Mediators of Inflammation

Cellular Senescence and Inflammaging in Age-Related Diseases

Lead Guest Editor: Carmela R. Balistreri
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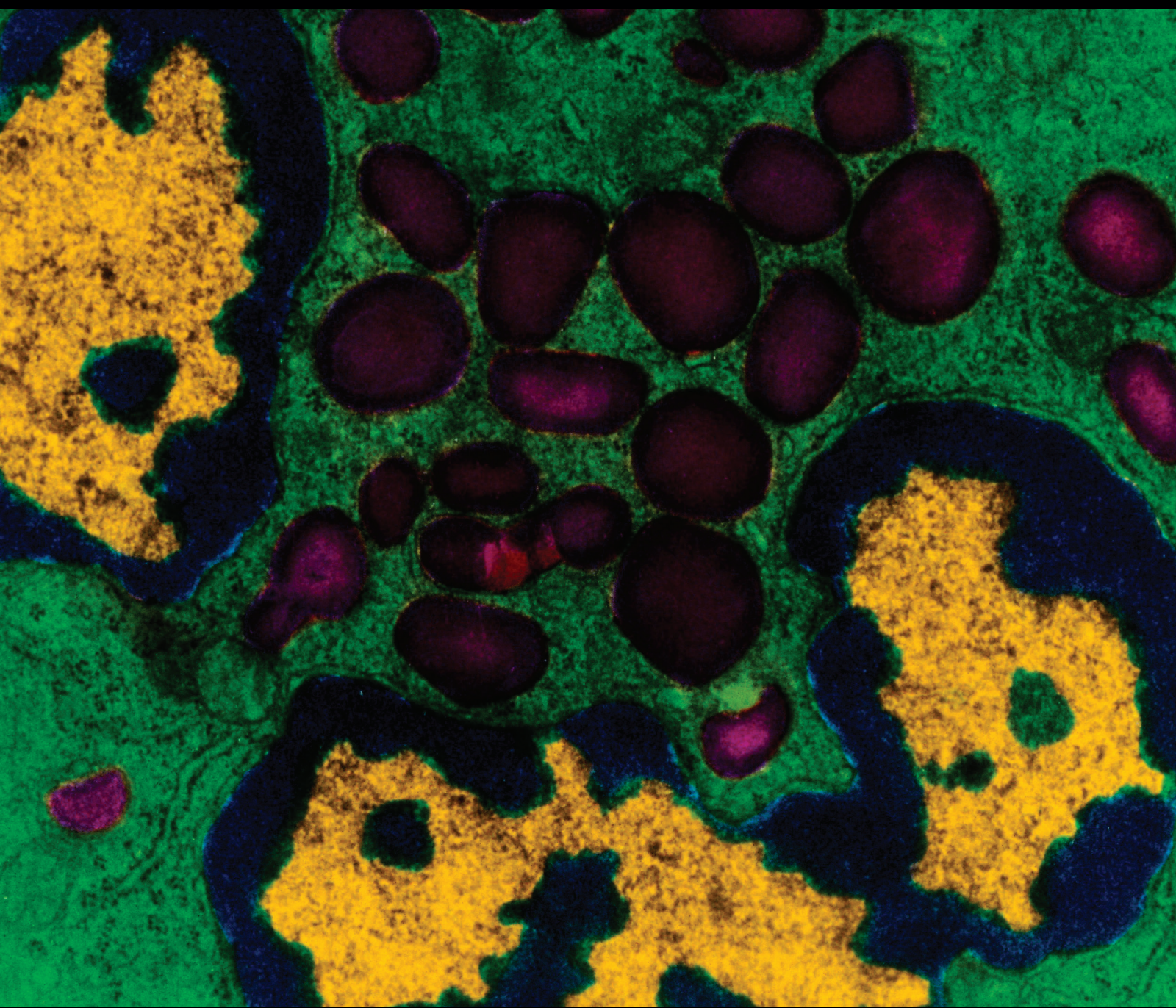

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Editorial Board

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Mediators of Inflammation
Volume 2018, Article ID 9076485, 6 pages
https://doi.org/10.1155/2018/9076485

Editorial

Cellular Senescence and Inflammaging in Age-Related Diseases

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Received 15 February 2018; Accepted 15 February 2018; Published 17 April 2018

The term *cellular senescence* was used in 1961 for the first time by Hayflick and Moorhead [1], to define the mechanism determining the irreversible loss of the proliferative activity of human somatic cells [1]. In this respect, cellular senescence has been usually viewed as one of the regulatory mechanisms able to stop the eventual uncontrolled proliferation of old and injured cells, mediating an activity likely similar to a tumour suppressor gatekeeper [2]. Accordingly, oncogene overexpression (i.e., *H-ras*) in primary cells is sufficient to trigger cellular senescence, a phenomenon defined *oncogene-induced senescence* [2]. Indeed, negation or bypass of oncogene-induced senescence is considered an obligatory step to develop a tumour. Later, other groups discovered that cellular senescence also occurs in differentiated cells exposed to several stressors, mainly DNA-damaging agents such as prooxidant molecules, radiation, and chemotherapeutics [3–5]. Increasing evidence has highlighted various physiological roles of cellular senescence, consequently providing a putative evolutionary explanation for this biological phenomenon. For instance, in 2013, Muñoz-Espín et al. and Storer et al. groups underlined a crucial role for cellular senescence in mammalian embryonic development by studying mouse, chick, and human embryos [6, 7]. In this context, senescence promotes tissue remodeling and it seems to represent the "*father evolutional mechanism*" of *damage-induced senescence*. In 2014, Demaria et al.'s group [8] showed that senescent fibroblasts and endothelial cells appear very early in response to a cutaneous wound, where they accelerate wound closure by inducing myofibroblast differentiation through the secretion of platelet-derived growth factor AA (PDGF-AA). In addition, it has been also demonstrated that *forced cellular reprogramming* induces senescence, and senescent cells (SCs) create a permissive environment for reprogramming itself [9]. This evidence leads to an interesting hypothesis based on the concept that SCs act as a trigger of tissue remodeling, following a precise regulated succession of events, characterized by the arrest of their proliferative activity and release of mediators to recruit innate immune cells, consequently promoting tissue regeneration and their elimination. These steps may result uncompleted in old tissues or in pathological circumstances, determining the accumulation of SCs [9, 10]. Accordingly, an increased number of SCs characterizes the old tissues during aging and age-related diseases (ARDs), including cardiovascular diseases (CVDs), type 2 diabetes (T2DM), musculoskeletal disorders, various types of cancer, and neurodegenerative diseases. Thus, cellular senescence happens in both physiological (i.e., embryonic development, wound healing, and tumour suppression) and pathological processes (i.e., ARDs), by providing beneficial effects early in life and deleterious consequences later in life, in association with the accumulation of SCs in different tissues and organs. The different age-tailored effects played by SCs can be explained by the *theory of antagonistic pleiotropy* formulated by Williams [11]. This vision is corroborated by the observation that selective removal of SCs is sufficient to sensibly increase the lifespan of normal, genetically heterogeneous

mice [12, 13]. These findings prompted the research of substances able to selectively promote the clearance of SCs, that is, senolytics (see below) [14, 15]. Some of these treatments have been demonstrated to delay the onset of ARDs and consequently to extend the healthspan (i.e., the length of time one lives in good health) in mice [15–18].

Despite the accumulating evidence regarding the contribution of SCs to the aged phenotype, the mechanisms through which SCs drive aging have not been fully elucidated, as well as their effective relationship with the major ARDs in humans. To this purpose, the lack of universal biomarkers for senescence *in vivo* has possibly contributed to hinder the detection of SCs in humans. However, growing evidence shows that SCs exert detrimental effects on the tissue microenvironment, generating pathological facilitators or aggravators (amply quoted in [19]). Accordingly, it has been suggested that SCs contribute to aging and ARD onset through the *senescence-associated secretory phenotype* (SASP), which consists in the secretion of a variety of soluble factors, such as proinflammatory mediators and matrix-degrading molecules [19]. SASP contributes to fuel a state of chronic, systemic, low-grade inflammation, called "*inflammaging*," which is one of the main risk factors for the development of the major ARDs [19]. The rate of reaching the threshold of proinflammatory status over which diseases/disabilities ensue depends on a complex interaction between genetic, environmental, and stochastic factors [19]. Immune cells, especially macrophages, emerge as key players in the induction and maintenance of inflammaging [20, 21]. As a result, it might be hypothesized a typical phenomenon of "*macrophaging*," which could contribute to reduce the clearance of SCs [19–22]. In turn, the age-related SC accumulation promotes immune system activation, and the consequent chronic immune induction is associated with a reduced SC clearance. As a result, this continuous response perpetuates a vicious circle that fuels inflammaging. Beyond immune cells and tissue cells, also adult stem cells from aged humans are affected (mesenchymal stem cells included). This evidence suggests that a senescent milieu could also reduce the stemness properties [22] or the differentiation capacity [23]. At the same time, these discoveries underline the specific actions and different features of SASP in the various tissues. However, the characterization of SASP is not complete yet: (i) what type of components are released like proteins (cytokines, chemokines, tissue remodelling factors, etc.), RNA, or DNA within or outside of extracellular vesicles, (ii) which are the SASP components derived from different cell types when senescent (fibroblasts, epithelial cells, endothelial cells, etc.), and finally (iii) which are the biological functions of various *secretomes* associated with senescence in different tissue microenvironments.

Altogether, this evidence suggests that inflammaging, sustained and perpetuated by a plethora of exogenous and endogenous stressors, is promoted by the accumulation of SCs during aging and perpetuated by both SASP spreading at systemic level and the SASP-associated bystander effects [24]. Importantly, SCs are abundant at all sites of ARDs, including not only malignancies but also degenerative disorders (above mentioned), suggesting that chronic inflammation induced by SCs might be a main driver of these pathologies [25, 26]. Accordingly, we recently investigated the "senescence" of endothelial cells (ECs) and the consequent endothelial dysfunction as one of the main trigger involved not only in the onset and progression of CVDs but also of other ARDs like osteoporosis [24, 27], since ECs are components of the stroma of all tissues and organs [28–30].

The abovementioned observations, about the clearing of senescent cells (SCs) from mice and its capacity to prolong their lifespan and healthspan, have opened a new era in the field of geroscience, with the creation of a new pharmacological branch devoted to *senotherapeutics*, that is, drugs able to affect (in a wide sense) the senescence process. Senotherapeutics currently includes three therapeutic approaches: (i) molecules able to selectively kill SCs, that is, *senolytics*; (ii) compounds with the capacity to attenuate the proinflammatory program of SCs, that is, SASP suppressors, or that modify the senescent phenotype, that is, senomorphics; and (iii) prevention of the accumulation of senescent cells. The latter is probably the "oldest" approach, since a plethora of antioxidants have been shown to delay the senescence process *in vitro* [31]. However, these findings have been hardly translated in promising findings, *in vivo* models, since no "usual" antioxidant (e.g., C and E vitamins) consistently increases lifespan or healthspan in mice models [31]. Moreover, data derived from human cohorts further suggest the inability of such compounds to prevent the major ARDs, even if consistent differences can be observed depending on the molecule tested [32]. Interesting preclinical findings have been reported for both senolytics and SASP-suppressing compounds. The development of senolytics has emerged thanks to both wide pharmacological screenings and differential gene expression studies. In addition, it has been optimized to identify *survival pathways* exploited by senescent cells to survive the proinflammatory milieu, where *they live in* (i.e., the SASP) [15, 33]. At present, five major senescent cell anti-apoptotic-pathways *(SCAPs)* have been identified and successfully targeted, that is, Bcl-family proteins, PI3K-Akt, p53, ephrin-tyrosine kinases, HIF-1α, and HSP90 pathways [33, 34]. The field is rapidly expanding, and the information provided by new technologies, for example, single-cell RNA-seq, is expected to provide more insights into the heterogeneity of SCs in order to facilitate the development of new senolytic drugs [35]. Interestingly, different pathological phenotypes associated with aging have been targeted with various senolytics in mice models, that is, atherosclerosis, osteoporosis, and osteoarthritis. On the other side, a valuable alternative is represented by SASP-suppressing drugs. The major antiaging molecules, with accepted lifespan and healthspan promoting activity in mice, have been found to suppress the SASP, that is, rapamycin and metformin. Precisely, the activity of rapamycin has been explained through the well-known role of mTOR pathway in regulating SASP-factor secretion, while the mechanisms underlying the metformin's action on the SASP are until now unclear. However, both molecules are increasingly recognized as broad anti-inflammatory compounds. Another class of molecules with peculiar SASP-suppressing activity is JAK inhibitor, with interesting results obtained in terms of alleviation of frailty

symptoms and amelioration of dysglycemia in old mice [36]. From a pharmacological point of view, senolytics have the advantage that noncontinuous, intermittent treatment appears as sufficient to produce a tangible effect, while with SASP suppressors, long-term treatment is often needed [33, 34]. However, at present, most of the drugs successfully tested in mice are compounds with an unfavourable profile of toxicity in humans (e.g., chemotherapeutics and immunosuppressors). Thus, their translation to clinical trial testing should be limited to specific situations, while a drug appropriate for a whole-population use is far to be discovered. At present, metformin appears as the most likely candidate for such use.

Preclinical data have uncovered a potential role for SCs in the promotion of a wide range of ARDs. Consistently, senolytics treatment has been associated with a plethora of beneficial effects: (1) improved cardiac ejection fraction in old mice; (2) enhanced vascular reactivity in old mice; (3) decreased vascular calcification, increased vascular reactivity, and reduced senescence burden in the plaque of apoE$^{-/-}$ mice; (4) decreased frailty, osteoporosis, and loss of intervertebral disc glycosaminoglycans in progeroid mice; (5) decreased gait disturbance in mice after radiation damage to a leg; (6) attenuated haematological dysfunction caused by whole body radiation; (7) increased coat density; and (8) improved pulmonary function and reduced pulmonary fibrosis in mice with bleomycin-induced lung damage [33, 34]. These observations are consistent with the hypothesis that SCs drive organism aging. Thus, their targeting must benefit more than one age-related phenotype, counteracting aging itself as a whole rather than one ARD at a time.

As mentioned above, newly discovered senolytics molecules are drugs already present in the market and used for the treatment of a wide range of life-threatening diseases, for example, various types of cancer. Thus, they are often accompanied by a range of potential side effects. This implies that the repurposing (or the extension of clinical indications) of these drugs should be limited to conditions where the possible benefits overcome the potential risks. Kirkland and colleagues have proposed a precise hierarchy of possible clinical settings, where to test such compounds [34]. Potential clinical trials are the following: (1) simultaneous attenuation of multimorbidity; (2) alleviation of potentially fatal diseases, for example, pulmonary fibrosis; (3) treatment of conditions with localized senescent cell accumulation, for example, osteoarthritis, possibly through local delivery of the drug to limit systemic toxicity; (4) treatment of conditions characterized by accelerated aging, for example, patients exposed to radio- and chemotherapy, HIV-infected patients, and progeroid syndromes; (5) increasing physiological resilience, that is, the capacity to recover after a stress, in the case that the stressor is known to promote the accumulation of senescent cells; and (6) alleviation of frailty [34]. The discoverers of senolytics have announced their intention to test these molecules in humans in specific setting in the next future [37]. However, at present time, the only scheduled antiaging trial is the Targeting Aging with Metformin (TAME) trial [38]. Despite the lack of a univocal mechanism of action for this drug, the evidence of a cardioprotective, cancer-preventive, and lifespan/healthspan-promoting activity for metformin is strong enough to justify an extension of its use to non-diabetic subjects. Data from human cohorts have clearly disclosed a marked anti-inflammatory effect of this compound, rendering metformin the ideal candidate to counteract inflammaging [38–40]. In addition, its favourable toxicological profile surely supports a "widespread" use of this drug, since the only serious adverse effect, that is, lactic acidosis, has an incidence of less than 10 episodes per 100,000 person-year (and it affects mainly people with renal impairment). The TAME trial will involve 3000 patients with at least one ARD, aged 65–79, and will measure a composite outcome that includes cardiovascular events, cancer incidence, dementia, and mortality. Functional and geriatric endpoints will be assessed too. While waiting for the results, it is worth mentioning that this trial represents the first case of intent to treat aging as a disease. In the case of positive results, this trial will pave the way for a revolution in the management of elderly subjects, with preventive medicine able to postpone ARD development instead of punctual medicine treating each disorder as a stand-alone entity.

In this issue, a large range of current directions in research about this topic will be described and discussed. Thus, we think that the papers of this issue could be of interest to the readers of this journal.

The group of E. Dozio et al. investigated, for the first time, the role of vitamin D status in patients with acute aortic dissection (AAD), since evidence sustains an inverse association between serum 25-hydroxy vitamin D (25OHD) levels and the onset of several cardiovascular conditions, such as aortic aneurysms and thoracic aortic dilatations. Interestingly, a condition of hypovitaminosis D associated with an increased osteocalcin levels was observed in the study population. Thus, these molecules may be helpful to identify individuals at high risk to develop AAD as well as to study preventing strategies.

The importance of 25OHD, in maintaining of healthy status of all our body's systems with advancing age, is also emphasized by L. Elizondo-Montemayor and coworkers. Precisely, they performed a longitudinal 12-month follow-up study in order to determine the vitamin D seasonal changes and their association with anthropometric parameters, lifestyle factors, and proinflammatory cytokines in an older adult Mexican population. Their results show a great prevalence of vitamin D deficiency and insufficiency across all seasons, with significantly greater prevalence of deficiency in winter compared with summer and autumn. Vitamin D levels were negatively correlated with BMI, waist circumference, and weight, as well as with gender differences and TNF-α levels. While WC explained almost half of the variations in vitamin D levels in women, BMI was the second significant predictor of vitamin D. However, neither dietary vitamin D intake nor sun exposure affected 25OHD levels.

The group of E. Dozio et al. also evidenced, in a cross-sectional study, that diabetic patients, with chronic kidney disease complication on dialysis (CKD-G5D), show significant increased levels of soluble receptor for advanced glycation end products (AGEs; sRAGE). Thus, they concluded that sRAGE may be a marker of cardiac remodeling. Indeed, its increase could be a potential protective mechanism

against the increased risk of cardiovascular complications related to AGEs and inflammation.

According to data above described, a dramatic increase of CVDs characterizes Western populations, because of aging population phenomenon. The group of L. Iop et al. summarizes the intrinsic and extrinsic causes related to cellular vascular senescence and their role in the onset of cardiovascular pathologies. Additionally, they dissect the effects of aging on the cardiac endogenous and exogenous reservoirs of stem cells. Finally, they offer an overview on the strategies of regenerative medicine that have been advanced in the quest for heart rejuvenation.

Among the exogenous risk factors linked to cellular vascular senescence, the diet represents an emergent CVD inductor. The group of M. Malavolta et al. examined, in a research study, the effects of postprandial sera derived from healthy adults and elderly volunteers who consumed meat meals on human coronary artery endothelial cell (HCAEC) oxidative stress, gene expression, DNA damage, and cellular senescence. They observed that a single exposure to postprandial serum induces a slight increase in ROS that is associated with modulation of gene expression pathways related to oxidative stress response and metabolism. The postprandial-induced increase in ROS is not associated with a measurable DNA oxidative damage. However, repeated exposure to postprandial serum induces an acceleration of cellular senescence. Taking into account the deleterious role of cellular senescence in ARDs, the results suggest a new mechanism by which excessive meat consumption and time spent in postprandial state may affect health status during aging.

The above-described effects are mediated through epigenetic factors. The group of A. Giuliani et al. focused its attention on the deep reshaping of microRNA expression and modulation of mitochondria activity, both master regulators of the SASP in tissue aging. In particular, they propose a network linking nuclear-encoded SA-miRNAs to mitochondrial gene regulation and function in aging cells. In this conceptual structure, SA-miRNAs can translocate to mitochondria (SA-mitomiRs) and may affect the energetic, oxidative, and inflammatory status of senescent cells. They discuss the potential role of several of SA-mitomiRs (i.e., let-7b, miR-1, miR-130a-3p, miR-133a, miR-146a-5p, miR-181c-5p, and miR-378-5p), using miR-146a as a proof-of-principle model. Finally, they suggest a comprehensive, metabolic, and epigenetic view of the senescence process, in order to amplify the range of possible approaches to target inflammaging, with the ultimate goal of decelerating the aging rate, postponing or blunting the development of age-related diseases.

The onset of ARDs, CVDs included, also is significantly linked to an impaired immune system (as mentioned above), which shows in old ages several changes, defined as immunosenescence. At present, all molecular and cellular mechanisms involved still remain to be identified. However, the human immunodeficiency virus (HIV) infection and the consequent acquired immune deficiency syndrome (AIDS) represent an optimal model for studying immunosenescence. T. Sokoya and coworkers describe in a review the role of systemic immune activation in the immunopathogenesis of HIV infection, its causes and the clinical implications linked to immunosenescence in adults, and the therapeutic interventions that have been investigated.

Furthermore, the group of F. Sizzano et al. from Nestlè Institute of Health Sciences investigated for the first time the response of the main lymphocyte subsets to the induced oxidative stress in semi-super-centenarians (CENT), their offspring (OFF), elderly 52 controls (CTRL), and young individuals (YO), by using flow cytometry. Results showed that the ratio of the ROS levels between the induced and noninduced (I/NI) oxidative stress conditions was higher in CTRL and OFF than in CENT and YO, in almost all T, B, and NK subsets. Moreover, the ratio of reduced glutathione levels between I/NI conditions was higher in OFF and CENT compared to the other groups in almost all the subsets. Finally, they observed significant correlations between the response to the induced oxidative stress and the degree of methylation in specific genes on the oxidative stress pathway. Globally, these data suggest that the capability to buffer dynamic changes in the oxidative environment could be a hallmark of longevity in humans.

According to immunosenescence evidence, the group of E. Costantini et al. reports in a review the link between immune and nervous system and how the immunosenescence and inflammaging can contribute to neurodegenerative diseases.

Changes in endocrine system also characterize old people and are significantly associated with ARD onset. In particular, thyroid dysfunction and its impact on cognition in older individuals have been demonstrated, even if some aspects remain unclear. The group of G. Traina et al. investigated the changes which accompany mouse thyroid gland in old age. The results obtained evidence changes in the height of the thyrocytes and in the amplitude of interfollicular spaces, anomalous expression/localization of thyrotropin, thyrotropin receptor, and thyroglobulin aging. Thyrotropin and thyrotropin receptor are upregulated and are distributed inside the colloid while thyroglobulin fills the interfollicular spaces. Furthermore, they found a higher expression of galectin-3 and a delocalization of neutral sphingomyelinase in the thyroid of old animals.

In order to counteract ARD onset and complications, several anti-inflammaging/anti-ARD treatments are emerging, ranging from pharmacological targeting of aging, basic biological assays, and big data analysis to the recent use of young blood, stem cells, cellular, genetic, and epigenetic reprogramming, or other techniques of regenerative medicine. C. R. Balistreri summarizes them in a review, stressing that only a little fraction of these approaches have the features for being tested in clinical applications. Thus, she describes new emerging molecules, drugs, and procedures, by evidencing potential benefits and limitations.

Among these approaches, the use of senolytics molecules emerges. Accordingly, the group of M. Malavolta et al. also summarizes in a review the i*n vitro* and *in vivo* effects of fifteen Nrf2- (nuclear factor erythroid-derived 2-related factor 2 pathway-) interacting natural compounds (tocotrienols, curcumin, epigallocatechin gallate, quercetin, genistein, resveratrol, silybin, phenethyl isothiocyanate, sulforaphane, triptolide, allicin, berberine, piperlongumine, fisetin, and

phloretin) on cellular senescence and discusses their use in adjuvant cancer therapy.

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Johannes Grillari
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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 6039171, 12 pages
https://doi.org/10.1155/2018/6039171

Review Article

The Role of Immunosenescence in Neurodegenerative Diseases

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Received 29 September 2017; Revised 15 December 2017; Accepted 17 January 2018; Published 8 March 2018

Academic Editor: Francesco Prattichizzo

Aging is characterized by the progressive decline of physiological function and tissue homeostasis leading to increased vulnerability, degeneration, and death. Aging-related changes of the innate and adaptive immune system include decline in the preservation and enhancement of many immune functions, such as changes in the number of circulating monocytic and dendritic cells, thymic involution, T cell polyfunctionality, or production of proinflammatory cytokines, and are defined as immunosenescence. Inflammatory functions are increased with age, causing the chronic low-grade inflammation, referred to as inflamm-aging, that contribute, together with immunosenescence, to neurodegenerative diseases. In this review, we discuss the link between the immune and nervous systems and how the immunosenescence and inflamm-aging can contribute to neurodegenerative diseases.

1. Immunosenescence

In the last century, the human lifespan has increased and so has the number of elderly people in the world. Aging is a complex process that occurs in every organism and is induced by genetic, epigenetic, and environmental factors [1]. It is characterized by changes at the molecular, cellular, and tissue levels [2]. The immune system is responsible for defending against pathogens such as bacteria, viruses, and fungi to eliminate broken and harmful cells, like senescent cells and toxic or allergenic substances [3]. In the immune system, there is an innate compartment, consisting of neutrophils monocytes/macrophages, natural killer (NK) cells, and dendritic cells (DC), and an adaptive compartment, composed of B and T lymphocytes, which have a well-orchestrated interaction. Immunosenescence is a term that describes a different state of the immune system in aged people, in association with detrimental clinical outcome, due to reduced ability to respond to new antigens [4]. Although immunosenescence is a phenomenon present in the majority of individuals, factors like genetic, environment, lifestyle, and nutrition are responsible for their heterogeneity among individuals and cause a higher susceptibility to develop infections and progression of disease pathology [5]. Studies on immunosenescence have been performed in vitro in human-derived cell lines, and in vivo in animal models, to evaluate their response to different stimuli. Furthermore, the age-related dysregulation of immune responses impacts the resistance to infections, diminishes responses to vaccination, increases the susceptibility to autoimmunity and cancer [6], and promotes the development of an inflammatory phenotype [7]. Franceschi et al. [8] have introduced the term "inflamm-aging ", related to the immunosenescence, to describe a low-grade, asymptomatic, chronic, and systemic inflammation, characterized by increased levels of circulating cytokines and other proinflammatory markers [6, 8, 9]. The relationship between aging and chronic disorders, including atherosclerosis, dementia, neurodegeneration, and many others, has its bases in senescent remodeling of immune system.

Although research is making significant progress, the impact of immunosenescence on the onset and progression of neurodegeneration remains incompletely clarified; in fact, not necessarily being able to favorably modulate the functions of immune cells, which results in a corresponding change in the clinical outcome. This will not be, by any means, a comprehensive review of the immunosenescence; in fact, this review is focused on changes

in the immune system relevant to several neurodegenerative diseases.

