexibility, aerobic endurance, bone density, tactile response latency, forced expiration volume, vision and hearing [27];

II. functioning at the cellular level monitored at the skin level by determining the cytoarchitecture changes of basal membrane, epidermal turn-over, sebaceous glands architecture, microvascular changes, collagen ratio, elasticity of the fibers [28];

III. the molecular level consider the growth and thyroid hormone level but also of coenzyme Q10, sensitivity to insulin, the heat shock proteins level, the oncogenes level in the blood, serum level of antioxidants [25];

IV. at DNA level is studied the position and size of telomeres (set at WBC level) and the accumulation level of mutations in the genetic material.

The researchers from the International Longevity Institute are currently working on a new laboratory chemistry test that should reflect the damage level of DNA and should bring science-based predictions regarding the therapeutic value of anti-aging interventions through the mutational changes occurring in the somatic genetic material [2, 28, 29].

Telomeres damage can be assessed indirectly by measuring some biomarkers that correlate with their shortening [28]. Thus, Jiang and Rudolph analyzed the telomeres shortening mechanism and the rate of aging in several organs and tissues, identifying four proteins whose expression increases in relation to telomere

shortening, as these: Cathelcidines from macrophages and polymorphonuclear leukocytes lysosomes activated by bacterial infection; Chitinaza 3 like3 (Chi 3L3) involved in the initiation of immune response; elongation factor 1α (EF-1 α) - which controls protein synthesis in human fibroblasts and Stathmins - which control the intracellular microtubules stability, cell motility and mitosis [2].

The concept of oxidative stress caused by free radicals or generation systems of such radicals in concentrations that exceed antioxidant defenses capacity, represents the basis of taking into account as biomarkers of aging, physiological and molecular indicators of oxidative stress. Carbonylate proteins represent, in this context, an example of the use of oxidative stress indicators as biomarkers of aging [2, 25].

Many scientists consider the accumulation of carbonyls proteins in erythrocyte membrane as an indirect marker of aging and the ferro-reduction antioxidant potential (FRAP) in blood plasma as an indicator of the body's antioxidant defense capacity [2]. The oxidative and reductive activity of enzymes that act on glutathione, thioredoxin and other substrates of interest in the oxidation-reduction process reflects not only the level of antioxidative protection but are probably relevant biomarkers for aging rate.

It has been suggested that microheteroplasmy could explain some of the pathological mechanisms related to aging such as diabetes, cardiovascular diseases, Parkinson, Alzheimer, cancer [2,23].

Mobile genetic elements (MGE) for whose description geneticist Barbara McClintock received the Nobel Prize in 1983 have been proposed as components of genetic instability and on these considerations as factors of cellular senescence for many species, with effects that include impaired gene expression and age-related disorders in cell physiology, blocking cell growth and eventually cell death or blastic transformation. According to this hypothesis, aging is correlated at the cellular level with the trasposonic activity whereby a DNA sequence copy remains at its predetermined site in nucleus while the second copy, called transposon, can move to different locations in the genome [2].

NADH and FADH coenzymes - critical components of energetic transport in mitochondria may represent, by their expression level, biomarkers of mitochondrial activity and indirectly of senescence and body age. Their measurement can be made currently by polarizing microscopy techniques or by fluorescence [2].

Heat shock proteins (HSP) are other biomarkers of aging, and there are studies showing that the change of these proteins expression correlates with changes in longevity, experimentally described in *Caenorhabditis elegans* [2].

Apolipoproteins synthesized in the liver and intestine are involved in the transport of cholesterol and triglycerides in the blood, are considered more eloquent predictors of death by coronary heart disease [23]. Particularly, apolipoprotein A1 that transports HDL cholesterol shows an inverse correlation with the risk of death by coronary heart disease, a high level being associated with a decreased risk while apolipoprotein B responsible for LDL-cholesterol transport has a direct correlation, the high level indicating a high risk of death. The ratio of the two apolipoproteins is, therefore, an influential indicator for the rate of aging [23].