2. Immunosenescence of Innate Immune Response Cells

The cells of the innate immune system form the first barrier against any pathogen. Neutrophils, monocytes/macrophages and DC, and NK are produced during fetal life and are continuously developed throughout the lifetime [10]. Microglia are tissue-resident macrophages in the CNS (central nervous system), derived from the yolk sac during embryogenesis, that colonize the developing brain where they stay during the individual's lifetime and, like macrophages in the periphery, act as the first line of defense. Phenotype and function of cells involved in innate immune response cells are profoundly influenced by aging, as described by Solana et al. [11]. Immunosenescence of the innate immune system has great complexity and seems to reflect dysregulation, rather than only impaired function. In fact, several responses in the innate immune system are reduced with aging, but, in contrast, also an age-associated hyperreactivity of innate immunity may be evidenced.

2.1. Neutrophils. They represent the first cells recruited in the presence of damage and during acute inflammation. Neutrophils are able to produce many degradative enzymes, antimicrobial peptides, and reactive oxygen species (ROS) for antibacterial activity. Many studies demonstrated that the activation of neutrophils, the free radical production signals, and the chemotactic ability are reduced in elderly people [7, 11–13].

The literature reports controversial results about the effect of aging on the amount of neutrophils [14] and their altered functionality. Minet-Quinard et al. demonstrated the presence of immature neutrophils, the production of high levels of intracellular reactive oxygen species, and the expression of activation markers such as CD11b and HLA-DR in the whole blood of advanced-age frail elderly [15]. In contrast with these findings, Sauce reported, in 2016, that ROS production by neutrophils is strictly dependent on priming their presence by proinflammatory mediators. At basal conditions, there are no differences between young and older people, but in the presence of a TNF-α (tumor necrosis factor) agonist, like bacterial fMLP (formyl peptides) or PMA (phorbol 12-myristate 13-acetate), ROS (reactive oxygen species) production is impaired across the two groups, with reduction in elderly population [16].

Most recently, Bartlett et al. underlined that the alterations of neutrophil functionality may be different between people of the same age. Chemotaxis, for example, which is a detrimental aspect for response in infection, contributing to the increase in proinflammatory insults persistence, could be positively editable by physical activity in older adults [17].

2.2. Macrophages. Monocytes play an important role as starters of the inflammatory response, and they can differentiate into macrophages, antigen-presenting cells, and dendritic cells [6, 12], although more complex differentiation pathways and cell origins have been proposed recently [18]. Macrophages that respond to inflammation stimuli may show two different phenotypes, the classical (M1) and the alternative (M2), depending on the local microenvironment [11]. M1 and M2 are balanced in healthy people, but in the presence of chronic inflammation, as in the presence of inflamm-aging, there is an imbalance, contributing to comorbidities and age-related disease development [19]. Franceschi et al. proposed macrophages as key cells in the induction and maintenance of inflamm-aging and have defined "macroph-aging" as the chronic macrophage activation that characterizes aging [8]. Macroph-aging and inflamm-aging happen in association with immunosenescence, and they reduce efficacy of immune cell activity. Accumulation of senescent macrophages contributes to the acceleration of the aging processes, and conflicting results on macrophage phagocytic function during aging have been reported [20, 21].

Many reports underline how the M2 phenotype develops in the spleen, retina, lymph nodes, and bone marrow in old mice compared to young mice, with higher production of IL- (interleukin-) 10 and reduced production of TNF- (tumor necrosis factor-) α [22]. Furthermore, in aged people, the expression of macrophage receptors, such as MHC (major histocompatibility complex) II and TLR (toll-like receptor), is declined with alteration of related activation mechanisms. In both human [11] and murine [23] models, a reduction of MHC II molecules expression was demonstrated, with decline in the ability to kill bacteria, phagocytic ability, and macrophage-specific cytokine and chemokine production [5, 6]. Altered expression and function in the context of TLR are linked to the aging process. Some studies demonstrated a reduced TLR expression together with changes in cytokine release and macrophage polarization [22]. TLR stimulation, mediated by LPS (lipopolysaccharides), is usually responsible for IL-6 and TNF-α secretion, but in macrophages from old mice, a reduction in IL-6 and TNF-α and an increased production of IL-10 [22, 24] were observed. The aging effect on macrophages is the reduced expression of TLR1, TLR2, and TLR4, with reduction in proinflammatory cytokine production [25, 26]. Moreover, the overexpression of TLR3 may be involved in the establishment of viral infections in elderly individuals [12]. Actually, studies investigating the impact of aging on human monocyte cytokine production did not provide concordant results; indeed increased, unchanged, or decreased LPS-induced cytokine secretion has been reported [27, 28].

2.3. Microglia. Microglia are the resident immune cells of the CNS. They have the ability to detect molecules of injured CNS cells or invade pathogen infiltration by pattern recognition receptors expressed on their surface and on the surface of infiltrating monocytes. During aging, senescent microglia display a higher production of proinflammatory cytokines and proliferative capacity, and a reduction of chemotaxis and phagocytosis of Aβ (amyloid-β) fibrils [29]. The number and density of microglial cells were higher in several aged brain areas, maybe to maintain the overall function. Replication of microglial cells can be very low in steady-state conditions and can be reactivated after perturbation by

harmful stimulation [30], which can culminate in the shortening of telomeres and the realization of replicative senescence. In aged microglia, accumulation of mtDNA (mitochondrial DNA) damages leads to ROS overproduction [31] and accelerates the switch in the senescent microglial phenotypes. Aged microglia show morphological changes such as cytoplasmic hypertrophy, fragmentation, and loss of ramifications [32]. Several mechanisms are responsible for microglial aging phenotype, such as the loss of inhibitory ligand-receptor interactions [33], accumulation of misfolded proteins [34], and the chronic exposure to TGF- (transforming growth factor-) β that reduces the capacity of microglia to secrete anti-inflammatory cytokines. Expression of TLR1, TLR2, TLR4, TLR5, TLR7, and CD14 is upregulated in microglial cells with increasing age [35], while in the signaling of CX3CL1 (chemokine (C-X3-C motif) ligand 1)-CX3CR1 (receptor 1), CD (cluster of differentiation) 200-CD200R, and CD200, CX3CR1 is decreased in aged microglia, driving activation and extension of proinflammatory responses [36, 37].

2.4. Dendritic Cells. Dendritic cells represent an important bridge between innate and adaptive immune response. The plasmacytoid and the myeloid DC are antigen-presenting cells that detect pathogens through the expression of PRRs (pattern recognition receptors) [38], composed of TLR, RLRs (RIG-I-like receptors), NLR (Nod-like receptors), and ALRs (AIM2-like receptors) [39]. TLRs are the most investigated receptors in the aged condition, and their expression changes at extracellular and intracellular levels [12]. On the other hand, Agrawal et al., in 2007, showed no altered expression of TLR in monocyte-derived DCs, from aged and young humans [40], in accord with Guo et al., who confirmed age-related changes in aged C57BL/6 mice [41]. Moreover, in aged people, the alterations of DC functionality may affect the immune regulation. Zacca et al. investigated DC's ability to prime and activate naïve CD8+ T cells, showing a lower capacity, with negative impact on immune response, in aged people [42], against viral and bacterial infections. The numerical reduction of DC and the decreased IL-12 production cause the higher susceptibility to immunosenescence of the adaptive immune system [43].

2.5. Natural Killer. NK cells are defined as the innate cytotoxic lymphocytes [44], responsible for the early defense against pathogens and cancerous cells [12]. NK cells can be classified into two groups based on their CD56 surface expression. $CD56^{bright}$ cells are the immature subset that showed a high proliferative activity and ability to release IFNγ, TNF-α, IL-10, RANTES, and MIP-1α, while $CD56^{dim}$ cells, the mature subset, showed a high cytotoxic activity and lower ability to produce cytokines [7]. During the aging process, the composition of NK subsets may experience some alterations in number and function, and impairments of cytotoxicity and secretion of immune-regulatory cytokines and chemokines, a phenomenon referred to as NK cell immunosenescence. Many studies have demonstrated the presence of an increased number of total NK in old subjects, with raised $CD56^{dim}$ [45–47], and lowered $CD56^{bright}$ NK cells [45–48], with respect to young subjects. This change reflects the dysregulation of innate and adaptive immunity interaction, with reduction in chemokine production and in cytokine-induced proliferation [11]. IL-2-induced NK cell proliferation is decreased in old subjects [49], while induction of cytotoxicity by IL-2, IL-12, or IFN is maintained [50]. NK receptor expression and activation seem to be involved in the aging process, and several studies have shown an age-related decline in the percentage of NK cells expressing NKp30 or NKp46 [45, 46], while others have reported no age-dependent effect on the proportions of NK cells bearing these receptors [48]. NKG2D, CD16, and KIR expressions have been shown to be either maintained or increased with age, while a reduction of KLRG-1 and NKG2A is age-associated [47, 51]. Recent studies have shown that the presence of senescent cells may be related to a reduced clearance activity of NK cells [52].

3. Immunosenescence of Adaptive Immune Response Cells

The adaptive immune system is more recent, in evolutionary terms, than the innate immune system. It is able to adapt to new threats developing specific strategies against every challenge. It fails when the cells responsible for maintaining immune memory overcome the cells capable of taking action. Profound age-related changes occur in the adaptive immune system, contributing to decreased immune protection against infections and responses to vaccination. Changes in cells of the adaptive immune system appear to have an important impact on the ability to respond to the immune challenges [53].

3.1. B Cells. B cells follow a well-defined developmental process, starting from naïve cells, that does not produce a specific antibody isotype, to the establishment of the mature peripheral B cell pool, kept by self-renewal [10, 54]. B cell immunosenescence induces alterations starting from the generation during haematopoiesis to the reduction of cell diversity [55] and lower antibody specificity. Antibody specificity, affinity, and isotype switch are affected by aging, determining the increased susceptibility of the elderly to infectious diseases and reducing the protective effects of vaccination. As demonstrated by Frasca et al., there is a great impact on B cell surface Ig switch, in old than in young people [56]. During the aging process, in mouse models and in humans, the B cell switch in IgM to IgG, IgE, or IgA is decreased [57]. A possible mechanism could be the presence of defects in the *E2A*-encoded transcription factor E47, responsible for defining the antibody diversity and downregulating AID (activation-induced cytidine deaminase) and CSR (class switch recombination) in the B cells of aged people [58].

Loss of Ig diversity has been related to the reduced percentages and numbers of mature B cells during aging [58]. Some studies showed an increased expression of the activation markers CD27 and CD38 in mature B cell compartments [5, 58, 59]. This is confirmed by the lower levels of IgM and IgD in the elderly, underlining a shift from the naïve (CD27−) B cell subset towards the memory (CD27+)

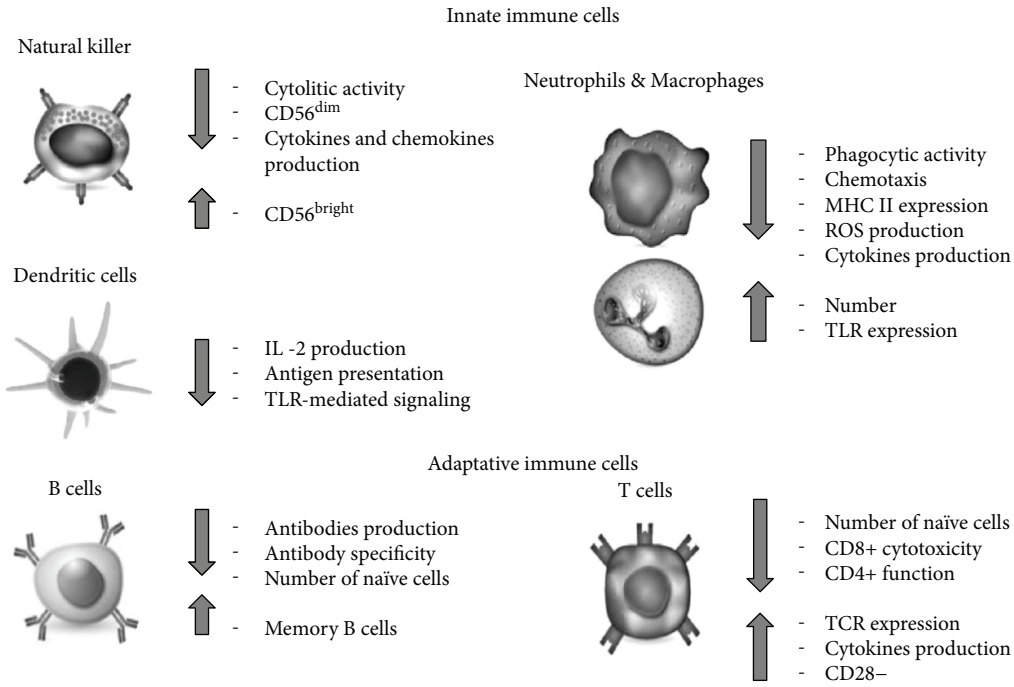

Figure 1: Schematic representation of immune cell age-related changes, leading to inflamm-aging, neurodegeneration, and neuroinflammation.

compartment [60]. B cell population is substantially altered in old age, contributing significantly to immunosenescence. The most important impairments affecting B cells during aging are reduction of the naïve B cell number, impaired capacity for response to new antigens, reduction of clonal expansion capability of memory cells related to reduction in circulating antibodies levels, and weakened antibodies function such as lower affinities and opsonizing abilities.

3.2. T Cells. Among the regulatory cells of the adaptive immunity, T cells are largely investigated in relation to immunosenescence. T cells are developed in the thymus, and distinct subsets are well recognized including the CD4+, CD8+, $\gamma\delta$, and NKT and the nonconventional T cells. All subsets have a specific role in the immune system [10, 61]. The thymus gland undergoes deterioration with aging, which starts after puberty and stabilizes after 65 years [10]. Thymus involution and reduced functionality are responsible for the reduction of naïve T cell frequency and number and for the increase in terminally differentiated T lymphocytes, with reduction in TCR (T-cell receptor) expression [62]. The numerical reduction in naïve T cells and TCR-reduced repertoire cause a decline in their functionality. In vitro studies showed that CD4+ naïve T cells, derived from old human and mice [63], have a reduced proliferation activity, an altered cytokine profile secretion, and a reduced responsiveness to TCR stimulation [63]. Otherwise, an increased number of memory CD4+ T cells is related to aging. Cytokine homeostasis results in alteration, favoring proinflammatory condition in aging. IL-6 increases in aged people and is responsible for inducing Th17 stimulation and related proinflammatory cytokine production [7, 64]. The CD8+ T cell subset is most affected by aging [65] with their accumulation. In particular T cell immunosenescence is characterized by increased number of highly differentiated memory CD8+, after chronic stimulation by viruses, like CMV (cytomegalovirus) infection [63]. As reported by Tu and Rao, CD8+ cells are able to persist after CMV infection, to prevent a virus reactivation. CD8+ subset presence during the time negatively impacts the immune system also in healthy CMV-infected individuals [66]. These alterations result in the impaired cellular immune response in infections and vaccinations [67].

In association with thymus involution, age-related changes of T lymphocytes include the reduced expression of costimulatory molecules (CD28, CD27, CD40L), and a progressive accumulation of CD28 highly differentiated T cells. CD28+ cells mediate the TCR-induced proliferation and differentiation of naïve T cells. In CD28 gene knockout mouse, the involvement of CD28− T lymphocytes in age-dependent immune decline was demonstrated. CD28− cells are accumulated during life, due to their resistance to apoptosis. The loss of CD28 in CD4+ and CD8+ cells is followed by altered secretion of second messengers and altered signal pathway activation [64, 68] and lowering in immune response to vaccination in older people. Thus, CD28 loss in T cells can be defined as one of the aging hallmarks (Figure 1).

4. Immunosenescence and Inflamm-aging in Neurodegenerative Diseases

The interaction between the nervous and immune systems during aging is characterized by bidirectional dependency and reciprocal causality of alterations. In elderly people, the increased systemic inflammatory condition, the

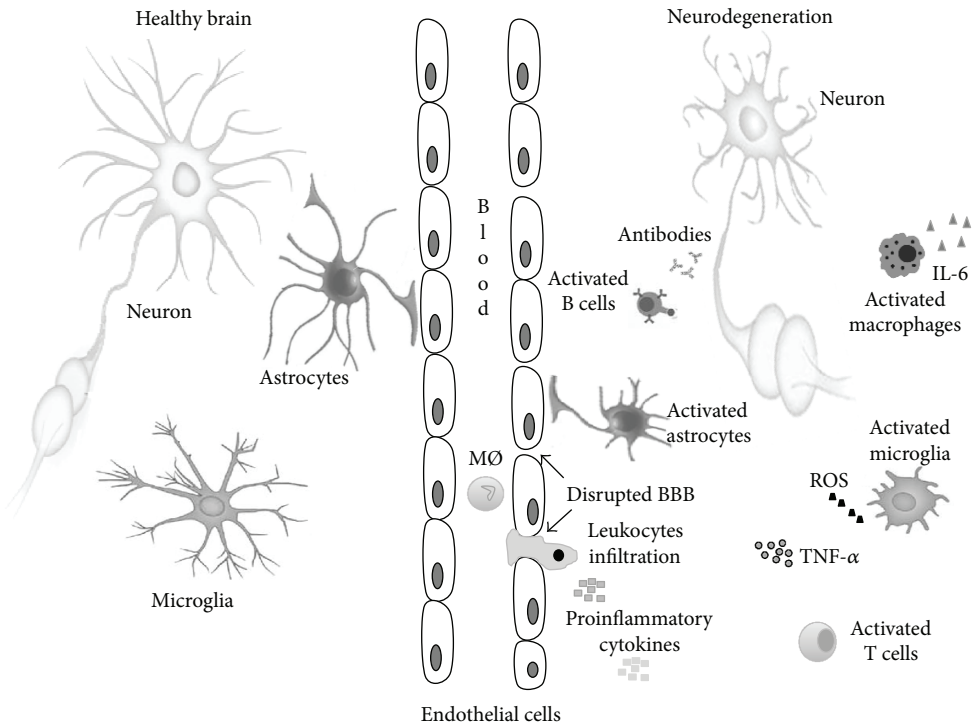

Figure 2: Immune activation in neurodegenerative diseases and healthy brain. The neurodegeneration is accompanied by immunosenescence and inflamm-aging. During aging, neuronal cells modify their morphology, and their overactivation leads to IL-6 and TNF-α abnormal production. The immune cells penetrate the damaged BBB and causes a further increase in proinflammatory cytokines, modulating, in turn, neuronal dysfunction.

inflamm-aging, and the peripheral immunosenescence can modulate neuronal immune cell activity and reactivity, leading to a chronic low-grade inflammation in the CNS, called neuro-inflamm-aging. Activation of glia by cytokines and glia proinflammatory productions are significantly involved in memory injury, and also in acute systemic inflammation, characterized by high levels of TNF-α and increase in the cognitive decline [69]. Autoreactive T cells, derived from the atrophied thymus, are a source of proinflammatory factors that strongly contribute to neurodegeneration. Immunosenescence and inflamm-aging induce brain aging, cognitive deficit, and memory loss; in fact, a bidirectional interconnection has been observed in neurodegenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Figure 2).

4.1. Alzheimer's Disease. Alzheimer's disease is the most common type of age-related neuronal disorder, affecting 44 million people in the world [70, 71]; with the improvement of life expectancy, this number is constantly growing. People that may develop AD in the late life, at the age of 65 and older [72], present deficit in memory, language, spatial vision, and physical equilibrium that together lead to cognitive impairment [70, 72].

Characteristics of AD are amyloid-beta (Aβ) deposition, NFTs (neurofibrillary tangles), NP (neuritic plaques), and the activation of immune cells of the CNS, microglia, and astrocytes [73]. Aβ deposition is the central event in AD pathogenesis and derived from larger and hyperactivation of the APP (amyloid precursor protein) [53]. APP is a transmembrane glycoprotein responsible for binding proteoglycans for regulating intracellular processes such as neuron-cell and cell-matrix interaction, cell growth, and synaptic plasticity [74]. The pathophysiological relation between Aβ and tau protein is still unclear [53, 70]. NFTs are composed of hyperphosphorylated tau protein, responsible for stabilizing microtubules in neurons, and their formation is secondary to neuronal damage and Aβ deposition. In regions of Aβ deposits and NFT, signs of oxidative stress and high levels of inflammatory mediators were observed. The inflammatory response is necessary and crucial to combat pathogen or dying cell, but dysregulated inflammatory responses are responsible for tissue damage such as in the CNS inflammation. To the inflammatory response in the CNS, cells of immune system, cells of the CNS, adhesion molecules, cytokines, and chemokines take part. Numerous studies have assessed the association between immune system activation and AD [71, 75]. This relation can be explained by the highly regulated communication between the brain and the immune system [76], and Sutherland et al., in 2015, explained the role of the immune cells in relation to the inflammatory condition inside and outside the CNS [77]. Aβ deposition-induced AD pathogenesis brings the activation of microglial and astrocytes cells, the phagocytosis and degradation of β-amyloid, and elimination of the debris of dead or dying cells, thereby reducing the likelihood of further cell loss through the release of toxic agents. Furthermore, reactive astrocytes isolate neurons from senile plaques and

release cytokines and growth factors that may help damaged neurons to survive and promote repair to inhibit Alzheimer disease [71]. The natural decline of CNS immune cells in adult and elderly people leads to a reduced state of health of the brain, contributing to AD. Microglial and astrocytes cell uncontrolled activation, increased in an age-dependent manner, leads to excessive inflammation [78]. Inflammation can lead to the injury or death of neurons, particularly if the response is chronic and uncontrolled. Neuronal loss in AD may be a direct effect, or due to the secretion of neurotoxins by activated microglia. Activated microglia may be involved in the generation of senile plaque either by the secretion of Aβ1–42 or through the release of agents such as iron, which aggregates soluble β-amyloid fragments. Microglia and astrocytes produced proinflammatory component, and also neurons, oligodendrocytes, and vascular endothelial cells may contribute to the maintenance of the inflammatory environment. Activated microglia may promote neurodegeneration but could also play a neuroprotective role dependently by the context, timing, and mediator of the inflammatory response. Cytokines are multifunctional mediators that act in a context-dependent manner and can promote or inhibit inflammatory processes. Thus, TNF-α may promote an inflammatory response, but it may protect neurons or even modulate neurotransmission, or TGF-β1 may promote inflammation and cellular infiltration early in an immune response, but it is critical later in downregulating inflammation [79]. In Dr. Chakrabarty's laboratory, using transgenic mice as AD model, it was highlighted that mIL-6-mediated reactive gliosis may be helpful early in the disease development by possibly improving Aβ plaque clearance rather than facilitating a neurotoxic feedback loop that aggravates Aβ plaque pathology [80], likely by inducing microglial phagocytosis and shifting towards the alternative M2 microglia with an anti-inflammatory phenotype.

The bidirectional communication between the nervous and immune systems, when properly orchestrated, resulted in body protection, and cytokines might be the key molecules that initiate the immune-to-brain communication, and activation of specific cytokine-to-brain pathways differentially mediates response to specific events. Peripherally released cytokines may reach the brain through permeable regions of the blood-brain barrier (BBB), by activation of nonneuronal cells in the BBB that can initiate a cascade of neural communication events, by active transporters that allow cytokines to cross the BBB, or by vagus nerve that acts as a neural route.

The chronicity of inflammation is characterized by activation of monocytes, as well as macrophages and infiltration in the CNS [81]. Peripheral immune system cell overactivation determines the increased proinflammatory cytokine and chemokine release, with upregulation of immune receptor expression (MHC II, CD68, CD14, CD11, and TLRs) [71, 81], promoting the brain tissue damage. Activated immune cells and their products reach the CNS crossing the BBB, physiologically responsible for isolating the brain from peripheral circulation. Lee and collaborators have shown an increased BBB permeability in aged mice, with a reduced expression of TJs (tight junctions) inhibiting endothelial cell interconnection [82]. In recent studies on 12-month-old wild-type C57BL/6J mice, BBB dysfunction was demonstrated in relation to TJs lost and heightened proinflammatory cytokine expression, in particular TNF-α [83], IFNγ [41], IL-1β, IL-6, and IL-18 [84]. These cytokines are produced not only by overactivated neuronal immune cells but also by peripheral immune cells, showing a relationship with neuronal dysfunction, increased inflammation in the brain parenchyma, and cognitive decline [85–87].

During the AD pathogenesis, the brain damage and the BBB higher permeability define a selective entry of peripheral immune cells in the CNS that activate the immune response [88], as the T cell infiltration in brain tissue reported by McGeer et al. [89], in a *postmortem* brain analysis [90].

In vitro studies demonstrate that Aβ stimulation of microglial cells and astrocytes is responsible for increasing the levels of TNF-α and TGF-β1 production [91], which are known to promote T cell transmigration [88]. The increased expression of MHC I and II represents the mechanism by which T cell numbers increase in the brain [53]. Moreover, there is a higher expression of CCR5 on B cell surface in the presence of Aβ that leads to inflammation and cytokine and chemokine production [92]. Marsh et al., in 2016, investigated the mechanism by which B cells are involved in AD progression using the immune-deficient transgenic model of AD, Rag-5xf AD mice, showing that Aβ plaque deposition is favored by nonamyloid-reactive IgG [93].

In mouse models, it has been observed that neurotoxicity could be mediated by activation of NLRP3 (NACHT, LRR, and PYD domains-containing protein 3), with release of classical proinflammatory molecules, such as IL-1β, IL-18, and IL-1α [94], and activation of macrophages. Monocytes/macrophages represent, not in pathological conditions, the responder cells to inflammatory stimuli and stressors by expressing protective molecules, such as IL-12 and IL-23, which contribute to homeostasis regulation, also in the perivascular space, through phagocytic activity [95]. Monocytes infiltrate the brain in AD, crossing the BBB and assuming macrophages or dendritic cell phenotype, with different states of activation [75]. In an Aβ-induced inflammatory microenvironment, macrophages change their protective phenotype M2 [96] to the proinflammatory M1, involved in the production of IL-1β and TNF-α and phagocytosis of dangerous molecules produced by stressors [75, 97]. These cells interact with microglia and Aβ plaques, considered as an inflammasome activator, producing cytokines and ROS, leading to neuronal loss and apoptosis [88]. To assess the immune system involvement, many authors evaluated the circulating levels of IL-6, TNF-α, and IFNγ production in AD patient serum/plasma [53, 98, 99], CSF (cerebrospinal fluid) [100], and derived peripheral blood mononuclear cells [101]. Circulating levels of proinflammatory cytokines are elevated and significantly associated with increased risk for AD cognitive decline. This increase was found also in triple-transgenic mice models of AD (3xTg-AD mice), considered as the most similar to human AD model [102]. Most recently in 3xTg-AD mice, an increased proinflammatory response with IL-6 and TNF-α increased production has

been observed, in association with immune cell infiltration in the brain [103].

4.2. Parkinson's Disease. Parkinson's disease is a neurodegenerative age-related disorder [104] affecting 1% of 60-aged human population and is considered the second most common neurodegenerative disease [105]. PD patient's clinical signs are bradykinesia, rigidity, and tremor, usually manifesting unilaterally or at least asymmetrically, in addition to sensory and neuropsychiatric features [106, 107].