Interleukin 6 - IL6 is a multifunctional cytokine that plays an essential role in acute inflammation stage. Although normally in the absence of inflammation can not be detected in blood samples over time by losing his regulation

expression, the expression level increases with age [22,23].

Growth hormone, secreted by the pituitary gland has a decreasing level with age of the body being registered a decrease by 14% every decade after the age of 20-25 years and reaches half around the age of 60 [2, 21,22,26].

Measuring the refractive index of a biological sample [96] is a relevant optical parameter used in the diagnosis and treatment [95], being an indicator of changes in the level of hydration, of the biological age of the sample or the level of intracellular dysfunction [93]. The refractive index measured in the serum of Wistar rats shows a time-dependence being measured an increase of 1,34136 from birth to 1.35175 (250g) to adulthood [94]. The effective refractive index of a single cell [101] is determined by the refractive index of cellular constituents, and its determination provides us data on the size and volume of the cell, nucleus volume, setting of certain dysfunction at the cellular level [97,99,100].

Using the Kramers-Kronig relations allows quantifying time dependent changes of the refractive index starting from reflectance measurement of samples that can be made both in biological serum samples or culture medium [99] but also at the skin surface [98]. Therefore, we can consider as a biomarker of aging the refractive index of blood serum and in the skin and will correlate the application of the developed treatments in the project with the change of this index.

Lifespan extension possibility was experimentally demonstrated for many geroprotector substances, including antioxidants [75], latirogene, chelating agents (carnosine [53]), adaptogens, neurotropes, monoamine oxidase inhibitors, dehydroepiandrosterone, sexual hormones, growth hormone, melatonin [50], preparations from the pineal gland, protease inhibitors, antidiabetic agents, thymic hormones, immunomodulators, superoxide dismutase and catalase mimetics, enterosorbents, etc.. [47]. According to several authors, there are not geroprotectors with antiaging effect with unquestioned efficiency, scientifically proved, there are not a few cases of supposed geroprotector substances whose long term effects have proved difficult to predict initially [2].

In the past years, a new area of the gerontology becomes important, that of studying the so called HORMESIS mechanisms by which substances classified as toxic or very harmful, in subliminal doses, may have geroprotector effects, currently being numerous scientific investigations undertaken this regard [2, 35].

Reducing calorie intake has been shown experimentally to be a certain antiaging behavior [31,58]. Studying the mechanisms by which calorie intake reduction influences senescence and longevity rate in experimental animals [82], researchers have correlated this required behavior with reduce plasma levels of insulin [81], increase insulin receptor sensitivity, low body temperature, cholesterol, triglycerides, alpha-lipoproteins, blood pressure and an increase in HDL cholesterol. Setting all these processes is experimentally correlated with the expression of some genes called sirtuins existing phylogenetic from yeast and to humans [39]. Sirtuins are enzymes such as deacetylase and ADP-ribosyltransferase, whose name derived from *Saccharomyces cerevisiae*, where they were discovered. In mammals sirtuins family consists of seven genes of this type, called Sirt1 to Sirt7. Recent researches seek to identify their role in cell physiology and mechanisms or molecular agents by which can be influenced their activity [31,32].

In 2011 there have been globally described 30 substances with geroprotector

properties, along side Gerovital - Romanian geroprotector product prepared by Prof. Ana Aslan in 1951, based on anesthetic procaine, noteworthy resveratrol and other polyphenols of plant origin [30], Rapamycin, antioxidants (vitamins A, C and E, carotenoids, lipoic acid, coenzyme Q, selenium, etc.), hormones (GH, thyroid hormones, adrenal and sexual hormones, melatonin [50]), bioregulatory peptides (timalin, epithalmin), biguanides (metformin, fenformin) [77], adaptogens (ginseng [48]).