The pathogenesis of PD is related to people's exposure to many environmental risk factors, such as pesticides, heavy metal, welding and chemical agent exposure [104, 108], and genetic and epigenetic factors [105], leading to oxidative stress, proteasomal system dysfunction, protein aggregation, and misfolding. These alterations are common to changes that occur during aging [108]. The effects of aging refer to the physiological changes of neuronal and nonneuronal cells [104], including progressive degeneration and loss of DA neurons in the midbrain substantia nigra, reduction of nigral pigmented neurons, accumulation of alpha-synuclein, and the inhibition of the UPS (ubiquitin proteasome system) [109]. The accumulation of α-synuclein leads to lamellated eosinophilic cytoplasmic inclusions, called "Lewy bodies" in the neuronal body, and to the insoluble polymers (Lewy neurites) in neuronal processes, astrocytes, and oligodendroglial cells [104, 67].

In the postmortem PD patients' substantia nigra, the content of DA (dopamine) is reduced by 10% compared to normal values, associated with dopaminergic neurons loss of cellular bodies [104].

It has been demonstrated that neurodegeneration and immune system activation are increased with age and contribute to PD onset [105, 108].

The common features of immune cell involvement are represented by the establishment of inflammation in the brain, recruitment of peripheral immune cells, proinflammatory mediators production, and increased ROS concentration. All of these may increase the neurodegenerative process in aged people [105], with acceleration and increased prevalence of PD [108].

Peripheral immune system activation affects brain neuroinflammation, for example, exacerbating the microglial function [110] and defining changes in DA neurons. In aged brain, microglial cells increase the MHC II and TLR expressions and adopt a proinflammatory phenotype to stimulate the peripheral immune cell migration in the CNS [106]. In addition, astrocytes show an increased proinflammatory profile with higher MCP-1 secretion for priming peripheral monocytes [111]. Peripheral immune cells, like macrophages and monocytes, in healthy brain, are responsible for the immuno-surveillance and the production of proinflammatory cytokines, such as TNF-α and IL-6, to solve the injury. The immunosenescence of these cells, with reduction of phagocytic activity and proinflammatory cytokine and chemokine production, seems to influence the disease progression because of the lack of surveillance [67]. Most recently, Lindenau et al. showed the involvement of TNF-α, which appears to be significantly released by monocytes/macrophage with proinflammatory phenotype. In elderly individuals, immunosenescent cells seems to contribute to the increased expression of TNF-α. The hypothesized mechanism is the methylation of the gene responsible for TNF production, which can contribute to the progression of inflammation and to the aging process [112].

Together, these data support the hypothesis that altered innate immune system activation, such as macrophages and monocytes, directly contributes to the pathology and biology of PD.

In PD patients, the most relevant sign is the alteration of the lymphocyte subsets. A reduction in the total number has been observed for CD19+ B cells and CD3+ T cells. Among T cells, their activation is related to the DC infiltration in lymph nodes and cell stimulation [105]. In PD patients, in association with immunosenescence, the reduced count and functionality of DC are responsible for the reduction in T cell activation [113]. The BBB dysfunction in PD patients determines the CD4+ and CD8+ infiltration in the CNS [106, 114]. Circulating levels of CD4+ T cells decrease in PD, while CD8+ T cells are unchanged, promoting the immune aging [115]. Baba et al. have demonstrated the selective reduction of CD4+CD45RA+ phenotype, naïve cells and an increase or a nonalteration in the expression of CD4+CD45RA− memory cells. This is supported by studies in animal models and in humans, highlighting the involvement of Treg (regulatory T cell) lymphocytes in the promotion of immune-mediated diseases in aged people [116]. In animal models, such as 6-hydroxydopamine (6-OHDA) PD rats, the increased expression of CD4+ T cells is responsible for increasing the inflammatory cytokine expression and facilitates NM (neuromelanin) activation and B cell production of autoantibody [117]. Additionally, the progressively increased neuroinflammation drives a high cognitive decline linked to vulnerability to virus and bacterial infection in aged people [67]. The reduced diversity in the T cell repertoire also represents one of the causal factors of the deregulation of the immune response in the elderly, with a significant increase of cytokine levels. Several studies, in which CSF and serum of PD patients were analyzed and correlated to PD progression, support this hypothesis. Increased levels of TNF-α, IL-1β, IL-2, IL-4, and IL-6 were detected in CSF of PD patients compared to age-matched controls [117]. Additionally, the increased levels of inflammatory markers were detected in serum from PD patients [105, 118, 119]. Moreover, circulating levels of cytokines were correlated to the overactivation of T and B lymphocytes, and upregulation of microglial cells, which are able to induce in turn IFNγ and TNF-α expressions [113, 115, 120]. It is well established that pathological changes in the CNS can be evaluated the in periphery. New proposed targets are circulating microvesicles that are generated in response to intracellular stimuli, representing an interconnection between normal and pathological tissues. Peripheral systems analysis of PD patients has led to highlighting the presence of brain damage, and microvesicle biology can be important for pathogenesis uptake.

Many authors evaluate the α-synuclein secretion outside or within exosomes [121, 122]. All of these studies support the hypothesis that α-synuclein associated with exosomes

contributes to the progression of brain disorders, but the mechanism is currently unclear. In addition, it was demonstrated that the exosome release in pathogenic form is upregulated in association with PD mutated genes, like LRRK2 and ATP13A2, that acts in modulating microvesicle biogenesis and trafficking.

5. Conclusions

Over the decades, there is enormous progress in the neural-immune crosstalk and mutual regulation of aging and age-related diseases, and in describing the innate and adaptive immune age-related alterations; however, investigations are necessary. Several studies have shown that changes in cell number, activity, and receptor expression lead, as consequence, to an increased susceptibility to infectious and inflammatory age-related diseases, in elderly population. Evidences showed the association between immunosenescence with a low-grade chronic inflammation, called the inflamm-aging, although inflamm-aging is necessary but not sufficient to cause age-related neurodegenerative diseases. The increased proinflammatory environment could be the major contributing factor to the development of aging-associated diseases. Given the well-established communication between the immune system and brain, the age-related immune dysregulation may bring neurodegeneration. Several studies have demonstrated that immunosenescence and inflamm-aging can induce an overactivation of CNS immune cells, promoting neuroinflammation. In AD patients, the microglial aging and dysfunction lead to Aβ accumulation and loss of peripheral immune response, contributing to disease pathogenesis. Furthermore, in PD, the interaction between aging and over time decreased immune response suggests a disease predisposition for neurodegeneration. Recently, several studies have reported the relationship between delayed immunological aging and reduced expansion of senescent late-stage differentiated T cells and active lifestyle and has been suggested that aerobic exercise training might attenuate cognitive impairment and reduce dementia risk. Although it is unknown whether effects of exercise are direct, such as a targeted removal of dysfunctional T cells, or indirect, such as lower inflammatory activity, it may be hypothesized that these changes can provide benefits for the health, including mitigate cognitive impairment. To mitigate the decline in the immune function, a practical and economic approach is represented by the nutritional intervention, without forgetting that difference exists between nutritional interventions and their immune-modulating activity. The use of both probiotics and prebiotics may reduce immunosenescence, improving Treg homeostasis, reducing the colonization potential of pathogens, and counteracting chronic inflammation, and may positively affect cognitive function [123, 124].

Caloric restriction partially retards or restores age-associated immunosenescence by oxidative stress energy metabolism regulation, and reduction of proinflammatory cytokine production and neuroendocrine homeostasis [125]. A healthy lifestyle may help to retard immunosenescence; in fact good sleep duration improves immune functions, and poor sleep may affect the body's ability to clear the amyloid-beta from the brain, while stress reduces the effectiveness of the immune system and can cause damage to the brain.

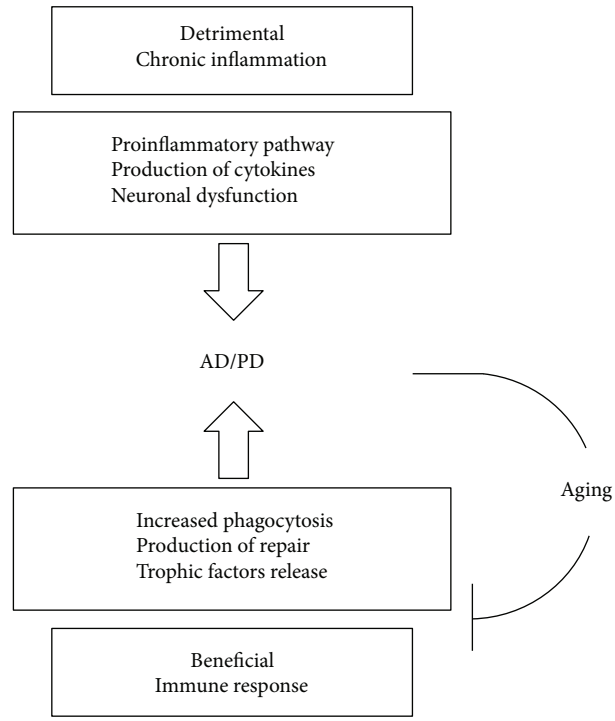

Figure 3: Advantages and disadvantages of an inflammatory response. Phagocytosis eliminates the debris of dead or dying cells, thereby reducing the likelihood of further cell loss through the release of toxic agents. Cytokines and growth factors may help damaged neurons to survive and promote repair. Chronic inflammation can lead to the injury or death of neurons.

New strategies to combat immunosenescence and neurodegeneration are focused on cellular and genetic therapies, such as genetic reprogramming and bone marrow transplantation, but cell reprogramming has still poor efficiency, and clinical translation shows several ethical and safety questions that may be answered.

Thus, a better understanding of immunosenescence mechanisms will be necessary to develop new, unconventional, or pharmacological therapy strategies, for peripheral and CNS immunosenescence delay. Additional studies are required to determine the effectiveness and optimal conditions to improve the function of the aged immune system and undertake the challenges of immunosenescence. Immunosenescence reversion can prevent, in elderly individuals, chronic inflammation and associated neurodegenerative diseases and can provide new and additional target for improving healthy lifespan and slow down age-related diseases incidence (Figure 3).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 7109312, 14 pages
https://doi.org/10.1155/2018/7109312

Research Article

Evaluation of Lymphocyte Response to the Induced Oxidative Stress in a Cohort of Ageing Subjects, including Semisupercentenarians and Their Offspring

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Received 20 July 2017; Revised 16 October 2017; Accepted 27 November 2017; Published 19 February 2018

Academic Editor: Elaine Hatanaka

The production of reactive oxygen species (ROS) may promote immunosenescence if not counterbalanced by the antioxidant systems. Cell membranes, proteins, and nucleic acids become the target of ROS and progressively lose their structure and functions. This process could lead to an impairment of the immune response. However, little is known about the capability of the immune cells of elderly individuals to dynamically counteract the oxidative stress. Here, the response of the main lymphocyte subsets to the induced oxidative stress in semisupercentenarians (CENT), their offspring (OFF), elderly controls (CTRL), and young individuals (YO) was analyzed using flow cytometry. The results showed that the ratio of the ROS levels between the induced and noninduced (I/NI) oxidative stress conditions was higher in CTRL and OFF than in CENT and YO, in almost all T, B, and NK subsets. Moreover, the ratio of reduced glutathione levels between I/NI conditions was higher in OFF and CENT compared to the other groups in almost all the subsets. Finally, we observed significant correlations between the response to the induced oxidative stress and the degree of methylation in specific genes on the oxidative stress pathway. Globally, these data suggest that the capability to buffer dynamic changes in the oxidative environment could be a hallmark of longevity in humans.

1. Introduction

Immunosenescence is characterized by age-associated changes in cell phenotype and function that ultimately leads to a general impairment of the immune response [1]. In the innate compartment, in mice as well as in humans, a decrease in neutrophil chemotaxis, phagocytosis, and oxidative burst has been observed along with a decrease in natural killer (NK) cells and macrophage cell functions [2, 3]. Changes in the acquired immunity during ageing are driven by the thymic involution, leading to a decreased production of naïve T cells capable of replenishing the peripheral pool [4]. Furthermore, homeostatic mechanisms as well as persistent infections (i.e., cytomegalovirus) push memory T cells towards several rounds of replication during the ageing process [5–7]. Once reached the replicative senescence, these cells show energy, resistance to apoptosis, and changes in cytokine production [8]. Moreover, the impairment of the immune function during the ageing process can be even promoted by its inability to restore a proper balance between prooxidant, such as reactive oxygen species (ROS), and antioxidant molecules, such as the enzymes superoxide dismutase and catalase or the reducing agent glutathione (GSH). This condition is commonly defined as oxidative stress [9, 10]. ROS are continuously produced in the mitochondria as a result of the reduction of a small percentage of molecular oxygen by leaked electrons in the electron transporting chain or are produced enzymatically by the NADPH oxidase, mediating the respiratory burst in phagocytes. Other sources of ROS include the xanthine oxidase or the nitric oxide synthase pathways as well as environmental ultraviolet or ionizing radiation [11]. ROS play an important role in the immune system other than the respiratory burst, participating in the T, B, NK, and dendritic cell signaling or coordinating the cytokine production [12–14]. Nevertheless, ROS mediate detrimental effects to different cellular components if not properly counterbalanced by the antioxidant system within the cell. Such effects include lipid peroxidation, DNA oxidative damage, and protein modifications like glutathionylation and carbonylation [15, 16]. As an example, in the immune system, it has been shown that the oxidative modifications of proteins can alter the tertiary structure of the self-molecules, thus representing a risk factor for the development of autoimmune reactions [17, 18]. Moreover, oxidative changes can impair the proteasome functions, resulting in an uncorrected processing of peptides for T cell presentation by MHC molecules [19]. Immunosenescence per se (thymic involution, memory T cell expansion, and replicative senescence) and oxidative stress are key stimuli for fueling the low grade, persistent, and systemic inflammatory status, known as "inflammaging" [20], that is one of the pillars of the ageing process and strongly contributes to the pathogenesis of the main age-related diseases in humans. In this framework, centenarians who have survived, escaped, or delayed the onset of major age-related diseases achieving the extreme limits of the human life-span appear to have reached a balanced status between the inflammaging and the so called *anti-inflammaging*; that is, the process, involving molecules and their related pathways, aimed to control and modulate the inflammation and/or the oxidative stress [21]. Data in literature show that centenarians have less amounts of plasma oxidative stress markers [22, 23] or increased amounts of antioxidant vitamins [24]. However, there is a lack of experimental data in human models on the oxidative stress measured directly in the cells, and especially in white blood cells, from centenarians. In fact, such determinations were performed using cells from elderly subjects who turned 95 yo as a maximum [25–27]. Moreover, these studies performed mainly the oxidative stress determination at the baseline, that is, without inducing the ROS production. As the organisms face dynamic changes of the oxidative environment [28], we questioned if the capability of a cell in resisting the induced oxidative stress could be a hallmark of an efficient *anti-inflammaging* and thus of healthy ageing and longevity. To the best of our knowledge, only limited data on the response to induced oxidative stress in centenarians is available in literature [29]. In this paper, in order to provide further evidence to this concept, we performed an *in-depth* cell immunophenotyping and oxidative stress analysis by means of polychromatic flow cytometry (PFC) in a cohort of semisupercentenarians (CENT), elderly controls (CTRL), and young (YO) individuals. Moreover, the present analysis includes cells from centenarians' offspring (OFF), individuals representing an informative model to identify trajectories of healthy aging and their determinants (genetic and environmental). PFC was used to analyze the response to induced oxidative stress as ROS and GSH relative fluorescence in the main subsets of T, B, and NK cells. The phenotypic changes were also evaluated by PFC in order to illustrate the ongoing process of immunosenescence in such cohort. DNA methylation profile of a list of genes involved in the response to oxidative stress was also analyzed, in order to determine whether changes in the ability to cope with oxidative stress may be related to epigenetic reasons.

2. Methods

2.1. Subject Cohort. The subjects described in this paper (CENT $n = 7$: 3 males (M)/4 females (F), median age/range 106 yrs/105–107 yrs; OFF $n = 6$: 3M/3F, median age/range 72 yrs/59–85 yrs; CTRL $n = 7$: 4M/3F, median age/range 72 yrs/59–77 yrs; and YO $n = 7$, 3M/4F, median age/range 35 yrs/25–37 yrs) were recruited all in Bologna and were part of a larger cohort described elsewhere [30]. The study protocol was approved by the Ethical Committee of Saint Orsola-Malpighi University Hospital (Bologna, Italy). Informed written consent for the participation was obtained at the time of blood withdrawal.

2.2. PBMC Separation. Diluted heparinized blood in PBS was carefully layered on Ficoll-Hypaque and centrifuged for 20 min at 600 g. After removal of platelets by centrifuging for 10 min at 200 g, PBMCs were resuspended in the frozen medium (FBS/10% DMSO), stored at −80°C for 24 hours and then transferred in liquid nitrogen until used.

2.3. Flow Cytometry. Cells were thawed in warm Iscove Modified Dulbecco's Medium (IMDM), centrifuged at 200*g* for 5 min, and resuspended in 100 μl of IMDM. Briefly, PBMCs (2 tubes for T cell and 2 for B/NK detection) were incubated in the presence or absence of ROS inducer tert-butylhydroperoxide (TBHP) for 30 min at 37°C in 5% CO_2, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Then, fluorescent probes for GSH (Thiol Tracker, TT, Invitrogen, ex: 405 nm, em: 525 nm) and ROS (CellROX green, Invitrogen, ex: 508 nm, em: 525 nm, specific for H_2O_2 and superoxide anion) were added at a final concentration of 5 μM and 500 nM, respectively. Cells were incubated for additional 30 min at 37°C in 5% CO_2. 50 μl of mixtures of mAbs targeting the antigens for specific T, B, and NK subsets was then added to the respective tube for 15 min. Mix for T cells includes the following mAbs all purchased from Beckman Coulter (Bree, CA, USA): anti-CD25-phycoerhytrin (PE), CD45RO-PE-Texas Red, CD3-PE-cyanine 7 (Cy7), CD45-Alexa 700, and CD4-allophycocyanin- (APC-) Cy7. Anti-CCR7-peridinin chlorophyll-Cy5.5, CD127-Alexa 647, and anti-CD8-BUV395 were acquired from Becton Dickinson, (Franklin Lakes, NJ, USA). Mix for B and NK cells includes anti-CD16-PE, anti-CD38-PE-Texas Red, anti-CD27 PE-Cy5.5, anti-CD3-PC7, anti-CD56-APC, anti-CD45 APC-Alexa 700, and CD20-APC-Cy7 purchased from Beckman Coulter and CD19-BUV395 from Becton Dickinson. Samples were then diluted by adding 300 μl of PBS and acquired by means of a LSORP Fortessa equipped with 5 laser lines (355 nm, 405 nm, 488 nm, 561 nm, and 640 nm) and 18 photomultipliers (PMTs). Dead cells were excluded using a viability dye (Live Dead, Invitrogen). All the reagents were titered according the internal SOPs. Moreover, to ensure consistent and reproducible results, we used the 8-peak Rainbow Beads (Spherotech, Lake Forest, IL, USA) as a fluorescent calibrator allowing us to trace the laser/PMT performance across the independent experimental runs. PBMC subsets were identified by a hierarchical gating strategy considering the shared (or not) CD antigens among the different populations. Data files were saved in the FCS 3.0 format and analyzed offline using the *Batch Analysis* function of the FCS Express 5.0 package (De Novo software, Glendale, CA, USA) allowing to export all percentages and fluorescence values in Excel format. The percentage of a given cell subset was referred to the parental gate (i.e., CD4 naïve in the CD4 gate or Tregs in CD4 memory gate). The level of ROS or GSH at the baseline was evaluated by the median fluorescence intensity (MFI) of the relative probe, whereas the response to induced oxidative stress was calculated as the ratio of MFIs of ROS (or GSH) between the I and NI conditions.

2.4. Methylation Profile. Whole-genome DNA methylation profile of PBMCs from the entire cohort has been described previously [30]. Briefly, genomic DNA was extracted from PBMCs with the AllPrep DNA/RNA/protein kit (QIAGEN, Hilden, Germany) and it was treated with sodium bisulphite using the EZ-96 DNA Methylation Kit (Zymo Research, Irvine, CA). Genome-wide DNA methylation level was assessed using the Infinium HumanMethylation450 BeadChip (Illumina, San Diego, CA) following the manufacturer's instructions, and the arrays were scanned by HiScan (Illumina).

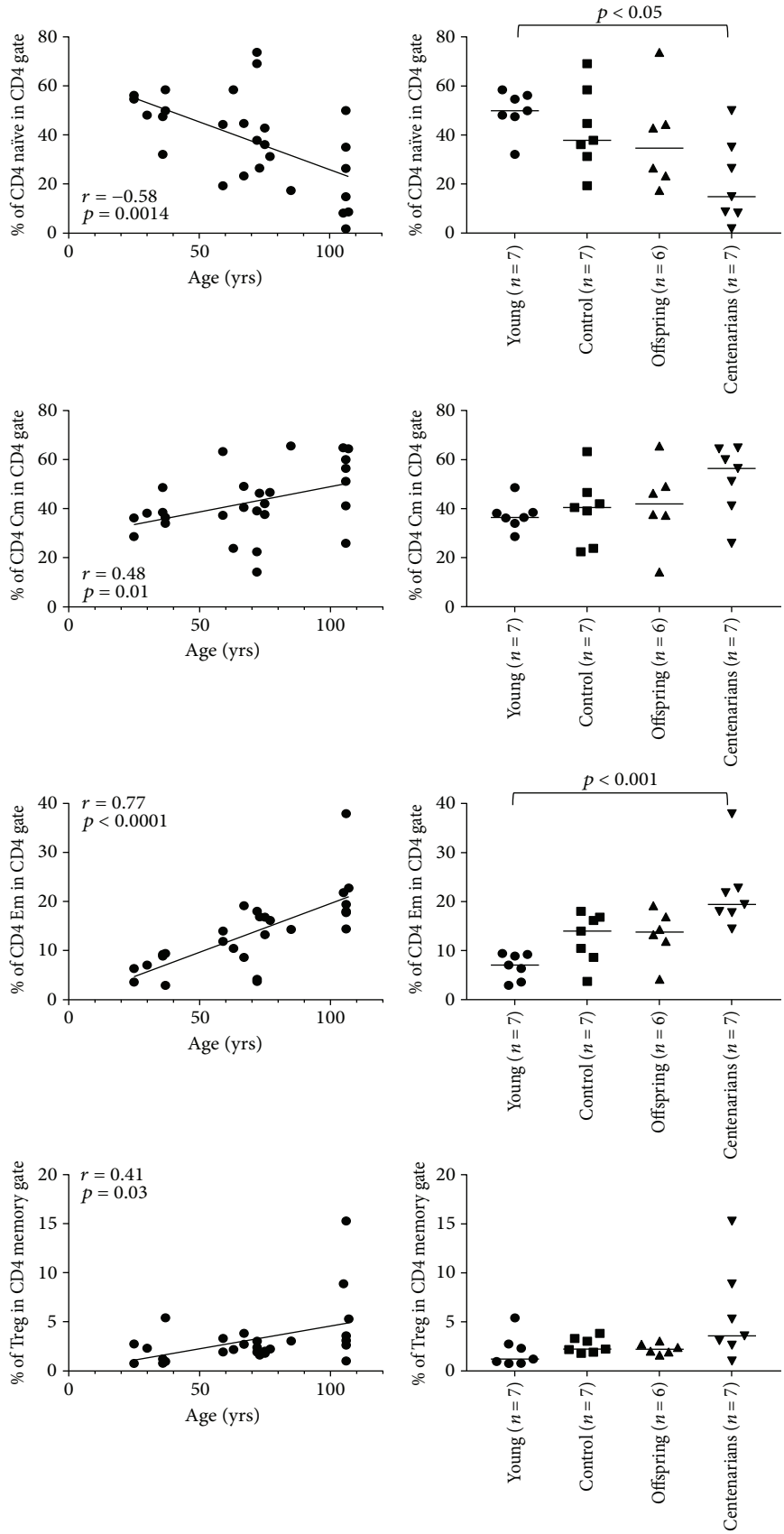
2.5. Statistical Analysis. Differences among groups in T, B, and NK subsets for cell percentages or MFI of ROS and GSH were assayed using the nonparametric Kruskal-Wallis one-way analysis of variance followed by Dunn's posttest. Correlations between cell percentage and age or between ROS MFI ratios and the degree of methylation were assayed using Spearman correlation. Prism 5.0 was used for both analyses (GraphPad, La Jolla, CA, USA).

For DNA methylation analysis, beta values were extracted using minfi Bioconductor package. The differences among CENT, CTRL, and OFF were assessed by ANOVA with R software.

3. Results

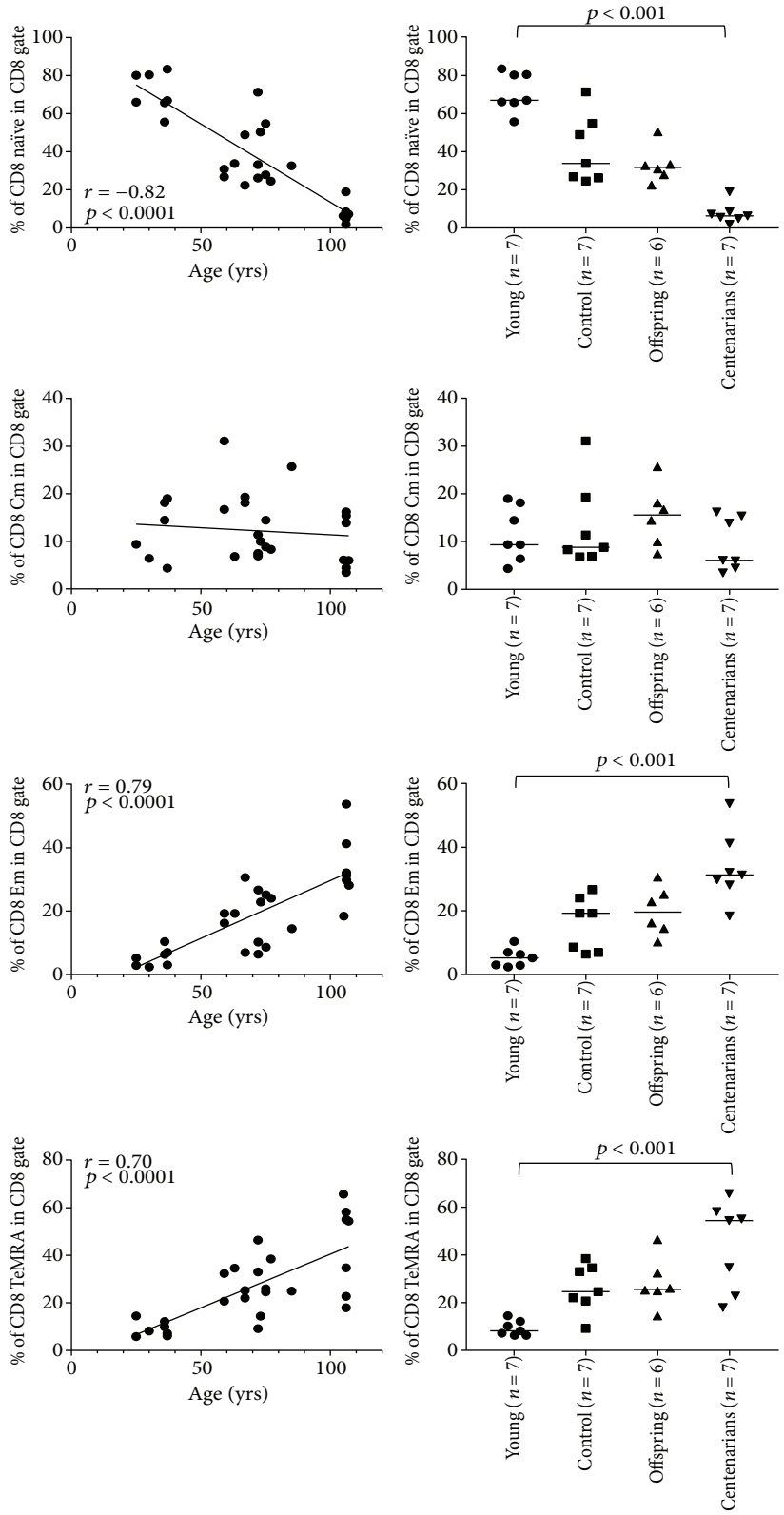
3.1. Percentages of PBMC Subsets across the Age Groups. As expected, correlation analysis showed a significant age-related decrease in the percentage of naïve subsets both in CD4 and CD8 T lymphocytes ($p = 0.0014$ and $p < 0.0001$, resp.). Conversely, a significant age-related increase in CD4 central memory (Cm; $p = 0.01$), effector memory (Em; $p < 0.0001$), and regulatory T cells (Tregs; $p = 0.03$) as well as in CD8 Em and terminally differentiated effector memory (TeMRA) cells was observed ($p < 0.0001$; Figures 1(a) and 1(b)). Analysis of variance (ANOVA) showed significant differences in the percentages of CD4 and CD8 subsets (except for CD4 and CD8 Cm and Tregs) between YO and CENT. No differences between OFF and CTRL in the different subsets were appreciated (Figures 1(a) and 1(b)). When considering B and NK cells, correlation analysis showed a significant inverse correlation with the age of the CD56bright subset, whereas no age correlation in B or NK CD56dim cells was observed (Figure 2). ANOVA showed statistically significant differences in B and NK CD56dim percentages across the age groups. Percentages of B cells in CTRL and OFF were significantly higher than those in CENT. Notably, CENT showed similar percentages of B lymphocytes compared with YO. Lastly, CTRL and OFF showed lower percentages of NK CD56dim cells compared with YO and CENT. Likewise, B cells, CENT and YO showed similar percentages of NK CD56dim (Figure 2).

3.2. Oxidative Stress and GSH Content at the Baseline in PBMC Subsets. The levels of ROS and GSH in noninduced (NI) condition were determined by means of the fluorescent probes. No significant differences among the age groups were observed, although a tendency towards higher levels of ROS in the YO was observed in several subsets (see Table S2). Results of GSH analysis showed a lower GSH level in OFF and CENT compared with YO and CTRL in all PBMC subsets considered, although ANOVA did not show significant differences among the age-groups (Figures 3 and 4 and Table S4).



(a)

Figure 1: Continued.



(b)

Figure 1: (a) Correlation between age and cell percentage and ANOVA for the cell percentage among the age groups in CD4 subsets. Overall ANOVA p values are as follows: CD4 naïve: $p = 0.03$; CD4 Cm: $p = 0.14$; CD4 Em: $p = 0.0012$; and Treg: $p = 0.10$. Significant results of Dunn's posttest are illustrated on the plots. (b) Correlation between age and cell percentage and ANOVA for the cell percentage among the age groups in CD8 subsets. Overall ANOVA p values for CD8 subsets are as follows: CD8 naïve: $p < 0.0001$; CD8 Cm: $p = 0.28$; CD8 Em: $p = 0.0004$; and CD8 TeMRA: $p = 0.0014$. Significant results of Dunn's posttest are illustrated on the plots.

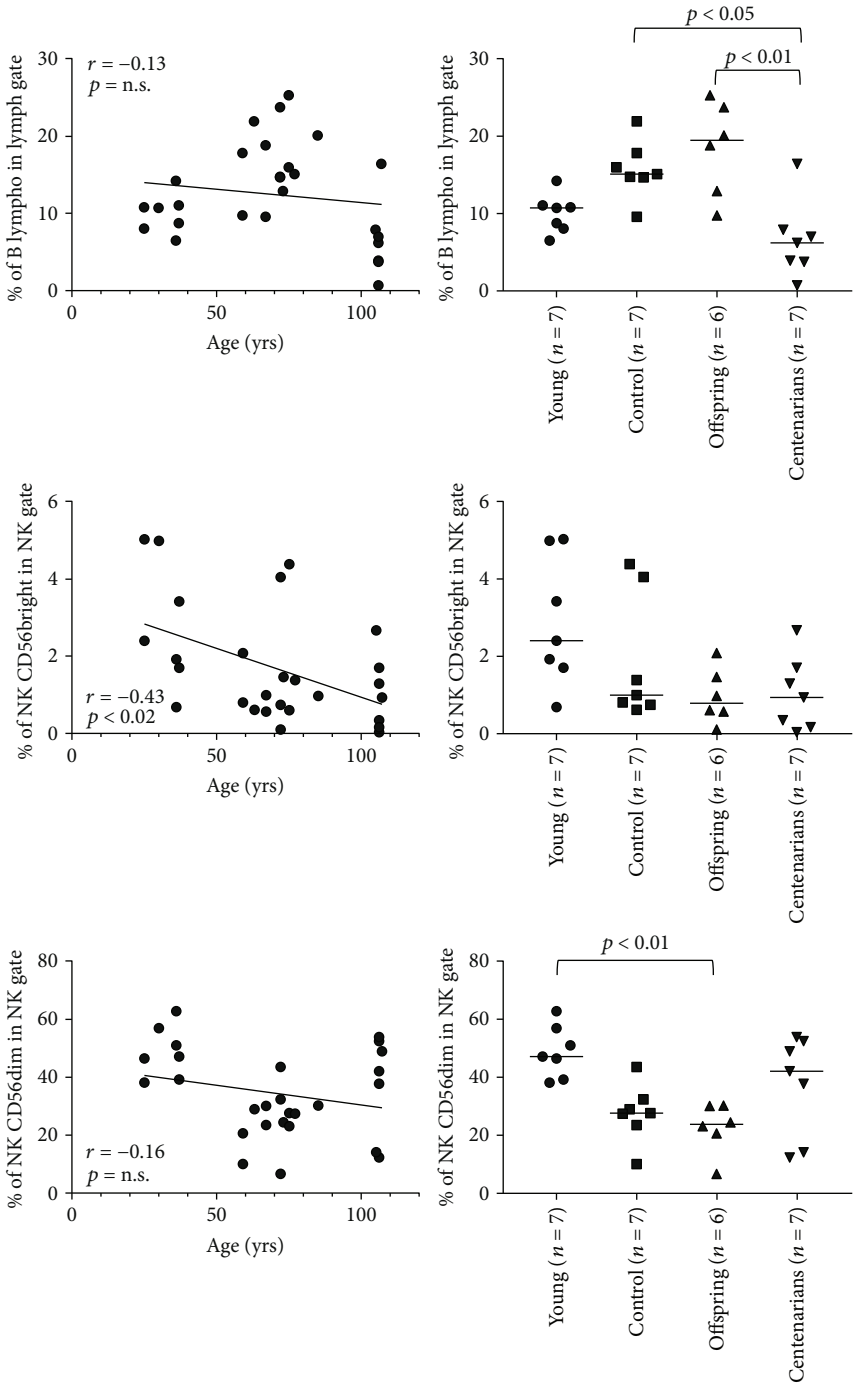


Figure 2: Correlation between age and percentage and ANOVA for the percentage among the age groups in B and NK subsets. Overall ANOVA p values for B cells: $p = 0.002$; NK CD56bright: $p = 0.06$; and NK CD56dim: $p = 0.007$. Significant results of Dunn's posttest are illustrated on the plots.

3.3. Response of the PBMC Subsets to Induced Oxidative Stress. To investigate the hypothesis that a different response to induced oxidative stress can be detected among the age groups, the ratio of ROS and GSH MFI between the induced (I) and NI oxidative stress conditions in the PBMC subsets was calculated (see Tables S3 and S5 for ROS and GSH MFI values in I condition). ANOVA showed significant overall differences in the CD4 and CD8 naïve subsets ($p = 0.0096$ and $p = 0.03$, resp.) and a trend for the CD8 TeMRA and NK subsets ($p = 0.0571$ and $p = 0.07$ for both NK CD56bright/dim, resp.; see Figures 5 and 6 and Table 1). Higher ROS I/NI MFI ratios were observed for CTRL and OFF in almost all the T, B, and NK subsets in comparison with YO and CENT, although such differences were not statistically significant at an alpha level of 5% (only in CD4 naïve, ROS I/NI ratio was significantly lower in YO compared to CTRL and

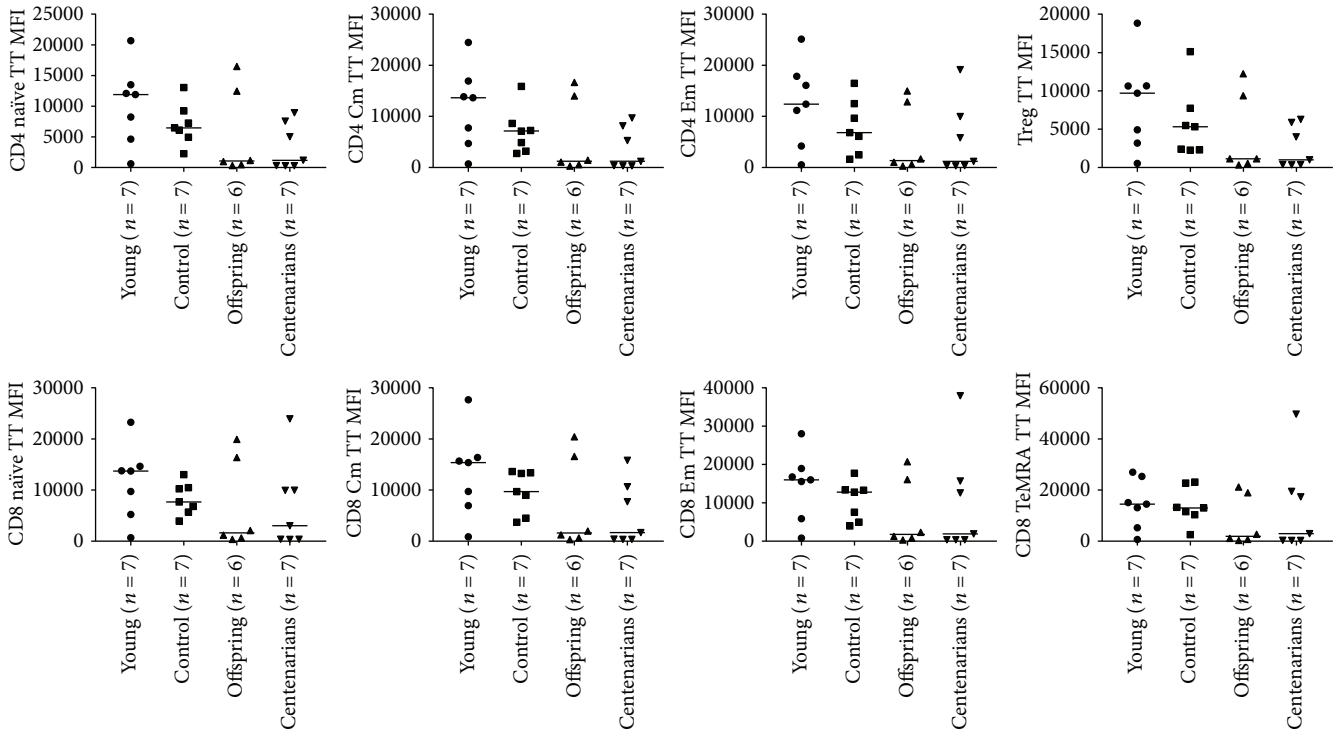


Figure 3: ANOVA for the levels of GSH expressed as MFI of the specific probe (Thiol Tracker, TT) among the age groups in CD4 and CD8 subsets. Overall ANOVA p values for CD4 subsets are as follows: CD4 naïve: $p = 0.13$; CD4 Cm: $p = 0.16$; CD4 Em: $p = 0.20$; and Treg: $p = 0.15$. Overall ANOVA p values for CD8 subsets are as follows: CD8 naïve: $p = 0.38$; CD8 Cm: $p = 0.23$; CD8 Em: $p = 0.28$; and CD8 TeMRA: $p = 0.50$.

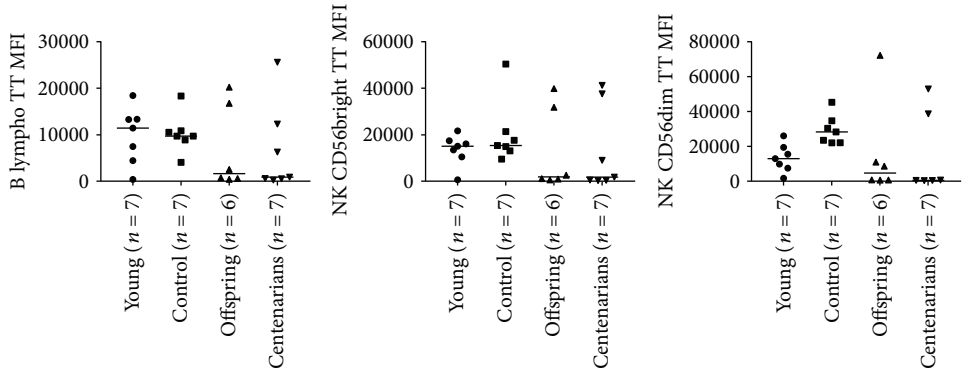


Figure 4: ANOVA for the levels of GSH expressed as MFI of the specific probe (Thiol Tracker, TT) among the age groups in B and NK subsets. Overall ANOVA p values for B cells: $p = 0.50$; NK CD56bright: $p = 0.35$; and NK CD56dim: $p = 0.07$.

CENT; see Figures 5 and 6 and Table 1). When we analyzed the GSH I/NI MFI ratios, ANOVA showed significant overall differences in CD4/8 naïve, CD4/8 Cm, CD4 Em, Treg, and B lymph. In general, a trend for increasing median values was observed across the 4 groups in almost all subsets, CENT and OFF showing the highest values. Notably, in CD4/8 naïve, CD4/8 Cm, CD4 Em, and Tregs, Dunn's posttest showed a significant difference between YO and CENT, whereas significant differences between CTRL and YO were detected in CD4 naïve and B lymphocytes (see Figures 7 and 8 and Table 2).

3.4. Correlation between the Response to Induced Oxidative Stress and the Degree of Methylation of Oxidative Stress-Related Genes. Finally, we evaluated if the differences in the response to induced oxidative stress described in the above paragraph could be related to epigenetic changes among the 4 groups. To this aim, we first considered the DNA methylation status of a list of genes involved in the response to oxidative stress (see Table S1) in a dataset including 47 CTRL, 63 OFF, and 82 CENT subjects. Several CpG sites showed significant DNA methylation differences in the three comparisons CENT versus CTRL, CENT versus OFF, and CTRL versus

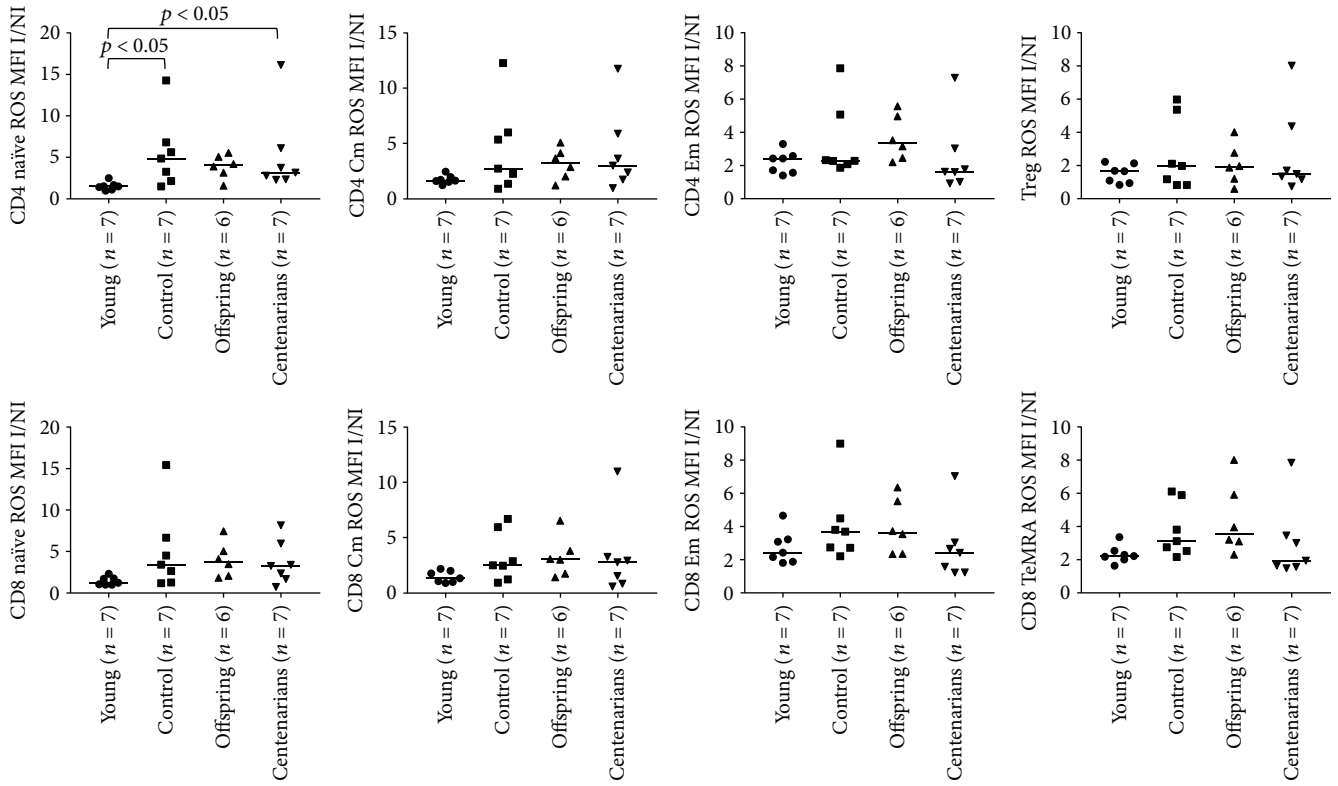


Figure 5: ANOVA for the ratios of the levels of ROS (expressed as MFI values of the ROS probe) between the I and NI oxidative stress conditions in CD4 and CD8 subsets among the age groups. See Table 1 for overall ANOVA p values. Significant results of Dunn's posttest are illustrated on the plots.

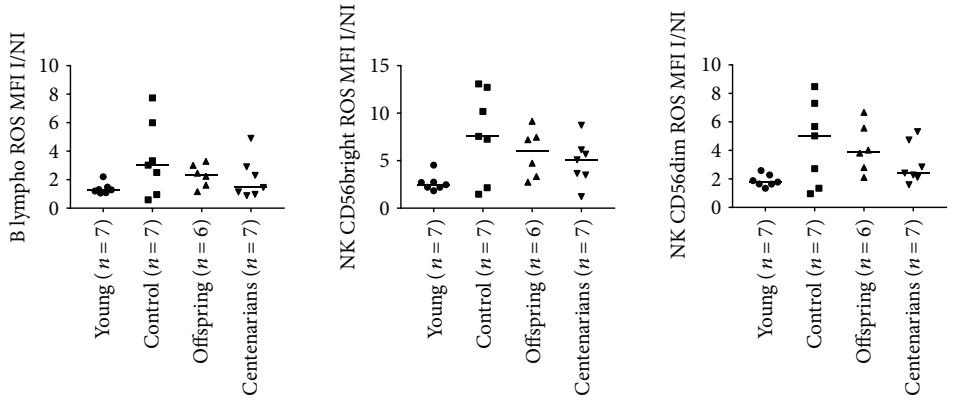


Figure 6: ANOVA for the ratios of the levels of ROS (expressed as MFI values of the ROS probe) between the I and NI conditions in B and NK subsets among the age groups. See Table 1 for overall ANOVA p values.

OFF (ANOVA p value < 0.05) (File S1). Several genes exhibited 2 or more adjacent differentially methylated CpG sites in the CENT versus CTRL and CENT versus OFF comparisons, suggesting a general epigenetic remodeling of these *loci* in the analyzed groups. These genes include GGT1, GGT6, GPX5, GPX6, GSTA 3-4, GSTM2, and LDHD (see Figures S1 and S2 sample plots). As a further step, we selected from the DNA methylation dataset the samples analyzed for the induced oxidative stress response (3 CTRL, 6 OFF, and 7 CENT) and correlated the degree of methylation of the abovementioned genes with the ROS I/NI ratio. Importantly, in this subcohort, the methylation profiles were comparable to those found in the whole cohort (see Figure S4 for an exemplative plot related to the GGT1 gene). As the methylation profile was assessed using PBMCs, we reanalyzed the ROS I/NI ratio gathering all the lymphocytes subsets together, including the monocyte population (percentage varying from 1 to 10% of the samples). The ROS I/NI ratio in CENT tended to be lower than in OFF and CTRL (Figure S3 and Table S6), confirming the above described

Table 1: Ratios of the MFI values of the ROS probe between the I and NI conditions in all the PBMC subsets among the age groups.

ROS I/NI ratio	YO	CTRL	OFF	CENT	Overall ANOVA p value
CD4 naïve	1.500	4.858	4.040	3.155	0.0096
CD4 Cm	1.631	2.724	3.256	2.992	0.2185
CD4 Em	2.423	2.279	3.336	1.622	0.1138
Treg	1.656	1.973	1.919	1.485	0.9240
CD8 naïve	1.247	3.432	3.798	3.281	0.0370
CD8 Cm	1.340	2.524	3.051	2.793	0.1657
CD8 Em	2.440	3.701	3.647	2.429	0.1745
CD8 TeMRA	2.225	3.129	3.585	1.953	0.0571
B lymph	1.289	3.023	2.346	1.457	0.2442
NK CD56bright	2.473	7.554	5.966	5.088	0.0727
NK CD56dim	1.782	5.021	3.915	2.410	0.0747

results on separated cell populations, although the differences were not statistically significant according to ANOVA test. This was probably due to the small sample size of the CTRL group, as methylation data were available only for 3 subjects analyzed with flow cytometry. Notwithstanding, Spearman correlation between the ROS I/NI ratio and the DNA methylation resulted significant for several CpG sites within GGT1 and LDHD genes (positive correlation) and GSTM2 (negative correlation) (Figure 9).

4. Discussion

In this paper, by means of PFC, we assessed the differences in the response towards induced oxidative stress in the main subsets of PBMC among the age groups, enumerating at the same time the relative amounts of each cell subset, in order to describe the process of immunosenescence. Notably, we had the opportunity to collect and analyze PBMC samples from the centenarians' offspring. This unique feature allowed us to reveal the cell phenotype of subjects sharing with CENT possible variants of genes involved in the healthy ageing and longevity and to further compare such phenotype with the one of their age-matched CTRL. Our data in T cell compartment, both in CD4 and CD8, show an age-related decrease in the percentage of naïve T cells and an increase in almost all the memory T cell subsets, including Tregs. Although the number of subjects analyzed in these cohorts was limited ($n = 27$), the differences observed are highly significant and these data are in line with the literature. Taken together, our results consistently describe the process of thymic involution and memory inflation, especially in the effector compartment [31–33, 35, 36]. Notably, in almost all T cell subsets, the percentage of cells in OFF and CTRL was comparable, suggesting that the immunosenescence of the T cell compartment follows the common age-dependent pattern, rather than being correlated to the genetic background. Moreover, we found increased percentages of B cells from YO to OFF, whereas in CENT, the percentages were comparable to YO. This is in contrast with previous observations in humans, in which a decrease or no changes were observed [31, 34]. This could be related to the decrease in CD3 T cell percentages observed in the present study from YO to OFF, already described in literature [31], whereas in CENT, the percentage was similar to YO (data not shown). As the B and CD3 gates were drawn under the same parent gate (lymphocyte scatter), the decrease in CD3 affected the B cell percentage. In this case, absolute counts would have provided more information on the amount of B cells among the groups, but only if using whole blood with stain-lyse-no wash methods, a condition that was not applicable in the present study. Finally, we observed a decreased percentage of NK CD56dim cells in CTRL and OFF, whereas in CENT, the percentage of such cells was similar to YO. As already suggested, the preservation of the number and *bona fide* function of the NK cytotoxic subset could represent a hallmark of healthy ageing [31, 34]. Once the relative amount of each PBMC subset in the age groups was determined, the response of such subsets to the induced oxidative stress was investigated. Our results showed no appreciable differences in the relative amount of ROS at the baseline in the different PBMC subsets among the age groups. Our results at the baseline are different from those available in literature, showing in general an age-related increase of the cellular oxidative stress parameters [25–27]. This could be due to different factors including the cell type investigated, the analytical methodology used, and the source of biological material, that is, frozen PBMC. Indeed, this represents a limitation of the study, as it has already been shown that the freeze-thaw process can modify the signal from ROS [27]. However, the main objective of this study was to define the response to the dynamic changes in the oxidative stress in subjects of different ages, rather than a baseline. This could represent a better hallmark of healthy ageing and at the same time offer a more reliable measurement than the baseline, potentially biased by the freeze-thaw process. Thus, we compared the ratio between the MFI of ROS in I and NI conditions. The results showed that CENT resists better to the induced oxidative stress compared with OFF and YO in almost all the PBMC subsets, having a lower ratio, in many subsets comparable to YO. Although the post hoc comparisons failed to provide significant statistical differences intergroups (possibly due to the limited number of subjects analyzed and then to the biological variability), our data suggests that CENT may have adopted efficient strategies to counteract possible sudden changes in the redox environment. This can limit the oxidative damage and, thus, preserve a better cellular response especially in the immune system. Notably, these results are in line with those obtained by Alonso-Fernández et al. measuring superoxide anion production in stimulated (but also not stimulated) neutrophils in young, middle-aged, and centenarian subjects [29]. In a previous work, using different methodologies, an age-related decreased susceptibility to the oxidative stress-induced apoptosis in nonactivated PBMC was demonstrated [37]. It is tempting to speculate that if, on the one hand, the chronic exposure to ROS decreases the susceptibility to apoptosis leading to the accumulation of particular subsets, on the other hand, CENT may be better suited to counteract dynamic variations in oxidative stress, preserving a better

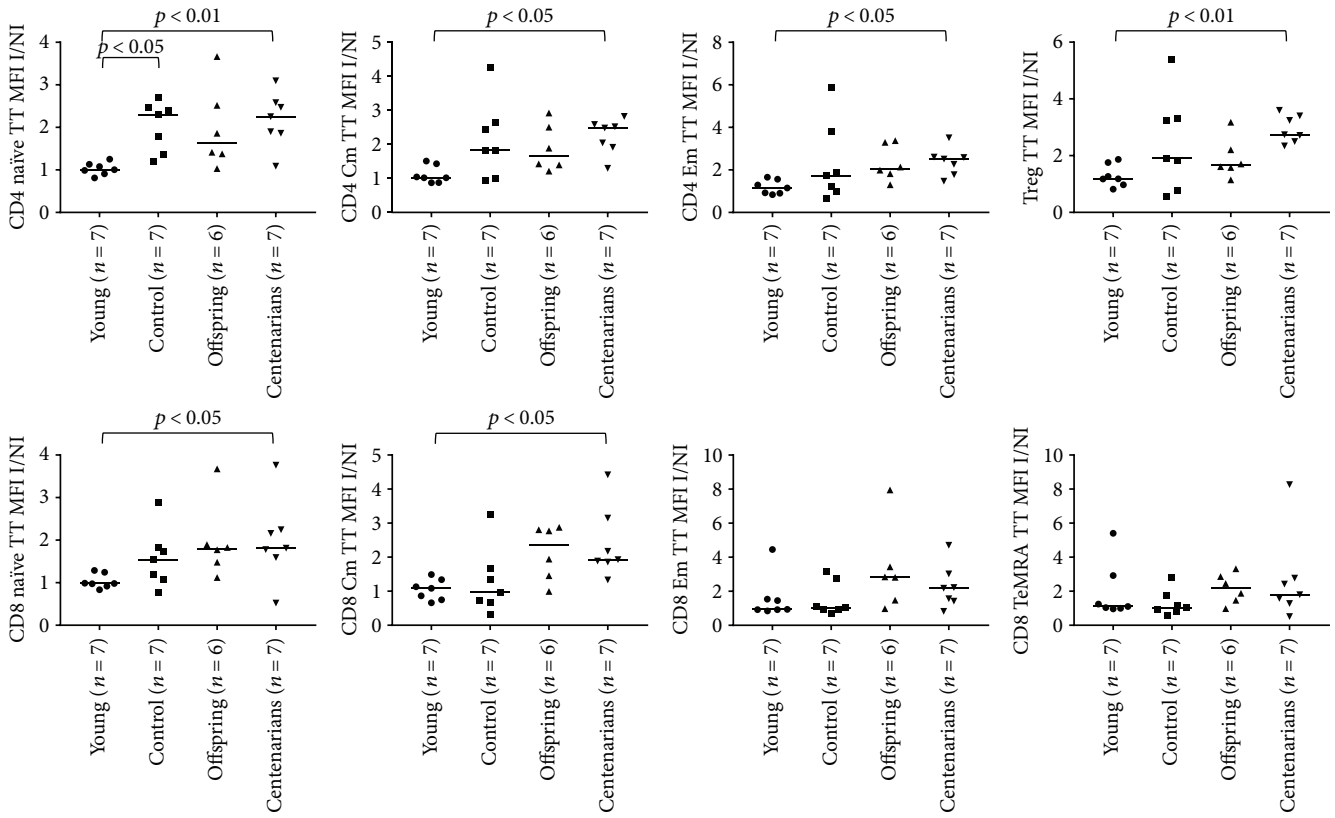


Figure 7: ANOVA for the ratios of the levels of GSH (expressed as MFI values of the TT probe) between the I and NI conditions in CD4 and CD8 subsets among the age groups. See Table 2 for overall ANOVA p values. Significant results of Dunn's posttest are illustrated on the plots.