Sirolimus or rapamycin, described as immunosuppressive and antitumoral drug has been shown to be also an anti-aging drug [41] whose goal is the TOR gene in yeast [43] and mTOR gene, its mammalian correspondent, also known as FK506 factor or FRAP1, a serine/threonine kinase that regulates cell growth, viability, mobility, protein synthesis and gene transcription [31,45, 78]. Rapamycin, according to recent studies, may act preventively in atherosclerosis, hypertonia and hypercoagulation, thus preventing myocardial infarction, cardiovascular accidents, osteoporosis, cancer, autoimmune diseases, arthritis, fat diabetes, Alzheimer and Parkinson diseases [31,42,45].

Data from the specific literature shows that biguanides, particularly metformin [56.77], used in the treatment of type 2 diabetes due to their ability to inhibit gluconeogenesis and to determine the increase of sensitivity to insulin may also have geroprotective effects [56].

2 deoxyglucose [78] is a glucose analog that absorbed by cells determine glucose inhibition resulting in decreased cellular energetic metabolism, situation that mimics calorie restriction. Data concerning the geroprotectection of this substance is not yet relevant.

Antioxidants such as α-tocopherol, ascorbic acid, retinol, ubiquinone, selenium, as endogenous compounds or numerous synthetic molecules, intervene in gerossuppresion based on the aging free radical theory [87].

Resveratrol is the best known current geroprotector, discovered in 2003, present in high concentrations in the epicarp, seeds and stalk of grapes and subsequently in red wine [30,33], for which Romania is famous and is specified in collections, among other things, or in roots of *Polygonum cuspidatum* [2]. It is a natural phytoalexin used by certain plants in defense mechanism against pathogens such as bacteria and fungi, currently extensively studied, and that can influence the expression of sirtuins [35,37]. Since its discovery in 2003 until today there were registered worldwide over 2,000 experimental studies on resveratrol, and there are laboratories focused on the study of this substance [2].

Preclinical studies on resveratrol demonstrated an increase in the longevity of *S. cerevisiae* of 70% by cultivation in a medium containing 10 mM resveratrol, of 20% in *C. elegans* and 29% in *Drosophila melanogaster* by treatment with 100 mM resveratrol [39, 40].

In studies on laboratory mice, resveratrol at doses around 20 mg/kg caused a statistically significant decrease of age-related parameters such as albuminuria, the inflammatory level, vascular endothelial apoptosis, decreases in the elasticity of the aorta, the incidence of cataract, etc., being inclusively registered data towards the decrease of genetic instability [2].

On MRC5 human fibroblasts, the resveratrol in concentration of 5 μm induced a significant protection of DNA oxidative deterioration, preventing the growth of nuclear volume, reducing the generation of acetylated forms of H3 and H4 histones and p53 protein. In another study on human fibroblasts, using a concentration of 10 μm and 25 μm have

been obtained data supporting the delay in the morphological alterations at the cellular level correlated with age [2].

It is supposed that polyphenols such as resveratrol inhibits senescence at the cellular level by activating genes such as p53 and AKT [39], sirtuins or inhibition of others, like mTOR [46]. These influence different intracellular signaling pathways by which are controlled the expression of genes involved in cell growth, the proliferation and cell viability [34].

Clinical trials regarding resveratrol action in oncology that used a commercial form of resveratrol called SRT501 showed a 39% increase in apoptosis of malignant cells in patients with metastatic colorectal cancer [2].

Neuroprotective effects of resveratrol [30,35] have been described in experimental studies on laboratory mice and were explained by researchers by the effect of resveratrol of increasing the cysteine level that can protect cells from oxidative stress by regulating proteic precursors of amylase plate [38]. Resveratrol acts also on manganese superoxide dismutase (MnSOD) a group of enzymes that degrades metabolic generated superoxide molecules thus having antioxidant effect [80].