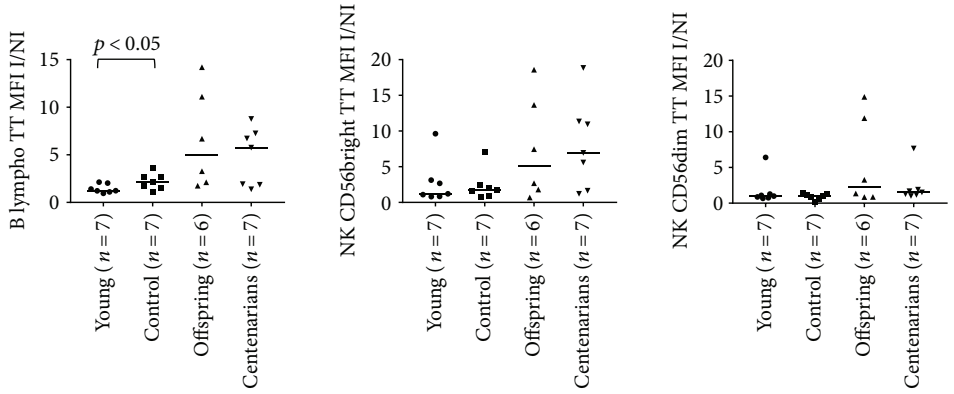


Figure 8: ANOVA for the ratios of the levels of GSH (expressed as MFI values of the TT probe) between the I and NI conditions in B and NK subsets among the age groups. See Table 2 for overall ANOVA p values. Significant results of Dunn's posttest are illustrated on the plots.

cellular response in such subsets. In this context, several lines of research put efforts in defining genetic variants in oxidative stress response genes that can be associated with exceptional longevity [38], and even more interest relies around the epigenetic changes, such as DNA methylation, that can underline the ageing process and be hallmarks of healthy ageing [39–41]. Presently, and with the limited number of subjects analyzed, we showed for the first time a significant correlation between the response to the oxidative stress in the PBMC population and the degree of methylation in several CpG positions of selected genes involved in the response to oxidative stress (LDHD, GGT1, and GSTM2; [42–44]). These findings could have relevant functional implications, as the differential degree of methylation could control the mRNA expression [45] leading, in turn, to different protein levels [46]. Thus, at least from a hypothetical perspective, different amount of proteins could impact differently the oxidative stress response. For limitations due to the availability of the specific PBMC samples, we have not been able to test such hypothesis. Thus,

Table 2: Ratios of the MFI values of the GSH probe (Thiol Tracker, TT) between the I and NI conditions in all the PBMC subsets among the age groups.

GSH I/NI ratio	YO	CTRL	OFF	CENT	Overall ANOVA p value
CD4 naïve	1.004	2.301	1.638	2.253	0.0050
CD4 Cm	1.019	1.833	1.660	2.482	0.0246
CD4 Em	1.150	1.736	2.063	2.513	0.0194
Treg	1.180	1.906	1.661	2.737	0.0146
CD8 naïve	0.9818	1.538	1.803	1.811	0.0295
CD8 Cm	1.086	0.9634	2.357	1.928	0.0114
CD8 Em	0.9410	1.019	2.832	2.176	0.1413
CD8 TeMRA	1.105	1.038	2.166	1.794	0.2438
B lymph	1.234	2.173	4.990	5.755	0.0171
NK CD56bright	1.207	1.712	5.077	6.949	0.1504
NK CD56dim	0.9887	0.9486	2.321	1.513	0.0913

additional experiments are needed, in order to ascertain whether differences in DNA methylation could be associated with variations in the gene (and protein) expression after dynamic changes in ROS level. In order to overcome possible limitations due to sample availability, PFC can be used for all the readouts, combining gene expression profiling with immunophenotyping and oxidative stress detection [47]. This would eventually allow a precise dissection of subset-related differences in the expression of selected genes, without the need to physically separate cells by cell sorting. When we approached the oxidative stress response from the antioxidant perspective, we observed an important decrease in GSH content at baseline in almost all subsets. An age-related decrease of GSH content has been already described in the lymphocyte population [26, 48], and the decline has been associated with a decrease in the γ-glutamil cysteine ligase (GCL), an important enzyme in the GSH biosynthesis, expression, and activity [49]. Notably, OFF showed the same GSH level as CENT, and much lower than the CTRL group. However, the induced oxidative stress promotes an increase in cellular GSH content [50] and, in our experimental model, we observed an age-related increase in GSH ratio after oxidative stress induction. In particular, CENT showed the highest fold-increase values in 6 out of 11 PBMC subsets analyzed, whereas OFF showed a GSH fold-increase value higher than CTRL in 8 out of 11 subsets. Strikingly, we observed a decrease in the baseline GSH content (ratio between I/NI condition < 1) after induction only in the YO and CTRL groups: in CD8 naïve, CD8Cm/Em, and in NK CD56dim subsets. This observation would suggest that CENT, consistent with the smaller fold-increase observed in ROS, promptly synthesizes and uses GSH to buffer the dynamic changes in oxidative stress level. Moreover, this could explain the fact that we did not observe drastic changes in the absolute fluorescence value of GSH. On the contrary, OFF produces GSH after oxidative stress induction but is not capable to efficiently use it to counteract the increase of cellular ROS. From a hypothetical point of view, we may speculate that CENT has a reduced capability to produce

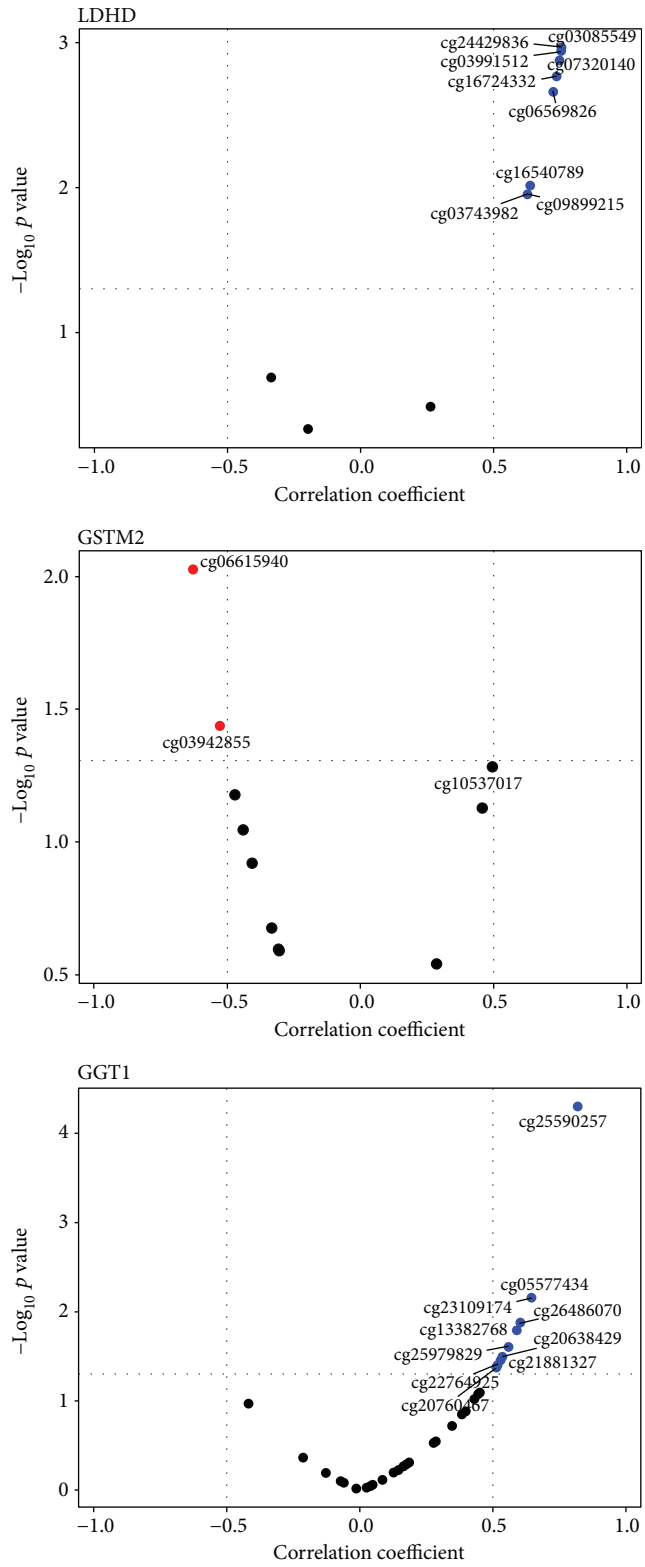


Figure 9: Analysis of correlation between the degree of methylation of the CpG sites and the response to induced oxidative stress (expressed as the ratio of MFI values of the ROS probe between I/NI conditions) for LDHD (top), GSTM2 (middle), and GGT1 (bottom) genes.

constitutive GSH but at the same time is more efficient in producing and using GSH under stress conditions. In contrast, YO and CTRL rely on their GSH stores to counteract the dynamic changes in the redox environment, whereas OFF promptly triggers GSH production but, unlike the CENT, appears not to be capable in using it to buffer the cellular ROS increase. Such observations, however, need to be confirmed in other independent cohorts of ageing subjects. Taken together, our results suggest that, at least in cultured PBMC, the resistance to changes in oxidative stress levels is a characteristic of the group of subjects who reached healthy ageing. In the immune system, this possibly implies that CENT could maintain good cell functions in order to efficiently fight infections. However, our observations must be placed into a more comprehensive framework as ageing involves complex processes shaped by genetic, epigenetic, and environmental factors. Thus, further investigations are needed to elucidate the mechanisms of the resistance to oxidative stress and to evaluate possible intervention (i.e., diet and lifestyle) to enhance such resistance in the ageing population.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Supplementary Materials

Table S1: list of the genes involved in response to the oxidative stress. Figure S1: percentage of methylation of the CpG sites throughout the GGT1 gene in the 3 ageing groups. Vertical bars represent the standard deviation of the mean of the methylation values for each CpG site in the 3 groups. Figure S2: percentage of methylation of the CpG sites throughout the GSTM2 gene in the 3 ageing groups. Vertical bars represent the standard deviation of the mean of the methylation values for each CpG site in the 3 groups. Figure S3: ratios of the MFI values of the ROS probe between the I and NI conditions in the whole PBMC population across 3 groups of ageing individuals (subcohort $n = 16$). Figure S4: percentage of methylation of the CpG sites throughout the GGT1 gene in the subcohort of 16 subjects. Vertical bars represent the standard deviation of the mean of the methylation values for each CpG site in the 3 groups. ANOVA between the three groups (CENT $n = 7$; OFF $n = 6$; CTRL $n = 3$) showed the following results: cg25590527: $p = 0.0042$; $q = 0.1181$; cg13825083: $p = 0.0406$; $q = 0.1800$; cg21881327: $p = 0.019$; $q = 0.1788$; cg09598276: $p = 0.0304$; $q = 0.1788$; cg01120527: $p = 0.005$; $q = 0.1181$; cg22123459: $p = 0.003$; $q = 0.1181$. Table S2: MFI values of the ROS probe in the NI condition among the age groups in the PBMC subsets. Table S3: MFI values of the ROS probe in the I condition among the age groups in the PBMC subsets. Table S4: MFI values of the GSH probe in the NI condition among the age groups in the PBMC subsets. Table S5: MFI values of the GSH probe in the I condition among the age groups in the PBMC subsets. Table S6: MFI values of the ROS and GSH probes in the NI, I, and I/NI conditions in the whole PBMC population among the age groups. *(Supplementary Materials)*

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 4159013, 32 pages
https://doi.org/10.1155/2018/4159013

Review Article

Inducers of Senescence, Toxic Compounds, and Senolytics: The Multiple Faces of Nrf2-Activating Phytochemicals in Cancer Adjuvant Therapy

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Received 6 October 2017; Accepted 19 December 2017; Published 12 February 2018

Academic Editor: Johannes Grillari

The reactivation of senescence in cancer and the subsequent clearance of senescent cells are suggested as therapeutic intervention in the eradication of cancer. Several natural compounds that activate Nrf2 (nuclear factor erythroid-derived 2-related factor 2) pathway, which is involved in complex cytoprotective responses, have been paradoxically shown to induce cell death or senescence in cancer. Promoting the cytoprotective Nrf2 pathway may be desirable for chemoprevention, but it might be detrimental in later stages and advanced cancers. However, senolytic activity shown by some Nrf2-activating compounds could be used to target senescent cancer cells (particularly in aged immune-depressed organisms) that escape immunosurveillance. We herein describe in vitro and in vivo effects of fifteen Nrf2-interacting natural compounds (tocotrienols, curcumin, epigallocatechin gallate, quercetin, genistein, resveratrol, silybin, phenethyl isothiocyanate, sulforaphane, triptolide, allicin, berberine, piperlongumine, fisetin, and phloretin) on cellular senescence and discuss their use in adjuvant cancer therapy. In light of available literature, it can be concluded that the meaning and the potential of adjuvant therapy with natural compounds in humans remain unclear, also taking into account the existence of few clinical trials mostly characterized by uncertain results. Further studies are needed to investigate the therapeutic potential of those compounds that display senolytic activity.

1. Introduction

Cellular senescence (CS) is a biological response to a variety of stresses that results in persistent growth arrest with a distinct morphological and biochemical phenotype [1–3]. It is currently considered a "barrier" to prevent malignant transformation and a potent anticancer mechanism as well as a hallmark of aging. Exploration of CS to drive towards antitumor adjuvant therapies by natural compounds is currently gaining increasing interest. Cancer cells can be forced to undergo senescence by natural compounds, with effects somewhat comparable to those obtained by genetic and epigenetic manipulations, anticancer drugs, and irradiation [4]. These effects have been shown after sustained exposure *in vitro* to a wide range of different substances that are also paradoxically used to obtain cytoprotective and chemopreventive adaptive responses in normal cells [5, 6]. Interestingly, most of these cytoprotective activities are likely to be mediated by Nrf2 (nuclear factor erythroid-derived 2 related factor 2) stress-responsive signaling [7–9]. Examples of these natural bioactive compounds include mostly phenols like curcumin, epigallocatechin gallate (EGCG), fisetin, genistein,

phloretin, quercetin, resveratrol, and silybin as well as other classes of compounds such as organosulfur compounds [i.e., allicin, phenethyl isothiocyanate (PEITC), and sulforaphane], metyl-tocols [i.e., tocotrienols], alkaloids (i.e., berberine, piperlongumine), and terpenoids (i.e., triptolide) [9–12]. Although, in certain cases, these compounds can specifically interact with the altered pathways of cancer cells [5]; the structural and physical differences of these compounds suggest that their ability to activate the antioxidant response elements (AREs) of many cytoprotective genes through the cytoplasmic oxidative stress system, Nrf2-Keap1 (Kelch-like ECH-associated protein 1), is perhaps a common mechanism of action. Considering that cancers with high Nrf2 levels are associated with poor prognosis because of radio and chemoresistance and aggressive proliferation, activating Nrf2 pathway is considered protective in the early stages of tumorigenesis but detrimental in the later stages [13]. Hence, it can be found a paradox on how Nrf2-activating compounds can be proposed to induce senescence in cancer cells and, eventually, as a tool for adjuvant therapy. Interestingly, it is becoming evident that some effects of Nrf2-Keap1 pathway may be mediated through crosstalk with additional pathways (i.e., the aryl hydrocarbon receptor (AhR) pathway) affecting aspects of cell fate that provide a multitiered, integrated response to chemical stresses [14] which, in turn, could eventually culminate in a senescent response. This could be promoted by defective pathways of cancer cells or by excess amounts of the bioactive compounds. Indeed, most of the prosenescence effects shown *in vitro* are obtained with relatively high concentrations of the bioactive compounds (micromolar ranges) that are likely to not be translated *in vivo* (usually nanomolar ranges) due to potential toxicity to healthy cells, unless the compound can be specifically targeted to cancer cells. Interestingly, selective accumulation of natural compounds (i.e. T3s) in cancer tissues has been reported [15] and would deserve appropriate investigation for the future development of adjuvant supplements in cancer therapy. The possibility to induce senescence in tumors with lower drug doses, especially if administered chronically, may potentially limit treatment-related toxic side effects. However, even in the cases where a sufficient degree of selectivity has been demonstrated, senescence escape systems of cancer cells [16] may hamper the efficacy and thus the clinical applications of these compounds. In addition, it is an emerging concept that immune responses against senescent cells are crucial to restrict disease progression in cancer pathologies [17]. Treatments aimed at inducing senescence in cancer are likely to fail in the complete clearance of senescent cancer cells if not supported by a proper senescence immune surveillance response [16]. Senescent cancer cells might later be able to revert their senescent phenotype [18] or promote new cancers in their microenvironment in the case of ineffective clearance mechanisms [19, 20]. In this case, the interaction of natural bioactive compounds with the senescent-associated secretory phenotype (SASP) might be crucial. The SASP may have positive or negative effects, depending on the context: it can cause local and potentially systemic inflammation, disrupt tissue architecture, and stimulate growth and survival of nearby malignant cells [17], but it can be eventually important to promote immune clearance of senescent cells. Hence, the anti-inflammatory activity of natural compounds should also be carefully evaluated in this context. Additional challenges in this field include the proper characterization of CS [21], a clear understanding of the role of senescent cells in physiological and pathological conditions [2] and the huge heterogeneity of CS models [22, 23] which, in turn, might make it difficult to compare the effects and understand the proper area of application of promising natural bioactive compounds. In this review, we summarize the most relevant studies focused on induction of CS *in vitro* by selected bioactive compounds and discuss critical aspects related to their putative mechanisms of action and eventual translation *in vivo*.

2. Cellular Senescence and Senolytic Compounds

CS is usually defined as a status of growth arrest mediated by insensibility to mitogen stimuli, chromatin and secretome changes, and upregulation of particular tumor suppressor pathways [2, 24, 25]. CS induction may occur by a variety of cell-intrinsic and cell-extrinsic stresses, including DNA damage, oxidative stress, critical telomere shortening and damage, chronic mitogenic signaling, oncogene activation and inactivation, loss of tumor suppressors, nucleolar stress, and epigenetic changes [25]. There is no unique and definitive marker to define the senescent status of a cell, and not all senescent cells show the same features. Hence, characterization of CS can be performed by assessing multiple markers such as an enlarged morphology, the activation of p53-p21 and/or p16-Rb tumor suppressor pathways, the presence of persistent DNA damage response (DDR), an increase in CS-associated beta-galactosidase (SA-β-Gal) activity, and the appearance of senescent-associated distension of satellites and telomere-associated DNA damage foci. In replicative senescence, critically shortened telomeres activate DDR and subsequent stabilization of p53 while oxidative stress and oncogene-induced senescence may work mainly through the activation of both p53 and p16 pathways [2]. Epigenetic derepression of CDKN2A (cyclin-dependent kinase inhibitor 2A) locus, which encode for both p16 and p14, is another trigger for senescence associated with aging and is responsible of the increased expression of p16 in aged tissues [26] and one of the most prominent indicators of the presence of senescent cells in aged tissues [27]. A common mediator of CS is the inhibition of Rb phosphorylation, which results in the inactivation of the E2F transcription factor, and its target genes involved in cell cycle progression [28]. The activation of growth pathways, via the mTOR (mammalian target of rapamycin), and the autophagic response appear as additional important players in establishing CS [29]. Senescent cells additionally display an increase in metabolic activity and, frequently, develop the SASP, which includes several proteins involved in inflammation processes, proteases, hemostatic, and growth factors [30].

Studies of human tissues and cancer-prone mice argue strongly that CS is one of the most important processes to suppress cancer *in vivo* [24], but the SASP produced by

senescent cells can induce deleterious effects in the microenvironment by damaging neighboring cells, thus facilitating tumor development and aggressiveness [31], mediating paracrine transmission of CS [32], and promoting age-related dysfunctions [25].

An important physiological function of the SASP is to promote clearance of senescent cells by the immune system (a process named senescence immunosurveillance). However, age-related immunodeficiency or the production of a less proinflammatory SASP by senescent cells accumulated in aged-tissues could hamper senescence immunosurveillance [25].

A relevant feature of some, but not all [33], senescent cells is their long-term survival and resistance to apoptosis [34], which likely contributes to their persistence and the respective deleterious consequences in aged tissues. A direct demonstration that senescent cells can drive age-related pathologies has been originally provided with the development of a transgenic mouse model, in which p16-expressing cells can be specifically eliminated upon drug treatment, with consequent prevention, delay, or attenuation of some age-related disorders [35]. This study prompted the birth of a field of research aimed to identify antiapoptotic mechanisms occurring in senescent cells and the relative compounds that are able to break this resistance to cell death with a high selectivity for senescent cells. The development of this area of research has been so fast that research around compounds able to selectively induce death in senescent cells (named senolytic drugs) represents now one of the most fruitful area of investigation [36, 37]. Preclinical studies have reported that senolytic compounds can improve cardiac function [38] in old mice, recover vascular function and decrease vascular calcification in atherosclerosis mice [39], and improve pulmonary function and physical health in mouse models of fibrotic pulmonary disease [40], as well as achieve partial rejuvenation in several tissues of progeroid mice [38, 41]. Besides, these are only part of the results achieved in age-related chronic conditions and others are expected to be shown soon. Importantly, senescent cells accumulate in mice treated with chemotherapy, causing a range of defects and promoting tumor recurrence [42]. Hence, it is not surprising that senolytic compounds have been proven to delay tumor recurrence and metastasis in mouse cancer models after chemotherapy [42] as well as to ameliorate side effects associated with the therapy [43]. However, senolytic compounds appear to be cell type restricted as a consequence of the heterogeneity of senescent cells and their different antiapoptotic pathways. The most important antiapoptotic pathways identified in senescent cells include the B-cell lymphoma 2 (BCL-2)/B-cell lymphoma-extra large (Bcl-xL), the PI3kδ/AKT, the p53/p21, the ephrins, the HIF-1α, the HSP-90, and various metabolic pathways [37]. These pathways may be differentially activated depending on the type of senescent cells (e.g., endothelial cells or fibroblasts) and the species of the donor (e.g., human or mice); thus, each senolytic compound displays its activity in some but not all types of senescent cells. In certain cases, the combination of two compounds is effective as senolytic in a wider range of cell type than the single compounds. This is the case of the combination of quercetin with dasatinib, which is effective in several models of senescent cells (endothelial, preadipocytes, and fibroblasts), whereas quercetin is only effective in radiation-induced endothelial cells and dasatinib in senescent preadipocytes [38].

Up to date, a small number of natural compounds have been shown to display senolytic activity, but it is likely that this is the tip of an iceberg that will be exposed in the coming years. These include quercetin [38], fisetin [44], phloretin [45], and piperlongumine [46], and there are preliminary indications that tocotrienols [47] and, eventually, cannabinoids [6] may also display senolytic activity in particular models of cellular senescence. Paradoxically, and very similar to the activity of senescence inducers described in the previous section, all these compounds can be accumulated by their potential to induce Nrf2 cellular response, which has a well-demonstrated cytoprotective and antiapoptotic effects. Indeed, the Nrf2/Keap1 pathway is activated by quercetin [48], fisetin [49], piperlongumine [50], and phloretin [51] as well as by different cannabinoids [52], tocotrienols [53], and a multitude of anticarcinogenic natural compounds that were found to cause cancer cell apoptosis or senescence under certain conditions (Figure 1).

The molecular mechanisms explaining how the Nrf2/Keap1 pathway is modulated during apoptosis and senescence are currently largely unknown, and this gap of knowledge may contribute to hamper the clinical translation of adjuvant therapies based on Nrf2-activating compounds.

3. The Nrf2-Keap1 Pathway

The Nrf2-Keap1 pathway is a key controller of cellular response to stress caused by reactive oxygen species (ROS) [13]. The Nrf2 antioxidant response is mediated by the activation of ARE/electrophile responsive element (ARE) in the regulatory region of target genes. Molecular details of this signaling pathway and its dysregulation in cancer have emerged over the last 10 years and are extensively reviewed elsewhere [13, 14, 54]. Oxidative signals induce changes in the sulfhydryl groups of Keap1, thus promoting Nrf2 dissociation from Keap1, Nrf2 nuclear translocation, and stimulate mRNA expression of Nrf2-targeted downstream genes. Genomic analyses indicate that gene families affected by Nrf2 display a multitude of responses with a defensive role against cellular senescence including detoxification, antioxidant, damage repair, and inhibition of inflammation. This response involves more than 200 antioxidant and protective genes that constitute the so-called phase II response. Among these enzymes, we can mention γ-glutamylcysteine ligase (γ-GCL), glutathione peroxidase (GPx), heme oxygenase 1 (HO-1), superoxide dismutase (SOD), glutathione S-transferase (GST), and NADPH-quinone oxidoreductase (NQO1) which have been frequently studied in the context of a protective response against cell death or senescence [55]. Hence, it is not surprising that a body of evidence supports the role of Nrf2 in mediating protection against stress-induced senescence [55–58]. Several studies have identified inactivating mutations in Keap1, leading to an increase in Nrf2 function, in human cancers [59–61]. Hence, inducing

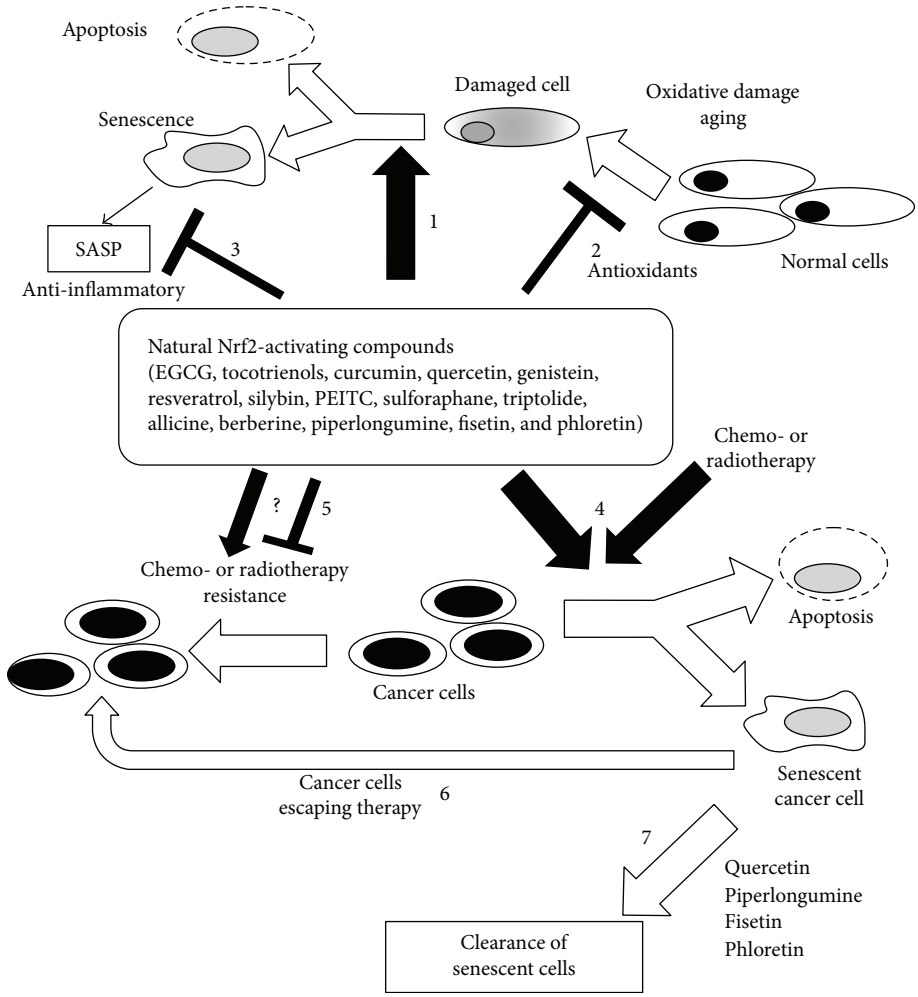

Figure 1: Potential effects and concerns of selected natural compounds as adjuvant in cancer therapy. Based on experiments "in vitro," epigallocatechin gallate (EGCG), tocotrienols, curcumin, quercetin, genistein, resveratrol, silybin, phenyl isothiocyanate (PEITC), sulforaphane, triptolide, allicin, berberine, piperlongumine, fisetin, phloretin might be useful in prevention and therapy of cancer. Gero- and cancer preventive activity include (1) induction senescence or apoptosis in normal damaged and potentially precancerous cells, (2) protection of normal cells by damage via modulation of antioxidant/cytoprotective pathways, and (3) anti-inflammatory activity that might reduce negative effects of the senescence-associated secretory phenotype (SASP) produced by senescent cells. In cancer therapy, natural bioactive compound might help (4) to induce apoptosis and senescence in cancer cells thus helping to reduce dosage of chemo- and radiotherapy while keeping efficacy. The major concern regards the possibility that these compounds might act as cytoprotective in some cancer cells (as in normal cells), thus aggravating the problem of resistance of cancer to therapy (5). However, failure to clearance senescent cells (6), as it might occur in immune-compromised subjects, might represent a serious challenge for these applications. Inclusion of additional strategies (7) with other natural compounds (i.e., phloretin, fisetin, piperlongumine, and quercetin) able to induce selective death of senescent cells should be evaluated in future preclinical studies to reduce relapses and side effects of chemo- or radiotherapy.

the Nrf2-regulated cytoprotective response could provide a selective advantage to tumor cells, raising the question of whether it is hazardous to elicit these changes in the context of interventions for cancer.

The role of Nrf2 in cellular senescence has been poorly studied. It is known by other models (cell lines and cancer cells) that Nrf2 upregulates most of the antiapoptotic mechanisms that have been shown to be repressed by senolytics, including natural compounds. In particular, HIF-1 alpha signaling is augmented by induction of the Nrf2 pathway, as demonstrated in hypoxia models [62]. Moreover, Nrf2 protein upregulates the antiapoptotic protein Bcl-2 [63] and interacts with p21, which promotes the activation of the antioxidant response mediated by Nrf2 [64]. Hence, it would have been expected that Nrf2 is upregulated in cellular senescence. In contrast with this rationale, Nrf2 has a declined function in senescence of human fibroblasts [65], whereas its silencing leads to premature senescence [65, 66]. Moreover, it appears to be downregulated in oncogene-induced senescence of transformed cell lines (in which senescence can be triggered by MEK activation) and upregulated when senescence is bypassed (GEO Accession: GDS1637, Profile GDS1637/201146_at) [67]. In contrast with the premature senescence induced by Nrf2, others have reported that deletion of Nrf2 in mouse embryonic fibroblasts is associated with immortalization [68]. Interestingly, these immortalized

cells display a positive staining for SA-β-Gal, thus suggesting that deficiency in proteosomal degradation induced by Nrf2 deletion could be compensated, at least in part, by induction of lysosomal hydrolytic enzymes [68] that are similarly activated in several models of cellular senescence.

In the next chapters, we describe the most studied Nrf2-inducing natural compounds that have been shown to act *in vitro* as toxic compounds in cancer cells and that can be used as senolytics or senescence inducers (or both). A particular focus on the doses used in the experiments and a short description of their eventual use in clinical trials is also provided as schematically represented in Figure 1.

4. Nrf2-Activating Phytochemicals: Senescence Inducers, Senolytics, or Toxic Compounds for Cancer Cells

4.1. Tocotrienols. Tocotrienols (T3s), members of the vitamin E family, are naturally occurring compounds composed of four different isomers: alpha (α), beta (β), gamma (γ), and delta (δ). These compounds are present in barley oil, coconut oil, corn oil, palm oil, rice bran oil, wheat germ, and annatto [69]. Cell culture studies suggest that T3s affects numerous pathways linked with tumorigenesis, including Nrf2 pathway [47, 53].

4.1.1. Tocotrienols as Toxic Compounds in Cancer. In the last years, T3s have been of increasing interest due to the discovery of their anticancer effects, not generally evident with tocopherol-rich vitamin E preparations [70]. Among the four isoforms of T3s, γ and δ are those which have proven greater effectiveness in countering the proliferation of tumor cells. T3s can induce apoptosis in various types of mammary cancer cells by acting on mitochondrial or death receptor-mediated pathways [71, 72].

Further anticancer mechanisms of T3s including down-regulation of mitogenic signal/survival factors and induction of paraptosis-like death have also been described in different cellular models.

4.1.2. Tocotrienols as Senescence Inducers. T3s have been additionally shown to induce cell cycle arrest and senescence-like phenotype in various cancer cells *in vitro*. Genes involved in cell cycle control, such as p21, p27, and p53, may represent the downstream effectors of T3s that affect the balance between signals that drive the cell into senescence or to death. In malignant mouse +SA mammary epithelial cells, 4 μM γ-tocotrienol significantly inhibited cell proliferation which was associated with reduction in cell cycle progression from G_1 to S, as evidenced by increased p27 level, and a corresponding decrease in cyclin D1, CDK (cyclin-dependent kinase) 2, CDK4, CDK6, and phospho-Rb levels [73]. Similar results have been shown in HER-2 (human epidermal growth factor receptor 2) overexpressing cell lines with the upregulation of p53, p21, and p16 induced by mixtures of γ-T3s and δ-T3s [72]. Interestingly, oral administration of 100 mg/kg annatto-T3 delayed the spontaneous onset of mammary tumor and reduced tumor number and size through enhancing in situ both apoptosis and senescence markers in a HER2/neu breast cancer mouse model [15], thus showing that the results obtained *in vitro* can be translated *in vivo*. In this mouse model, T3s have been shown to specifically accumulate in cancer tissues of HER2/neu mice at a very high rate than observed in normal tissues.

T3s (10–20 μM) have also been shown to inhibit telomerase by affecting hTERT (human telomerase reverse transcriptase) and c-Myc expression through PKC (protein kinase C) activity suppression in human colorectal adenocarcinoma cell lines [74]. By the way, c-Myc is known to induce epigenetic changes leading to transcriptional activation of genes that suppress key drivers of CS. The involvement of PKC, whose isoforms are known to selectively mediate certain malignant phenotype including HER2-positive breast tumors [75], in the mechanisms of action of T3s might also contribute to explain why T3s can induce opposite effects (antisenescence) in normal human fibroblasts [76]. Another upstream target of T3s that could mediate senescent-like response or apoptosis in breast cancer cells is ERs (estrogen receptors) [77]. T3s display high affinity for ERβ and increase its translocation into the nucleus which, in turn, activates the expression of estrogen-responsive genes [MIC-1 (macrophage inhibitory cytokine-1), EGR-1 (early growth response protein 1), and cathepsin D] involved in growth arrest, altered morphology, and apoptosis of ERβ expressing breast cancer cells (MDA-MB-231 and MCF-7) [78]. Hence, the idea that these compounds might promote senescence in cancer cells while displaying antisenescence effects in normal cells sounds very promising in view of its potential clinical applications.

4.1.3. Tocotrienols as Potential Senolytics. Senolytic activity has not been tested for T3s. However, some metabolic and apoptotic pathways affected by these compounds in cancer cells overlap with those of other compounds that have been shown to display senolytic activity, such as quercetin [47]. Moreover, T3s have been shown to display rejuvenating effects which might in the end represent the net results of a senolytic activity on senescent cells and a selective survival of a subpopulation of nonsenescent cells in the culture.

4.1.4. Tocotrienols in Cancer Adjuvant Therapy. Despite the number of clinical trials conducted to examine the multifaceted health benefits of T3s [79, 80], very little is known about the efficacy of T3s as adjuvant supplements in cancer therapy. Pilot clinical trials on the synergistic effect of T3s and chemotherapy have been mainly addressed to test safety without any clear advantage for survival or other clinical endpoints [81, 82]. However, measurements of T3s in malign and benign adipose breast tissues of a Malaysian population found that total T3s levels were lower in the malignant tissues compared to the benign ones [83]. These data reinforce the idea that T3s may provide some kind of protection against breast cancer but the circumstances and the modality of intervention would require further studies.

4.2. Curcumin. Curcumin, a component of turmeric rhizome, is another example of Nrf2-activating compound [84, 85]

that, in certain circumstances, acts as cytotoxic or prosenescence compound in cancer cells.

4.2.1. Curcumin as Toxic Compound in Cancer. Curcumin affects various biochemical and molecular cascades involved in cancer by acting on a multitude of molecular targets including NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), Akt, MAPK (mitogen-activated protein kinases), p53, Nrf2, Notch-1, JAK (Janus kinase)/STAT (signal transducer and activator of transcription), β-catenin, and AMPK (5′ adenosine monophosphate-activated protein kinase) [86]. A direct inhibition of mTORC1 (mammalian target of rapamycin complex 1) signaling [87] and induction of autophagic cell death [88] have also been claimed to explain the cytotoxic effects of curcumin in various cancer cells. However, the same mechanisms can also be responsible for the reversion of senescence and appearance of proliferating cells in irradiated apoptosis-resistant cells [89].

4.2.2. Curcumin as Senescence Inducer. Notwithstanding senescence-suppressive activity, there is substantial evidence that curcumin can induce senescence in different cancer models. This has been clearly shown in MCF-7 breast cancer cell line [90, 91], human colon cancer cells, [90, 92] and breast cancer-associated stromal fibroblasts [93]. Inhibition of telomerase activity, induction of p53, p21, and p16, and an increased autophagic response have been reported as the main mediators of this prosenescence activity of curcumin.

4.2.3. Curcumin as Potential Senolytic. Curcumin has been recently tested for a potential senolytic activity in senescent fibroblasts from Ercc1−/− mice showing no effect on senescent cells [41]. However, this kind of activity would deserve further experiments in different type of cells.

4.2.4. Curcumin in Cancer Adjuvant Therapy. The most likely explanation to this multitude of proposed mechanisms is that curcumin can display cell-specific effects, thus suggesting that adjuvant therapy with this compound could be most effective in certain type of cancer using appropriate delivery systems. Some promising effects have been observed in breast, prostate, lung, pancreatic, and colorectal cancer as well as in multiple myeloma [94]. Consistent with *in vitro* studies, curcumin administration has been shown to affect molecular targets involved in cancer. Presurgery curcumin administration in patients with colorectal cancer decreased serum TNF-alpha levels and increased cancer cell apoptosis, observed as enhanced p53 and Bcl-2, and decreased Bax expression in tumor tissues compared with control [95]. A decrease in NF-κB and cyclooxygenase-2 (COX-2) expression and pSTAT3 activation was shown in peripheral blood mononuclear cells (PBMC) from patients with advanced pancreatic cancer receiving curcumin oral administration contemporary to gemcitabine-based chemotherapy [96]. Also, curcumin seems to be effective in protecting from side effects associated to chemo- and radiotherapy [97], though no biological evidence has been provided. In spite of these promising results, the paucity of well-controlled clinical trials, the poor bioavailability of curcumin and the limited effects reported by some investigators are currently a major limitation to the therapeutic use of curcumin.

4.3. Epigallocatechin Gallate. Epigallocatechin gallate (EGCG), the most active and major component of polyphenols in green tea, is known to be the principal contributor to the potential benefits of green tea to human health [98]. Hence, it is not surprising that EGCG and other tea catechins have been claimed of anticarcinogenic and antimutagenic activities [99]. The use of EGCG as a possible chemopreventive agent is supported by a number of studies regarding the ability of EGCG to modulate Nrf2-mediated cellular events [100, 101]. There is also substantial evidence that EGCG can display antisenescence effects, as observed in mesenchymal stem cells [101, 102].

4.3.1. EGCG as Toxic Compound in Cancer. The cytoprotective effect of EGCG is apparently in contrast with a number of studies in cancer cells suggesting that induction of apoptosis could be the main mechanism of green tea to suppress cancer cell growth [103]. This paradoxical effect and the different effects shown in cancer versus primary cells could be related to their different metabolism including defects in the regulatory feedback that involves mTOR, p53, and AMPK [5] which are related to the epigenetic differences between cancer and normal cells [104, 105]. EGCG has been shown to be able to induce dose-dependent (5–80 μM) apoptotic cell death in estrogen receptor- (ER-) independent breast cancer cells via an increased Bax to Bcl-2 protein ratio and p53 expression [106]. Moreover, EGCG appears to be critical for cancer cell metabolism due to the inhibition of mitochondrial functions and the generation of a starvation-like condition that activates AMPK and its downstream effects, including inhibition of mTOR signaling [107] and the activation of a sustained autophagic response that can promote autophagic cell death [108].

4.3.2. EGCG as Senescence Inducer. Nontoxic concentrations (15 μM) of EGCG shortened telomeres, increased SA-β-Gal staining, induced chromosomal abnormalities, and, most importantly, limited the lifespan of U937 monoblastoid leukemia and colon adenocarcinoma cell lines (HT29) [109]. Experiments in breast cancer (MCF-7) and promyelocytic leukemia (HL60) cell lines have confirmed an inhibitory activity on telomerase activity by EGCG [110]. Alterations in histone modifications, decreased methylation of hTERT promoter, and increased binding of the hTERT repressor E2F-1 at the promoter were proposed as mediators of the observed bioactivity [110].

4.3.3. EGCG as Potential Senolytic. Although there are studies that have indicated EGCG as a senomorphic (suppressor of senescent phenotype) compound *in vitro* with potential lifespan-extending effects in animal models [111], there is currently no evidence that EGCG can exert senolytic activity in selected type of senescent cells.

4.3.4. EGCG in Cancer Adjuvant Therapy. EGCG has also been proven to synergize with some anticancer agents and to ameliorate their deleterious side effects, which makes

EGCG a suitable adjuvant in chemotherapy [112]. However, most of the studies on this topic are preclinical, and several limitations in terms of stability, efficacy, and bioavailability have currently hampered the application of EGCG in clinical settings [113]. There are contrasting results depending on the type of cancer and therapy. For example, EGCG was shown to provide regression of esophagitis in patients with unresectable stage III non-small-cell lung cancer under chemo- and radiotherapy [114]. Conversely, green tea polyphenols may have the potential to negate the therapeutic efficacy of the boronic acid-based synthetic anticancer drug bortezomib, thus suggesting that EGCG may be contraindicated during cancer therapy with bortezomib [115]. Finally, it is important to note that the concentrations of EGCG used *in vitro* (tens of micromolar) are usually far from levels observed in serum after drinking few cups of tea as biologically achievable concentrations were generally reported to be below 1 μM [116].

4.4. Quercetin. Quercetin is a member of flavonoid found in many dietary plants such as apple, apricot, broccoli, Brussels sprout, cauliflower, grape, lettuce, onion, strawberry, tomato, and wolfberry [117]. Quercetin has been reported to have anti-inflammatory, antidiabetic, antiobesity, and anticancer activities [118, 119]. Quercetin is also widely known to exert antioxidative stress activity via activating Nrf2 signaling pathway [120–122]. It has been demonstrated that quercetin can display antisenescence activity in normal cells. Senescent fibroblasts treated with about 6 μM of quercetin for 5 consecutive days were shown to restart proliferation compared to the control cultures [123].

4.4.1. Quercetin as Toxic Compound in Cancer. There are several studies that propose the use of quercetin to induce apoptotic and nonapoptotic forms of cell death in cancer cells [124, 125]. Various mechanisms have been claimed to explain the ability of quercetin to bypass apoptotic resistance of cancer cells. Most of the studies report that quercetin can target antiapoptotic kinases and selective oncogenes (such as Mcl-1, Ras, MEK, and PI3K) or upregulate tumor suppressor genes (p53, p21), which lead to the selective elimination of cancer cells [126]. There is also evidence for an involvement of heat shock response proteins (HSP) in the toxicity of quercetin for cancer cells. Various quercetin-treated tumor cell lines were not induced to show aggregation of HSP70 in the nuclei in response to heat shock, resulting in apoptosis [127].

4.4.2. Quercetin as Senescence Inducer. As shown for most Nrf2-activating compounds, the finding that, in certain circumstances, it is also possible to use quercetin to induce senescence in cancer cells is not surprising. Chronic administration of 25 μM quercetin plus 10 μM resveratrol was shown to induce a senescent-like growth arrest in human glioma cells [128]. The prosenescence activity of quercetin in the glioma cellular models is compatible, at least in part, with the inhibition of HDAC (histone deacetylases) [129]. Interestingly, this inhibitory activity on HDAC was not observed in normal astrocytes. Quercetin was also shown to activate and stabilize p53 by inhibiting its RNA degradation and protein ubiquitination in liver carcinoma cells (HepG2), thus promoting p21 expression and cyclin D1 suppression in favor of cell cycle arrest [130]. Hence, circumstances where p53 is not stabilized or where HDAC is over activated pave the way to a potential use of quercetin to induce senescence in cancer.

4.4.3. Quercetin as Potential Senolytic. Quercetin (10 μM) was proven to induce death in radiation-induced senescent endothelial cells and senescent bone marrow-derived mouse mesenchymal stem cells [38]. Conversely, quercetin was found to lack senolytic efficacy in senescent preadipocytes and mouse embryonic fibroblasts. The combination of quercetin with the anticancer drug dasatinib was shown to be effective as senolytic in several types of senescent cells [38]. In the context of cancer therapy, the potential of quercetin (or its combination with dasatinib or other compounds) to induce death in cancer cells after therapy-induced senescence should deserve appropriate investigation. This could be useful to reduce adverse effects of chemotherapy and cancer relapse, which are promoted by therapy-induced cellular senescence [42].

4.4.4. Quercetin in Cancer Adjuvant Therapy. Excluding studies designed to test safety, availability, and metabolism of quercetin [131], its use in clinical trials as adjuvant therapy for cancer patients still need to be appropriately investigated. Interesting results have been observed regarding the modulation of cancer-related biomarkers in few patients with ovarian cancer and hepatoma [132]. Critical points that hamper the use of quercetin in these trials include the side effects of the pharmacological dose that need to be administered, the lack of specificity, and the identification of direct cellular targets.

4.5. Genistein. Genistein is an isoflavonoid compound present in some edible plants such as alfalfa, soybean, fava bean, psoralea, pea, green lentil, and lupine [133]. Genistein is known for antioxidant, anticancer, anti-inflammatory, antiobesity, and antidiabetes activities [134–136]. In addition, this compound can protect cells from injury, toxicity, and oxidative stress by activating Nrf2 [12, 137, 138]. At relatively low concentrations (1–10 μM), genistein has been shown to delay senescence in vascular smooth muscle cells [139] and to enhance telomerase activity in prostate cancer cells [140].

4.5.1. Genistein as Toxic Compound in Cancer. Genistein can induce apoptotic and nonapoptotic cell death in several models of cancer cells [141]. For example, in H460 non-small lung and MDA-MB-231 breast cancer cells as well as in HT29 colon cancer cells, genistein inhibits cell growth and induces apoptosis at concentration from 30 to 50 μM [142–144]. Genistein at lower concentration (10 μM) can also sensitize sarcoma and breast cancer cells to X-ray-induced cell death by inhibiting the double-strand break (DSB) repair pathways [145, 146].

4.5.2. Genistein as Senescence Inducer. Numerous studies have shown that genistein can induce the expression of tumor suppressor genes p53, p21, and p16 in cancer [134, 147–150]

that mediate cell cycle arrest and senescent response. It has also been reported that genistein at pharmacological concentrations (50 μM) inhibited telomerase activity in brain [KNS60, U251MG(KO), and ONS76], ovarian (SKOV-3), breast (MCF-7), and prostate (DU-145, LNCaP) cancer cells [140, 151].

4.5.3. Genistein as Potential Senolytic. There is no information about senolytic activity of genistein but its inhibitory effect on tyrosine kinase [152] (the same target of dasatinib) would deserve appropriate consideration.

4.5.4. Genistein in Cancer Adjuvant Therapy. The contrasting results obtained in a relatively narrow range of concentration suggest that the use *in vivo* of this compound might deserve particular caution. Genistein aglycone can eventually stimulate tumor cell proliferation and growth in mice that exhibit a deficient immune system [153]. Moreover, epidemiological studies have shown an inverse correlation between genistein intake and breast cancer risk [153].

4.6. Resveratrol. Resveratrol is a naturally occurring polyphenolic compound present in grapes, mulberries, peanuts, and red wine. It has been identified as a cancer chemopreventive agent, based on its safety and efficacy in experimental models of carcinogenesis [154]. The antitumor activity of resveratrol has been attributed to the inhibition of diverse cellular events associated with tumor initiation, promotion, and progression [155]. Inhibition of carcinogenesis and the chemopreventive effects of resveratrol might be related to the induction of Nrf2-mediated protective pathways [156].

4.6.1. Resveratrol as Toxic Compound in Cancer. In vitro studies suggest that resveratrol is able to induce growth inhibition and apoptosis in several tumor cell lines [157–159]. The IC_{50} value in five cell lines (Seg-1, HCE7, SW480, MCF7, and HL60) was attributed to be in the range of 70–150 μM [160] and only three of these cell lines (MCF7, HL60, and Seg-1) started to show a significant reduction in cell viability at 50 μM.

4.6.2. Resveratrol as Senescence Inducer. Resveratrol represents also one of the most active natural compounds in inducing senescence in cancer cells, in particular at concentrations equal or below 50 μM. The increase in the activity and expression of senescence-associated effectors (e.g., p53 and p21) was observed in various cancer cells treated with resveratrol. Resveratrol has shown to be able to exert SIRT1-dependent inhibitory effects on gastric cancer by inducing senescence in cellular models, as evidenced by the increased protein levels of inhibitors of CDKs (p21 and p16) and SA-β-Gal staining in resveratrol-treated samples [161]. The inhibitory effect on gastric cancer was also confirmed *in vivo* using a nude mice xenograft model. This effect was abrogated after SIRT1 (sirtuin 1) depletion probably through an indirect regulation of involved genes. Evidence of the involvement of senescence-associated effectors in the resveratrol-mediated antitumor action has been shown in many other tumor cell lines [162–164]. As it happens for some drugs, even resveratrol would seem to hijack the fate of tumor cells towards antiproliferative pathways depending on the dose of treatment and this phenomenon appears to be important also in cancer prevention [165]. In particular experimental settings, there is evidence that resveratrol may act as a potent senescence inducer. It has been shown that micromolar doses (10–50 μM) of resveratrol-treatment in non-small-cell lung cancer cells can lead to a significant increase in SA-β-Gal staining and enhanced p53 and p21 expression, suggesting that the anticancer effect of resveratrol is largely attributable to the induction of senescence [166]. Similar concentrations of resveratrol have been effective in reducing the telomerase activity in MCF-7 breast cancer cells, probably affecting posttranscriptional phosphorylation and nuclear translocation of the catalytic subunit hTERT [167]. In accordance with these results, inhibition of transcriptional hTERT expression was proposed as a mechanism to explain resveratrol-mediated inhibition of human colorectal carcinoma cell proliferation [168]. All the above results highlight the ability of resveratrol to modulate different pathways related with the complex machinery of CS depending on the tumor types and treatment conditions.