Cardioprotective effects of resveratrol [30,36], as well as the other polyphenolic antioxidants such as quercetin or catechins, was observed in *in vitro* studies that showed a reduction of cardiomyocyte apoptosis by decreasing the level of caspase-3 and other cytokines, including NF-KB2, E-selectin, TNF-α or troponin [2].

Resveratrol also has an anti-inflammatory effect [30,33], resulting in decreased activity of cyclo-oxygenase with a key role in the synthesis of other cytokines such as IL17.

The theory of using antidiabetics like resveratrol [30] is explained by activating SIRT1 and subsequent increase in sensitivity to insulin [32], improving microcirculation and peripheral nerve function [2].

Resveratrol acts on the cellular mechanisms involved in photo-aging correlated with UV action, including MAP kinases, NF-kB nuclear factor and matrix metalloproteins. External applications of resveratrol in SKH-1 hairless mice model prior to UV exposure caused a significant reduction of cell proliferation, protection mRNA and phosphorylation [30].

The pharmacology of resveratrol [34] is still marked by a number of limitations, having a low solubility in water and consistently a low bioavailability and stability, being easily oxidized in the presence of light or heat. There are even data that contradict the fact that resveratrol would determine the extent of longevity, obtained on *Drosophila melanogaster* and *C. elegans* and disseminated by some authors [36].

Among the many hormones above mentioned with geroprotector effects [49], greater relevance for this project have the substances with similar actions of sexual hormones. Substances such estrogen like, testosterone like, which stimulate or inhibit various neuroendocrine mechanisms [49] are detected in the organic component of therapeutical muds and their geroprotective action is currently intensively studied.

The testosterone serum level gradually decreases along with the advancing in age which caused a progressive muscle atrophy, fatigue, osteoporosis, reduced sexual function and increased fat levels, all this can be treated by administration of testosterone [22], which has as effect, in addition to a recovery of lipid levels also a decrease of concentration of plasma glucose correlated with increased sensitivity to insulin.

The last half of the century allowed due to numerous scientific results, the use of estrogen in postmenopausal women to

prevent signs of aging, such as osteoporosis, cardiovascular diseases, decrease sexual and cognitive functions [52].

However, the enthusiasm was diminished by the recent publication of the results of some studies that show the risks of using estrogen, including breast cancer, pulmonary embolism, stroke and coronary heart disease. In this context, we can discuss by the importance of phytoestrogens and estrogen like substances from peloids composition whose effects does not reach the mentioned risks [2, 55].

Investigating the physiological effects of Procaine, an anesthetic [79,83,54] from Gerovital composition on bee neurons cultures, showed that this acts on Na^+ voltage- dependent channels or ligand-dependent channels [51].

Procaine has known neuroprotective properties, preventing the formation of amyloid plaques and preserving ATP depletion, most likely by inhibiting neurotoxic effect of glutamate, like has been shown in PC12 rat cells cultures [76].

Procaine is used in cardioplegia and in stabilizing the postischemic cardiac rhythm after the aortic decompression in cardiac surgery [84]. Anti-atherogenic effects of Gerovital and Aslavital were confirmed by recent studies regarding the LDL oxidation and inhibition of the generation of superoxide radicals, thus having antioxidant properties by which interfere with cellular and molecular mechanisms of aging [85,86].

The amount of procaine hydrochloride from Gerovital H3 - tablets / vials and Aslavital H3 - tablets / vial is 100 mg plus 6 mg of the benzoic acid, 5 mg of potassium metabisulfite and 0.5 mg biphasic sodium phosphate. The preventive treatment involves taking two tablets / day at 2-3 hours after meals, for 12 days. The treatment is resumed after a break of one month. Parenteral the treatment is made 3 times/week for 4 weeks. Treatment is resumed after a break of 1-2 months. A combination therapy is recommended - 4 weeks injection (12 vials) 30 days break followed by a 12 days treatment with tablets. After 24 days, a new course of treatment can be started.

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