4.6.3. Resveratrol as Potential Senolytic. There is no reported senolytic activity for resveratrol. A recent high throughput screening of senotherapeutics in senescent Ercc1−/− mouse embryonic fibroblasts showed no effect (neither senolytic nor senomorphic) of resveratrol, but this could be due to the concentration tested (not clearly specified, but likely at 1 μM as declared for other compounds) and the specific model used [41].

4.6.4. Resveratrol in Cancer Adjuvant Therapy. Lack of specificity, efficacy, and poor bioavailability is the major limitation for the use of resveratrol as adjuvant therapy in cancer. While *in vitro* resveratrol seems to be highly effective in overcoming chemoresistance (at concentration of 25–50 μM), for example, in the case of multiple myeloma cells [169], the clinical translation of these doses in clinical settings appears problematic. Indeed, an unacceptable safety profile and minimal efficacy were shown in a clinical trial performed with 5 g/day of SRT50 (a micronized oral formulation of resveratrol developed to improve bioavailability) combined with bortezomib in patients with relapsed/refractory multiple myeloma [170]. However, the same dose and formulation resulted safe when administered in patients with colorectal cancer and hepatic metastases [171], suggesting that a thorough patient cohort study should be defined before clinical applications.

4.7. Silybin. Silybin, a major active constituent of silymarin (extract of the milk thistle seeds), has been shown to have antioxidant and cytoprotective as well as antitumor effects. Moreover, several studies performed in *C. elgans* suggest that silybin may display antiaging activity, mainly based on counteracting age-related loss of proteostasis [172–174]. In analogy with other flavonoids, also in this case, the antioxidant and cytoprotective effects seem to be related to the activation of Nrf2 pathway [175].

4.7.1. Silybin as Toxic Compound in Cancer. Silybin was found to induce growth inhibition and apoptosis in different human and murine tumor cell lines and to potentiate the effects of doxorubicin, cisplatin, and carboplatin *in vitro* [176–178]. Fewer studies have been conducted on the antitumor effect exerted by *in vivo* supplementation with silybin or silymarin. Most data on the *in vivo* effects of these compounds, confirming a general anticancer activity, have been drawn from studies done in mice treated with carcinogens or in nude mice bearing human xenografts [4].

4.7.2. Silybin as Senescence Inducer. Study reported that IdB 1016 (silipide), a silybin-phosphatidylcholine complex with improved bioavailability, induced cellular senescence in mammary tumor cells of mice at 450 mg/Kg, as demonstrated by SA-β-gal staining in cancer tissues. According to the same study, this complex (at concentration in the range of 10–50 μM) also induced cellular senescence and apoptosis in human breast SKBR3 cancer cell line, which were associated with increased expression of p53 [179].

4.7.3. Silybin as Potential Senolytic. There is currently no evidence for a senolytic activity of silybin. However, the cooccurrence of markers of apoptosis and senescence in breast cancer cells treated with silybin [179] would suggest appropriate investigation in this field.

4.7.4. Silybin in Cancer Adjuvant Therapy. Conversely to the lack of cancer tissue penetration observed in prostate cancer patients (receiving 13 g per day of silybin-phytosome) [180], administration of silybin-phosphatidylcholine, 2.8 g daily, 1 month before surgery, to patients with early breast cancer showed a selective accumulation of silybin in breast tumor tissue [181]. However, clear proof of clinical efficacy as adjuvant in cancer therapy is still lacking. A pilot study (administration of 2 g per day in 3 patients) in advanced hepatocellular carcinoma demonstrated the complete lack of benefits [182].

4.8. Phenethyl Isothiocyanate. Phenethyl isothiocyanate (PEITC) is a member of isothiocyanate distributed as gluconasturtiin in some cruciferous plants including broccoli, cabbage, cauliflower, horseradish, and watercress [105]. This compound has multiple pharmacological activities including anticancer activity [183]. It has been reported that PEITC exhibits antioxidant activity by affecting Nrf2 signaling pathway [11, 184].

4.8.1. PEITC as Toxic Compound in Cancer. PEITC (at 5–10 μM) induces apoptosis in several cell lines by a cancer cell-specific generation of ROS [185] that is related to mitochondrial deregulation and modulation of proteins like Bcl2, BID, BIM, and BAX, causing the release of cytochrome c into cytosol leading to apoptosis [183]. Other mechanisms by which PEITC induces apoptosis (at 50 μM) include the increase of DDB2 (damaged DNA-binding protein 2) expression, as observed in colon cancer cells (HCT 116) *in vitro* and *in vivo* [186], as well as the activation of the extrinsic apoptotic pathway (death receptor-mediated apoptosis), as observed in oral and cervical cancer cells [187].

4.8.2. PEITC as Senescence Inducer. Modulation of the senescence effectors p16, p53, and p21 as well as increased staining for SA-β-Gal by PEITC was observed in cancer cells at concentration from 4 μM to 20 μM [186, 188–190]. PEITC also downregulated telomerase in cervical cancer cells (HeLa) [189].

4.8.3. PEITC as Potential Senolytic. The potential for PEITC as senolytic agent has been tested in radiation-induced senescent WI-38 fibroblasts without any evidence of selective death in normal versus senescent cells (LD50 ratio = 1) [191].

4.8.4. Efficacy of PEITC in Cancer Adjuvant Therapy. Preclinical evidence suggests that combination of PEITC with conventional anticancer agents is also highly effective in improving overall efficacy [183]. There is a clinical trial showing that PEITC can be an inhibitor of lung carcinogenesis [192], but its relevance in adjuvant cancer therapy is still unknown.

4.9. Sulforaphane. Sulforaphane is one of the most potent phase II enzyme inducer isolated from edible cruciferous vegetables with potent activity against cancer progression [193]. This activity has been demonstrated at the level of chemoprevention, as well as at the level of therapy at various stages of cancer. Sulforaphane represents a strong activator of Nrf2-Keap1 signaling pathway, enabling Nrf2 to escape Keap1-dependent degradation and leading to stabilization and nuclear accumulation of Nrf2 [194]. Acting through the Nrf2 pathway, sulforaphane inhibited 7,12-dimethylbenz(a)anthracene-induced skin tumorigenesis in mice [195]. At the same time, the Nrf2 pathway seems to be involved in the sulforaphane-mediated protection from apoptosis in different cellular models [196, 197]. Oral administration of sulforaphane was able to inhibit DMBA-induced mammary carcinogenesis in rats [198]. In this animal model, an accumulation of sulforaphane metabolites, followed by an increased expression in NQO1 and HO-1 cytoprotective mRNAs, was observed in mammary gland after a single oral 150 μM dose of sulforaphane. Interestingly, a local increase of SFN metabolites was observed in epithelial cells from human breast tissue after a single oral sulforaphane dose (200 μM) in healthy women undergoing reduction mammoplasty. Hence, the specific intracellular accumulation and retention of this compound in mammary epithelium might contribute to protecting normal cells from tumor initiation and progression, though more large-scale clinical trials are needed to verify the effectiveness of sulforaphane as anticancer agent.

4.9.1. Sulforaphane as Toxic Compound in Cancer. Sulforaphane has been defined as "hormetic" dietary compound, because of its ability to induce different/opposite biological effects at different doses [199]. Treatment of mesenchymal stem cells (MSC) with low doses of sulforaphane (0.25–1 μM) increased cell proliferation protecting from apoptosis and senescence, and conversely higher doses (5–20 μM) induced cytotoxicity together with HDAC inhibition and increasing number of apoptotic and senescent cells [199]. However, it has been frequently reported a different IC_{50}

for normal and cancer cells. For example, IC_{50} values from 14.0 to 19.3 μM were found in MCF-7, MDA-MB-231, and SK-BR-3 breast cancer cell lines, whereas the IC_{50} for normal human mammary epithelial cells was 81.24 μM [200]. Sulforaphane-induced HDAC inhibition and induction of cell death has been shown in various cancer cells [201]. In colorectal cancer cells, sulforaphane treatment (15 μM) induced alteration of histone acetylation status and a specific increase in acetylated histone H4 bound to the promoter region of *P21* leading at an increased p21Cip1/Waf1 protein expression [202]. Consistently, *in vivo* administration of sulforaphane inhibited HDAC activity in mouse colonic mucosa after six hours from the oral treatment with concomitant increase of acetylated H3 and H4 histone. Changes in histone acetylation status were also observed after long-term (10 weeks) administration of sulforaphane diet that resulted in augmented acetylated histones and p21 expression in the ileum, colon, prostate, and PBMC cells. Dietary sulforaphane was also able to suppress polyp formation in Apc^{min} mice [203]. In addition, changes in the histone modifications of the hTERT promoter and DNA demethylation of hTERT exon 1 were observed in human breast cancer cells in response to sulforaphane [204]. Acting as HDAC inhibitor, sulforaphane may be useful in the treatment of many types of cancer in which HDAC activity and hypoacetylation contribute to malignant progression.

4.9.2. Sulforaphane as Senescence Inducer. As mentioned above, the dose and duration of sulforaphane treatment result in a divergent cell fate also in cancer cells. Sulforaphane at 5–10 μM promotes cell cycle arrest, elevation in the levels of p21 and p27, and cellular senescence in breast cancer cells (MCF-7, MDA-MB-231, and SK-BR-3), whereas at the concentration of 20 μM, apoptosis was induced [200]. The effects were mediated by upregulation of sixty microRNAs and downregulation of thirty-two microRNAs, global hypomethylation, and decreased levels of DNA methyltransferases (DNMT1, DNMT3B), as well as nitrooxidative stress, genotoxicity, and diminished AKT signaling. Transient sulforaphane exposure for up to 6 hours induced reversible G2/M growth arrest, while exposures of 12 to 72 hours resulted in irreversible G2/M arrest and apoptosis of human colon cancer cell line [205]. Cell cycle arrest in G1 phase and induction of key effector molecules related to cellular senescence, such as p21, p27, Rb, and PAI-1, has been observed by treatment with sulforaphane of adipocytes at early stage of differentiation [206].

4.9.3. Sulforaphane as Potential Senolytic. There is currently no evidence of senolytic effects of sulforaphane.

4.9.4. Efficacy of Sulforaphane in Cancer Adjuvant Therapy. Sulforaphane is considered a good candidate in adjuvant therapy of cancer due to its proapoptotic, antiangiogenesis, and antimetastasis activities shown in preclinical settings [207]. However, clinical studies in men with recurrent prostate cancer have shown limited efficacy with significant effects only on secondary endpoints [208, 209].

4.10. Triptolide. Triptolide is a natural diterpenoid abundant in thunder god vine (*Tripterygium wilfordii*). It has gained importance because of its potential for prevention and treatment of cancer [210]. Triptolide is able to induce toxic cellular effects, which induce Nrf2 and its target genes, as it has been shown in hepatic cell lines [104].

4.10.1. Triptolide as Toxic Compound in Cancer. Triptolide (50–100 nM) is able to decrease mitochondrial respiration and increase ROS and apoptosis in p53-deficient non-small-cell lung cancer and consequently to upregulate Nrf2 and its target gene HO-1 and NQO1 [10]. This compound was shown to induce apoptosis and cell cycle arrest in various cancers by targeting the p53/p21 and BCL-2 pathway [211–213].

Conversely, in resistant myeloid leukemia cell lines, triptolide enhanced the sensitivity to doxorubicin-induced and imatinib-induced apoptosis through a downregulation of Nrf2 and its target genes [214].

4.10.2. Triptolide as Senescence Inducer. Triptolide has the ability to induce senescence. Treatment of liver cancer cells (HepG2) with nanomolar concentrations of triptolide (2.5–10 nM) induced senescence via Akt and hTERT pathway [215]. Triptolide (3 nM) induced senescence of primary prostate adenocarcinoma cells, as demonstrated by SA-β-gal activity [216].

4.10.3. Triptolide as Potential Senolytic. There is currently no evidence of senolytic effects of triptolide. Triptolide (0.25 mg/kg i.v., twice weekly for 1, 2, and 3 months) is able to mitigate radiation-induced pulmonary fibrosis in rats [217] but conversely to senolytic drug, which can be given when fibrosis is permanent [218]; triptolide beneficial effects have been demonstrated when given before irradiation. Anyway, further studies could be performed in this area due to the important proapoptotic effects shown by triptolide in cancer cells.

4.10.4. Triptolide in Cancer Adjuvant Therapy. A phase I and pharmacological study of F60008 (a semisynthetic derivate of triptolide, which is converted to triptolide) given intravenously in patients with advanced solid tumors displayed various adverse effects without any clear proof of efficacy [219].

4.11. Allicin. Allicin, an organosulfur compound, is mainly present in garlic (*Allium sativum*). The compound is reported to have antimicrobial, anticancer, and cardioprotective activities [220]. Allicin inhibited lipopolysaccharide-induced vascular oxidative stress and inflammation in human umbilical vein endothelial cells, which were associated with activation of Nrf2 and reduction of TNF-α (tumor necrosis factor α) and IL-8 (interleukin 8) production [221]. By activating Nrf2, allicin protected spinal cord tissue from traumatic injury in rats [222].

4.11.1. Allicin as Toxic Compound in Cancer. Allicin (10–30 μM) reduced cell viability and proliferation in several mammalian lines with higher efficacy to induce apoptosis in 3T3 and MCF-7 cell lines [223]. Treatment of liver cancer cells with allicin induced apoptotic cell death via p53 modulation [224].

4.11.2. Allicin as Senescence Inducer. Allicin has been shown to inhibit telomerase activity and to induce apoptosis in gastric cancer adenocarcinoma cells (SGC-7901) [225]. While conventional senescent markers have not been measured in this study, cells treated with allicin (100 μM) showed typical morphological changes (enlarged and irregular) that have been reported in several models of senescence. However, cells treated with allicin underwent rapid apoptosis after morphological changes, and these changes were more likely related to cell death events rather than senescence.

4.11.3. Allicin as Potential Senolytic. Allicin has not been investigated in this area.

4.11.4. Allicin in Cancer Adjuvant Therapy. Clinical trial with allicin as adjuvant in cancer therapy is still lacking. There is a report of partial efficacy (mild increase of apoptosis in cancer tissues) of a local application of allicin, via gastroscopy (48 h before surgical intervention), in patients with progressive gastric carcinoma.

4.12. Berberine. Berberine is a naturally occurring isoquinoline alkaloid present in barberry (*Berberis vulgaris*), tree turmeric (*B. aristata*), oregon grape (*B. aquifolium*), goldenseal (*Hydrastis canadensis*), and goldethread (*Coptis chinensis*) [226]. Berberine has been shown to possess a wide range of pharmacological activities [227], including antidiabetic, antihyperlipidemic, antiarrhythmic, and antioxidat activities that find a common rationale in the upregulation of Nrf2-related pathways [228, 229].

4.12.1. Berberine as Toxic Compound in Cancer. Berberine displays hormetic effects "in vitro." It has been shown that berberine at low-dose range (1.25 ~ 5 μM) can promote cell proliferation while at high-dose range (10 ~ 80 μM) can inhibit cell proliferation [230]. *In vitro* treatment with berberine can inhibit cell growth and induce cell cycle arrest and apoptosis (IC_{50} from 7 to 20 μM) of various cancer cells, for example, prostatic, gastrointestinal, hepatic, and mammary human cancer cells (reviewed in [231]), as well as skin- [232] and hematological-derived cancer cells [233]. Activation of AMPK, inhibition of mTOR pathway, and induction of apoptosis or autophagic cell death are the best-characterized cascade of events by which berberine exerts anticancer activity [234, 235].

4.12.2. Berberine as Senescence Inducer. However, there is a series of scientific evidence about the ability of berberine to exert cell type-specific effects that, in certain circumstances, include cell cycle arrest and induction of a senescent-like phenotype. Indeed, chronic treatment with berberine (15 μM) for one week was shown to induce senescence in human glioblastoma cells by downregulation of EGFR-MEK-ERK signaling pathway [236]. Moreover, the antitumor effects of berberine and berberine derivatives in human HER-2/neu overexpressing breast cancer cells are mediated not only by apoptotic cell death but also by increased expression of p53, p21, p16, and PAI-1 mRNAs, thus suggesting that the mechanism of action of berberine may also include the induction of CS [237]. Another potential mechanism that could explain this role of berberine in CS regards the inhibition of telomerase activity forming a G-quadruplex with telomeric DNA [238]. Finally, treatment of promyelocytic leukemia HL-60 cell line with 150 μM berberine induced a time-dependent reduction in the activity of telomerase [239].

Berberine was also shown to trigger the transcriptional activity and the inhibition of the degradation of p53 in human breast cancer MCF7 cells [240]. All these observations suggest that berberine is another example of natural Nrf2-activating compound that exerts different and even contrasting, that is, gerosupressive [241] and prosenescence [236, 237], effects likely depending on cell type, time of exposure, and dosage. It is important to consider that most, if not all, studies *in vitro* with berberine tested doses in the micromolar range which is far higher than levels achievable in blood plasma after oral dosing. These observations prompt further investigation to clarify the conditions that might allow to use safely berberine in prosenescence therapy for cancer.

4.12.3. Berberine as Potential Senolytic. A compound with the ability to modulate FLIP in senescent cells may potentially be used as a senolytic drug. Berberine is among those compounds that modulates FLIP and has been included in a recent patent as potential senolytic [242]. Studies are currently in progress around this topic.

4.12.4. Berberine in Cancer Adjuvant Therapy. Recent applications related to berberine's possible therapeutic use are focused on metabolic syndrome, type 2 diabetes, and dyslipidemia. However, the use of berberine as adjuvant therapy in cancer appears to be promising as well. Berberine reduced radiation-induced lung injury (RILI) and pulmonary fibrosis in non-small-cell lung cancer (NSCLC) patients treated with radiotherapy [243]. Moreover, there is evidence that oral administration of berberine can reduce the familial adenomatous polyposis patients' polyp size [244]. Additional trial with berberine as chemopreventive agent as well as in reducing recurrence rates of colorectal adenoma (CRA) is currently ongoing.

4.13. Piperlongumine. Piperlongumine is a natural alkaloid isolated from the long pepper. It is a potent inducer of Nrf2 response and of its target genes including heme oxygenase-1 (HO-1) [50]. Interestingly, HO-1 has antitumor functions in cancer cells, but cytoprotective functions in normal cells.

4.13.1. Piperlongumine as Toxic Compound in Cancer. Piperlongumine displays a high degree of selective toxicity to cancer cells. It has been identified as strong inhibitor ($IC_{50} = 1.7$ μM) of signal transducer and activator of transcription 3 (STAT3) by a recent high throughput drug-repository screening [245]. STAT3 is a validated drug target for cancer therapy and thus it is not surprising that piperlongumine was found to be able to induce apoptosis at low doses (IC_{50} from 0.16 up to 5.1 μM) in multiple breast cancer cell lines having increased STAT3. This proapoptotic activity is associated with the modulation of several antiapoptotic genes including Bcl-2, BcL-xL, survivin, X-linked inhibitor of apoptosis (XIAP), and cellular inhibitor of apoptosis proteins

(cIAP). Alone and in combination with cisplatin, piperlongumine (2.5–15 μM) is able to dysregulate the oxidative stress response and kill head and neck cancer cells independently by their p53 mutational status [246] as well as a multitude of pancreatic, kidney, breast, lung, and pancreatic cell lines (Panc1, L3.6pL, A549, kidney, and SKBR3) [247]. In human oral squamous cell carcinoma, piperlongumine induces increased ROS and subsequent caspase-dependent apoptosis at 7.5–10 μM [248]. However, another study found no evidence of dose-response relationship between cellular ROS, induced by piperlongumine, and its cytotoxicity [249], thus suggesting the presence of different mechanisms related to induction of cell death.

4.13.2. Piperlongumine as Senescence Inducer. Piperlongumine has been shown to suppress proliferation and to induce p21-mediated senescence (2.5–7.5 μM) [248] in human oral squamous cell carcinoma cells.

4.13.3. Piperlongumine as Potential Senolytic. A recent library screening for compounds with senolytic activity identified piperlongumine as a promising compound. It has been shown to preferentially induce cell death in irradiation, replicative, and oncogene-induced senescent WI-38 fibroblasts (EC_{50} 6–8 μM) compared to nonsenescent fibroblasts (EC_{50} 20 μM) [46]. However, apoptotic mechanisms of piperlongumine in senescent cells were found to be independent by the generation of ROS [46].

4.13.4. Piperlongumine in Cancer Adjuvant Therapy. Piperlongumine was found to be nontoxic in mice up to a dose of 30 mg/kg/day for 14 days and caused regression of breast cancer cell line xenografts in nude mice. These results, in addition to the recently discovered activity as senolytic compound, hold promises for a potential translation in human trials.

4.14. Fisetin. Fisetin is an organic flavonoid present in numerous fruits and vegetables such as strawberries, mangoes, and cucumbers that exhibits antioxidant, neurotrophic, anti-inflammatory, and anticancer effects. Attention on fisetin in the context of aging research and chemopreventive therapy is mostly related to its ability to increase transcriptional activity of Nrf2 [250] and its target gene HO-1 [251] and also to inhibit the activity of mTOR kinase [252].

4.14.1. Fisetin as Toxic Compound in Cancer. In prostate cancer cells with upregulated activity of pathway upstream mTOR, high concentration of fisetin (40 μM and above) induces autophagic cell death [253]. Death induction in monocytic leukemia cells by fisetin ($IC_{50} = 50\,\mu$M) was mediated by an increase in NO resulting in the inhibition of the downstream pathways of mTOR, double-strand DNA breaks, and caspase activation [254]. Fisetin can induce apoptosis and suppress the growth of colon cancer cells (HCT116 and HT29) with an IC_{50} comprised from 50 to 132 μM after 72 h of exposure [255], and similar effects were observed in prostate cancer cells (PrEC, LNCaP, and CWR22Rv1) with an IC_{50} comprised from 20 to 60 μM after 48 h of exposure.

4.14.2. Fisetin as Senescence Inducer. The mechanism of accelerated cellular senescence was not observed among those involved in the antiproliferative effects of fisetin (1–50 μM) in PC3 or lymph node carcinoma of the prostate (LNCaP) cells [256]. There is no further investigation about a potential prosenescence effect of fisetin.

4.14.3. Fisetin as Potential Senolytic. Fisetin selectively induces apoptosis (at 5–10 μM) in senescent, but not in proliferating, HUVECs. However, it is not senolytic in senescent IMR90 fibroblasts or in primary human preadipocytes [44].

4.14.4. Fisetin in Cancer Adjuvant Therapy. Although preclinical data appear to be convincing, well-designed clinical trials in humans are needed to conclusively determine the efficacy across various cancers as well as senolytic adjuvant therapy.

4.15. Phloretin. Phloretin is a dihydrochalcone flavonoid, which can be found in apple tree leaves. Phloretin has been shown to protect hepatocytes against oxidative stress [58] as well as HEI-OC1 auditory cells against cisplatin-induced apoptosis [51] by upregulating Nrf2 defensive pathway. Importantly, the cytoprotective effects of phloretin were also observed at relatively low doses (2.5–5 μM) also in H9c2 cardiomyoblasts exposed to arsenic trioxide, a drug used in the treatment of acute promyelocytic leukemia that is associated to cardiotoxic side effects [257].

4.15.1. Phloretin as Toxic Compound in Cancer. Phloretin is known to inhibit glucose transporter (GLUT) 2, a process which results in the induction of apoptosis in cells with high metabolic requirement, as shown in human liver cancer cells HepG2 treated with 200 μM phloretin [258]. At the dose of 10 mg/kg, phloretin was found to exert antitumor effects in immune deficiency mice carrying a HepG2 xenograft [258].

Moreover, phloretin was shown to induce apoptosis of non-small-cell lung cancer (NSCLC) cell line A549, Calu-1, H838, and H520 (IC_{50} approx. from 50 to 100 μM) through deregulation of Bcl-2 [259] and other ROS-related pathways, such as P38 MAPK and JNK1/2 [260] which are related to the rise of ROS. Interestingly, the anticancer effects were enhanced in presence of cisplatin, which suggest a potential in adjuvant cancer therapy. Similar proapoptotic effects, associated with increased ROS and ROS-related pathways, were observed after treatment with very high (200–300 μM) concentrations of phloretin [261].

4.15.2. Phloretin as Senescence Inducer. While there is evidence that phloretin can induce cell cycle arrest in cancer cells [261], this process seems to be unrelated to senescence induction as there are no clear data about the possibility to induce senescence with phloretin.

4.15.3. Phloretin as Potential Senolytic. Phloretin at 50 μM was found to specifically reduce the viability of therapy-induced senescent lymphoma cells [45]. These cells were also shown to be sensitive to another blocker of glucose transporters, cytochalasin B, thus suggesting that the mechanism

by which phloretin induces cell death in senescent cells is related to their increased metabolic requirement.

4.15.4. Phloretin in Cancer Adjuvant Therapy. The recent observation related to the senolytic activity of phloretin as well as its potential in combination with withaferin A to suppress gefitinib-resistant adenocarcinoma cell line growth [262] appears to be promising therapeutic strategy to overcome the occurrence of cancer relapse and the resistance to chemotherapy. However, these senescent and glucose-targeting therapeutic strategies have still not been tested in clinical settings.

5. Mechanisms Mediating the Cytoprotective, Cytotoxic, or Prosenescence Effects of Nrf2-Activating Compounds

Activation of Nrf2-regulated cytoprotective response could provide a selective advantage to tumor cells which is clearly in contrast to the majority of effects described in the above chapters. In line with the hypothesis that Nrf2 response is an advantage for cancer cells, some types of polyphenols (usually belonging to the flavone class) have been shown to sensitize different cancer cells to chemotherapy via an inhibitory activity on Nrf2 signaling pathway. These compounds include luteolin [263], chrysin [264], and apigenin [265] which, in turn, was shown to induce senescence in IMR-90 cells [266]. These results might form a good rationale to use these compounds as adjuvant in cancer therapy. However, a multitude of data, exposed in the chapters above, supports the opposite concept that most polyphenols and other natural bioactive compounds activating the Nrf2 pathway can display cytotoxic effects or promote senescence in cancer cells. This dichotomy could be explained by the activity of pathways unrelated to Nrf2. Moreover, tissue and cell type specificity of Nrf2 downstream targets appear to be still poorly understood and the range of stress-response phenotypes observed when components of the pathway are genetically disrupted in mice is not completely explained. Keap1 KO mice hepatocytes experience a different signaling and gene expression compared with controls treated with an Nrf2-inducing agent [267]. Some of the induced factors can even antagonize Nrf2 thus suggesting that in the presence of unrepaired damage, such as in cancer cells, the complex response to Nrf2 includes damage-sensing factors that may activate apoptotic or senescence mechanisms. Cytoprotective effects of Nrf2 against oxidative stress are related to the presence of a functional aryl hydrocarbon receptor (AhR), a transcription factor that display pleiotropic activity in the context of carcinogenesis [13] and while some AhR ligands can suppress senescence acting as tumor promoters [268], recent work suggests that Ahr gene can function as a tumor suppressor gene by inhibiting cell proliferation and promoting senescent-like phenotype thus counteracting cancer progression [269–271]. Bidirectional interactions of Nrf2 and AhR have been reported, thus suggesting that Nrf2 can directly modulate AhR signaling [272]. This signaling network includes Cyp1A1 and Cyp1B1 that have been reported to induce upregulation of the cyclin-dependent kinase inhibitors p27 and p21 [273] that are regarded as key effectors of cellular senescence.

Another interaction of Nrf2 that deserves attention in the context of cellular senescence regards p53. The transcription of some Nrf2 target genes involved in the antioxidant response can be suppressed by p53 [274], but their mutual interaction still remains unclear. It has been reported the Nrf2 can increase the expression of the p53 inhibitor mouse double minute 2 homolog (MDM2), which is an ARE-regulated target gene [275]. However, p53 can be stabilized by Nrf2 target genes, that is, NQO1, suggesting both a positive and negative coregulation between p53 and Nrf2 [274] that is likely affected by the specific genomic and epigenomic profile of the target cell as well as by the duration of the stress. Epigenetic changes induced by natural compounds targeting Nrf2 could be likely involved in this process. For example, EGCG, a known Nrf2-activating polyphenol, can reduce the expression of miRNAs that target and suppress p53 [276], while epigenetic depression of one of these miRNAs (miR-200a) was reported to contribute to the dysregulation of Nrf2 activity in breast cancer [277].

Nrf2 can also interact downstream of p53 with its target gene p21 [64] or with p16 pathway by activating Notch-1 signaling. One or more functional ARE sequences exist in the promoter of Notch1, thus it is not surprising that Notch1 signaling can be triggered by Nrf2 [14]. Interestingly, Notch-1 may act as an oncogene or a tumor suppressor gene even within the same tumor type, and recently, it has been implicated in induction of cellular senescence mediated by p16 [278] and p21 [279].

Finally, the Jun dimerization protein 2 (JDP2), an important player in the senescence program [280], has a critical role as a cofactor for Nrf2 in the regulation of the antioxidant-responsive genes and production of ROS [281].

These data point towards a possible extension of Nrf2 to a more complex response that include damage-sensing prosenescence pathways, likely activated with a different timing in the case of persistent damage (Figure 2). However, it is also important to consider the multitarget ability of natural compounds, which might be able to interfere with senescence or apoptosis-related pathways, usually not directly related to Nrf2. These include multiple targets that have been frequently observed to be altered in cancer and senescent cells and that form the rationale to explain the different response from normal cells to the studied compounds [5]. Among the most critical targets that are noteworthy to mention are as follows:

(a) The reprogrammed metabolic pathway in cancer (Warburg effect or aerobic glycolysis) and some therapy-induced senescent cells, consisting in the switch of normal metabolism to support proliferation of cancer cells or production of SASP in senescent cells targeting glycolytic and other metabolic pathways, makes some cancer [282] and senescent cells [45] more susceptible to cell death than normal cells

(b) The defects in checkpoint kinases and repair genes that make cancer cells more susceptible to cell death following HDAC inhibition [104]

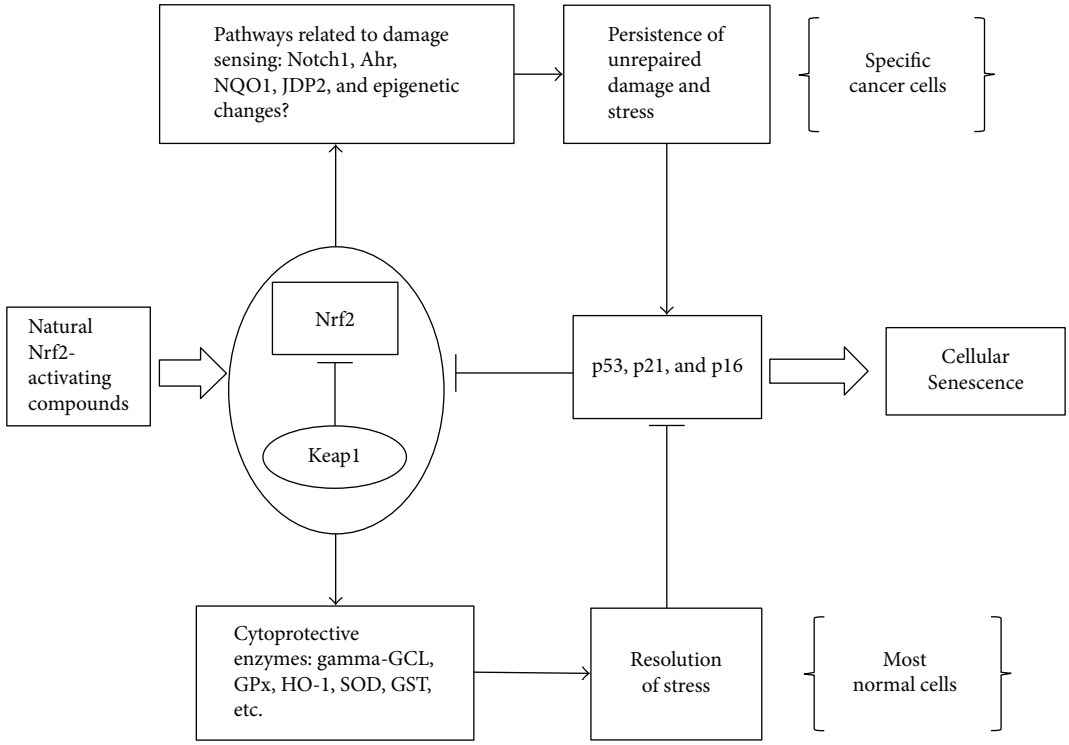

Figure 2: Potential mechanisms leading to senescence by NRF2-activating compounds in cancer cells. The response under NRF2 signaling involve the activation of glutamylcysteine ligase (γ-GCL), glutathione peroxidase (GPx), heme oxygenase 1 (HO-1), superoxide dismutase (SOD), glutathione S-transferase (GST), and many other enzymes involved in the antioxidant cytoprotective response that lead to suppression of senescence-related pathways (i.e., p53, p21, and p16). However, this response include and interact with additional genes, such as Notch-1, NADPH-quinone oxidoreductase (NQO1), the aryl hydrocarbon receptor (AhR), the Jun dimerization protein 2 (JDP2), and perhaps epigenetic changes that may be involved in sensing stress and damage and that are known to participate in processes leading to cellular senescence. In the case of (particular) cancer cells, the persistence of unresolved damage can eventually lead these pathways to the reactivation of the senescence program.

(c) The upregulation of antiapoptotic factors including Bcl-2/Bcl-xL and others mentioned above (shared by some cancer and senescent cells) which makes these cells susceptible to selective inhibitor

(d) The inhibition of autophagy (as consequence of high PI3K/Akt/mTor signaling) that makes cancer cells susceptible to cell death by AMPK inhibitors and starvation [283]

(e) The upregulation of both ROS and stress response pathway in cancer cells which make them susceptible to cytostasis by antioxidant treatment (due to unbalanced stress response) [284]

6. Nrf2-Activating Compounds and the Clearance of Senescent Cells

Senescent cells are an important source of inflammatory factors (SASP-related factors) for tumor progression [19] and can promote the development of cancer in the surrounding environment. In certain cases, it has been hypothesized that senescent cancer cells might even be able to overcome the "permanent" cell cycle arrest, revert their phenotype, and restart proliferation, thus suggesting that senescence may be used as therapy escape system by some cancer cells [16]. Taking into account that senescent cells can display long-term survival and resistance to apoptosis [285], finding ways to eliminate senescent cells might deserve a great impact on future adjuvant therapeutic strategies against cancer. This kind of senolytic adjuvant therapy could be important to reduce the incidence of relapses and the adverse effects of the therapy, as recently demonstrated in animal models [42]. In the last five years, at least 12 compounds have been identified as agents with senolytic activity *in vitro*, and more than half of them have been shown to be effective *in vivo* in animal models. Four of these candidate senolytics are natural bioactive compounds that have been described in the chapters above, namely, fisetin, quercetin, piperlongumine, and phloretin, and the estimation of their IC_{50} for senescent and nonsenescent cells is described in Table 1. The finding that some natural compounds that can be found in fruit and vegetables have been found to reduce specifically the viability of senescent cells is remarkable. Apparently, this could improve and accelerate the translation of these findings into clinical trials, as these compounds are available as supplement or included in extracts that are commercially available. However, there are some limitations that deserve to be discussed. First of all, it is important to bear in mind that a

Table 1: Estimated IC_{50} for senescent and nonsenescent cells of natural bioactive compounds with reported senolytic activity observed in vitro after the exposure time reported in brackets.

IC_{50}	Senescent cells							Nonsenescent cells					Reference
Compound	IRS IMR-90	IRS WI-38	RIS WI-38	OIS WI-38	IRS HUVEC	IRS ADP	TIS LYMP	IMR-90	WI-38	HUVEC	ADP	LYMP	
Fisetin	50 μM (72 h)				**30 μM (72 h)**	>>60 μM* (72 h)		>50 μM (72 h)		>60 μM* (72 h)	>>60 μM* (72 h)		[44]
Quercetin					**10 μM (72 h)**	>50 μM (72 h)				30 μM (72 h)	>50 μM (72 h)		[38]
Piperlongumine		**7.97 μM (72 h)**	**6.24 μM (72 h)**	**7.09 μM (72 h)**					20.3 μM (72 h)				[46]
Phloretin							**50 μM# (120 h)**					>>50 μM# (120 h)	[45]

*The highest concentration tested in the respective reference (IC_{50} not measured); IRS = irradiation-induced senescence; OIS = oncogene-induced senescence; RIS = replicative-induced senescence; ADP = preadipocytes; LYMP = lymphoma; #unique dose studied. The IC_{50} highlighted in bold are those supporting a senolytic activity (based on the difference with nonsenescent cells).

natural compound is not necessarily less toxic than a synthetic compound, especially when the doses required to achieve a therapeutic efficacy are high. If we look at the estimated IC_{50} reported in Table 1, it appears that the differences between the IC_{50} of nonsenescent and senescent cells are comprised in a relatively narrow range and that their ratio is comprised from 2 to 3. This means that at high dosage, these compounds could be toxic also for normal cells especially assuming a nonuniform distribution through the body. Moreover, experiments *in vitro* cannot take into account the effects of multitude of metabolites that originates from a bioactive compound as a consequence of the microflora and the enzymatic activity of our body. In the case these compounds should be used as senolytics, the therapeutic regimen would consist of a single or few more treatments with very high doses. In the case of quercetin, there is no example of senolytic therapy given alone, but its combination with dasatinib has been tested in mice. The combination of dasatinib 5 mg/Kg and quercetin 50 mg/Kg has been given as weekly single dose (by gavage) in $ERRC1^{-/\Delta}$ mice for several weeks [38] as well as in the form of single monthly dose (dasatinib 5 mg/Kg and quercetin 10 mg/Kg) for 3 months in normal mice [39] with several reported benefits and without any report of side effects. A similar dose in humans would approximately correspond to a dose around 1.5–2 g, while common oral dosage of quercetin supplements is in the range 50–500 mg. Moreover, when 2 g/day (corresponding to 25 and 36 mg/Kg for individuals of 80 and 55 Kg body weight, resp.) was given to healthy men, the serum quercetin concentration was found to be 5 μM [286], which is below the IC_{50} observed for senescent cells *in vitro* Table 1. In addition, the efficacy of quercetin as senolytic has been proven only in irradiation-induced senescent HUVEC while there is no evidence of this activity for any other model of senescence.

Also, fisetin displays a restricted activity that is limited to irradiation-induced senescent HUVEC at concentration higher than those reported for quercetin. If the IC_{50} of fisetin in vitro is 3-fold, those described for quercetin, we should expect senolytic doses that are very near to a toxic dosage. In mice, the LD_{50} dose was 180 mg/Kg given intravenously, which may suggest that doses above 500 mg in humans may be unsafe. For piperlongumine and phloretin, there is not a clear LD_{50}. However, piperlongumine displays senolytic activity at concentration below 10 μM in vitro which may suggest a potential to achieve these concentrations *in vivo*.

Anyway, at this moment, it is unclear if bioactive natural compounds can be safely used as adjuvant senolytic agents after cancer therapy. Moreover, it is unclear why it should be preferred a natural compound to a peptide (FOXO4-DRI) or a drug (Navitoclax) that have a substantially higher ratio between the respective IC_{50} of nonsenescent to senescent cells [43, 287] and thus are more likely, at least theoretically, to display less side effects at senolytic doses.

7. Concluding Remarks

CS likely evolved as a tumor-suppressing mechanism to prevent proliferation of cells that are at risk for acquiring potentially hazardous and transforming mutations. The possibility that putative cytoprotective natural compounds can reactivate pathways that induce apoptosis or senescence in cancer cells or that display senolytic activity appears a realistic perspective *in vitro* and a promising field of research. Emerging evidence has demonstrated that therapy-induced senescence can be achieved at much lower doses of chemotherapy and that it is a critical mechanism through which many anticancer agents inhibit the growth of tumor cells [288]. However, there are still lots of problems that need to be addressed in planning clinical trials. The first question that needs to be addressed is can we efficiently use adjuvant natural compounds to reduce the dose of chemotherapy while keeping the same efficacy and/or reducing undesired side effects associated with the therapy. The most likely answer is that it may depend on the cancer type and on the therapy scheme. In clear antagonism with excellent in vitro findings, most clinical trials performed up to now show extremely limited efficacy of any adjuvant therapy with these bioactive compounds (Table 2). There is already some (but limited) evidence that adjustments in the therapy scheme, especially in the case of curcumin, might help to achieve promising results. However, most applications are directed to elucidate the safety and bioavailability of the compound in oncologic patients. Even though a close correlation with the induction of CS has not been directly highlighted, some biological effects have been demonstrated as a direct effect of curcumin treatment.

In spite of these promises, we should acknowledge that now the benefit of the use of nutraceuticals as adjuvant in cancer therapies seems to be limited by a number of factors. These include issues related to the effective bioavailability of such active compound (in safety condition of treatment) but also concerning the heterogeneity of the population cohort in terms of age, type of cancer, comorbidity, and schedule of treatment. There are always concerns that the concentrations frequently used *in vitro* are excessive (tens to thousands of micromolar) and may not therefore reflect what happens with more physiological exposures (nanomolar ranges). For example, even if most of published studies in cell culture systems used 10–100 μM of EGCG [5], the blood level of EGCG after consumption of 2-3 cups of green tea was reported to be 0.1–0.6 μM [116]. Similarly, for berberine, in spite of the use of micromolar ranges in cell culture, the C max in humans after an oral dose of 400 mg was reported to be 0.1 nM [289], while higher doses (above 0.9 g/day) were shown to induce gastrointestinal side effects (Table 2). Moreover, the oxygen partial pressure in a cell culture system is much higher than that in the blood or tissues and the metabolism that occurs after (a limited) intestinal absorption suggests that the real "actors" are likely to be metabolites rather than the original compound tested in cell culture. Last, but not least, the possibility that these Nrf2-activating compounds can promote an antioxidant cytoprotective response in cancer cells thus aggravating resistance to chemotherapy should not be under evaluated. This problem could be afforded with more studies around the complex response of Nrf2 pathways in presence of cancer-specific alterations so that a personalized strategy can be developed. Moreover, induction of CS does not ensure that these cells

Table 2: Clinical trials of adjuvant (or alternative) therapies with natural bioactive compounds in cancer.

n	Compound	Dose	Drug*	Patients and scheme	Results of the trial	Ref.	Established link with the drug and induction of cellular senescence**
1	Tocotrienols	200 mg twice per day of tocotrienol-rich fraction	Tamoxifen	Double-blinded placebo-controlled trial in 240 women with early breast cancer subdivided in 2 groups: tocotrienol-rich fraction (TRF) plus tamoxifen and placebo plus tamoxifen	No association between adjuvant tocotrienol therapy and breast cancer-specific survival (risk of mortality due to breast cancer was 60% lower in tocotrienol group but not statistically significant)	[81]	[290]
2	Curcumin	Tablets containing 500 mg Meriva, a proprietary lecithin delivery system containing 100 mg curcuminoids (33 parts of curcumin, 8 parts of demethoxycurcumin and 1 part of bis-demethoxycurcumin)	Patients were under different chemotherapy or radiotherapy regimen	A controlled study on the possibility to alleviate adverse effects of cancer treatment with Meriva. Half of patients ($n = 80$) received Meriva plus the "best available treatment" and the other half ($n = 80$, control groups) received only the "best available treatment"	Results showed that lecithinized curcumin might alleviate the burden of side effects associated to chemo- and radiotherapy	[97]	—
2	Curcumin	2.0 grams of curcumin or placebo orally three times per day (i.e., 6.0 grams daily)	Radiotherapy	Randomized, double-blind, placebo-controlled clinical trial to assess the ability of curcumin to reduce radiation dermatitis severity in 30 breast cancer patients	Curcumin significantly reduced the severity of radiation dermatitis (fewer moist desquamation)	[291]	[292]
2	Curcumin	Oral administration of highly bioavailable curcumin (Theracurmin) containing 200 mg or 400 mg of curcumin	Gemcitabine-based chemotherapy	A phase I clinical study on 16 pancreatic or biliary tract cancer patients who failed standard chemotherapy	No unexpected adverse events were observed and 3 patients safely continued Theracurmin administration for >9 months	[293]	[294]
2	Curcumin	360 mg curcumin three times daily presurgery (10–30 days)	Radiotherapy, chemotherapy, chemoradiotherapy, or no additional therapy	A placebo-controlled clinical trial randomized 126 patients with colorectal cancer to either receive curcumin or placebo	Curcumin administration increased body weight, decreased serum TNF-alpha levels, increased apoptotic tumor cells, enhanced expression of p53 molecule in tumor tissue, and modulated tumor cell apoptotic pathway	[95]	—

Table 2: Continued.

n	Compound	Dose	Drug*	Patients and scheme	Results of the trial	Ref.	Established link with the drug and induction of cellular senescence**
2	Curcumin	Oral curcumin 8 g/day	Gemcitabine-based chemotherapy	A preliminary study in 17 patients with advanced pancreatic cancer	5 patients (29%) discontinued curcumin within few weeks due to abdominal fullness or pain (observed in 7 patients), and the dose of curcumin was reduced to 4000 mg/day. One of 11 evaluable patients (9%) had partial response, 4 (36%) had stable disease, and 6 (55%) had tumor progression	[295]	[294]
2	Curcumin	Oral curcumin 8 g/day	Gemcitabine-based chemotherapy	A phase I study in 21 gemcitabine-resistant patients with pancreatic cancer	No patients were withdrawn from this study because of the intolerability of curcumin	[96]	[294]
2	Curcumin	Curcumin was orally given from 500 mg/d for seven consecutive d by cycle (from d-4 to d + 2) and escalated until a dose-limiting toxicity should occur	Docetaxel chemotherapy	An open-label phase I trial in 14 patients with advanced and metastatic breast cancer	Maximal-tolerated dose of curcumin was established to 8000 mg/d. Some improvements as biological and clinical responses were observed in most patients	[296]	[297]
2	Curcumin	Patients received 8 g curcumin by mouth daily until disease progression, with restaging every 2 months	Individuals who underwent radiotherapy or chemotherapy <4 weeks beforehand were excluded from the study	A nonrandomized, open-label, phase II trial of curcumin in 25 patients with histologically confirmed adenocarcinoma of the pancreas	One patient had ongoing stable disease for >18 months and another had a brief, but marked tumor regression (73%) accompanied by significant increases (4-fold to 35-fold) in serum cytokine levels (IL-6, IL-8, IL-10, and IL-1 receptor antagonists). No toxicities were observed	[298]	—
2	Curcumin	Curcuma extract in proprietary capsule form was given at doses between 440 and 2200 mg/day, containing 36–180 mg of curcumin	Patients received various previous chemotherapy or radiotherapy	A preliminary study in 15 patients with advanced colorectal cancer refractory to standard chemotherapies	Curcuma extract was administered safely to patients at doses of up to 2.2 g daily, equivalent to 180 mg of curcumin but a low bioavailability of curcumin in humans was also observed	[299]	—
3	EGCG	Dose escalation proceeded according to a standard phase I design from 40 μM to 440 μM	Cisplatin and etoposide	Single-arm phase I study in 24 patients with unresectable stage III non-small-cell lung cancer	Dramatic regression of esophagitis was observed in 22 of 24 patients and mean pain score was reduced	[114]	[300, 301]

Table 2: Continued.

n	Compound	Dose	Drug*	Patients and scheme	Results of the trial	Ref.	Established link with the drug and induction of cellular senescence**
3	EGCG	Double-brewed green tea at 500 ml day	Platinum/taxane	Maintenance treatment after chemotherapy in 16 women with advanced stage ovarian cancer	Only 5 of 16 women remained free of recurrence after 18 months	[302]	[303]
3	EGCG	2000 mg twice a day	—	Single-arm phase II trial in 42 patients with asymptomatic, Rai stage 0 to II chronic lymphocytic leukemia who did not meet criteria to initiate conventional chemotherapy treatment	29 patients (69%) showed decline ≥ 20% in the lymphocyte count and/or a reduction ≥ 30% in the sum of the products of all lymph node areas during the 6 months of treatment	[304]	—
3	EGCG	Green tea extract 375 mg per day	—	Single-arm study in 19 hormone-refractory prostate cancer patients	No patient had a prostate-specific antigen response (i.e., at least 50% decrease from baseline), and all 19 patients were deemed to have progressive disease within 1 to 5 months	[305]	—
3	EGCG	6 g of powdered green tea extract daily	—	Phase II trial of green tea in the treatment of 42 patients with androgen-independent metastatic prostate carcinoma	Limited antineoplastic activity, as defined by a decline in PSA levels (decline > 50% in the baseline PSA value, occurred in a single patient) and 4 episodes of toxicity, was observed	[306]	—
4	Quercetin	Quercetin 20 mg orally 3 times a day in combination with curcumin (480 mg)	—	A phase I trial in patients with familial adenomatous polyposis with prior colectomy	All 5 patients had a decreased polyp number and size from baseline after a mean of 6 months of treatment with curcumin and quercetin	[307]	—
4	Quercetin	Short i.v. infusion at escalating doses from (1400 mg/m2 was recommended)	Previous chemotherapy was reported for 40/51 patients	A phase I and phase II trial in patients with various cancer no longer amenable to standard therapies	In 9 of 11 patients, lymphocyte protein tyrosine phosphorylation was inhibited. In one patient with ovarian cancer and in another patient with hepatoma, circulating tumor markers were decreased	[132]	
5	Genistein	In escalating doses (400 mg–1600 mg daily) of a multicomponent crystalline form	Concomitant gemcitabine treatment (1000 mg/m^2)	A phase I study in 16 patients with inoperable pancreatic carcinoma	No signs of toxicity observed. The median overall survival time was 4.9 months (range 1.5–19.5 months)	[308]	—

Table 2: Continued.

n	Compound	Dose	Drug*	Patients and scheme	Results of the trial	Ref.	Established link with the drug and induction of cellular senescence**
5	Genistein	30 mg genistein or placebo capsules daily for 3–6 weeks before radical prostatectomy	—	A phase II placebo-controlled, randomized, double-blind clinical trial was conducted in 47 patients with prostate cancer	No significant effects on proliferation-, cell cycle-, apoptosis- or neuroendocrine biomarkers. Modulation of the expression of some biomarkers related to prediction and progression	([309]; [310])	
5	Genistein	Oral genistein (300 or 600 mg/d as the purified soy extract G-2535) for 14 to 21 days before surgery	—	A phase II randomized, placebo-controlled trial in 59 subjects diagnosed with urothelial bladder cancer	Reduction in bladder cancer tissue p-EGFR staining at dose of 300 mg. No other significant differences in the multitude of clinical molecular parameters measured		
6	Resveratrol	Micronized resveratrol at 5 g daily	Bortezomid	A phase II study of SRT501 (resveratrol) with bortezomib in 24 patients with relapsed and or refractory multiple myeloma	Unacceptable safety profile and minimal efficacy in patients with relapsed/refractory multiple myeloma	[170]	[311]
6	Resveratrol	Micronized resveratrol at 5 g daily	—	Phase I randomized, double-blind pilot in patients with colorectal cancer and hepatic metastases	Administration was safe. A small but significant increase in cleaved caspase-3 immunoreactivity in tumor tissue compared to placebo was detected	[171]	—
7	Silybin	Dose escalation from 2 to 12 g per day with silybin-phosphatidylcholine	—	Phase I dose-preliminary study in 3 patients with advanced hepatocellular carcinoma not eligible for other therapies	All patients died soon after enrolment and were not possible to conclude about the effects	[182]	—
7	Silybin	Dose escalation from 2.5 to 20 g per day with silybin-phytosome	—	Phase I pharmacokinetic study in 13 patients (over 70 years) with prostate cancer	No response on prostate-specific antigen was observed and one patient displayed grade 3 toxicity	[312]	—
8	PEITC	10 mg in 1 ml of olive oil, 4 times per day, for 1 week	—	Not an adjuvant therapy, but a clinical trial with a crossover design versus placebo in 18 smokers	Metabolic activation of one carcinogen was reduced by treatment	[192]	
9	Sulforaphane	200 μmoles/day of sulforaphane-rich extracts for a maximum period of 20 weeks	—	Phase II study in 20 patients with recurrent prostate cancer	Treatment did not lead to ≥50% PSA declines in the majority of patients. A significant lengthening of the on-treatment PSA doubling time was observed compared with the pretreatment PSA doubling time. Administration was safe with no grade 3 adverse events	[208]	—

Table 2: Continued.

n	Compound	Dose	Drug*	Patients and scheme	Results of the trial	Ref.	Established link with the drug and induction of cellular senescence**
10	Triptolide	2 weekly infusions for 3 weeks with 2 mg of a semisynthetic derivate of triptolide, which is converted to triptolide in vivo (F60008)		Phase I trial in 20 patients with advanced solid malignancy for whom standard therapy options did not exist	Treatment-induced mild grade 1-2 anaemia, fatigue, nausea, vomiting, diarrhea, and constipation. Two lethal events were observed	[219]	
11	Allicin	Local application of allicin, via gastroscopy (48 h before surgical intervention)	—	Trial on 40 patients with progressive gastric carcinoma versus 40 controls	In cancer tissues removed by surgery, cell apoptosis rate was 9.60 versus 2.20 in the control group. There were additional differences in the expression of proapoptotic genes and in cell cycle progression	[313]	
12	Berberine	20 mg kg− 1 once a day	Radiation therapy	Two arm study in 90 patients with non-small-cell lung cancer. The trial group received radiation therapy plus berberine, and the control group received radiation therapy plus a placebo for 6 weeks	Reduced the incidence of radiation-induced lung injury and improved pulmonary function	[243]	[292]
13	Piperlongumine	No clinical trial	—	—	—	—	—
14	Fisetin	No clinical trial	—	—	—	—	—
15	Phloretin	No clinical trial	—	—	—	—	—

*When the natural bioactive compound was used as adjuvant therapy, otherwise not applicable. **Explicative references that identify mechanisms related to senescence induction of the chemotherapy drugs or treatments reported in the adjuvant therapy.

can be cleared off by the immune system and they might eventually use senescence as a system to escape death with the potential to revert their phenotype later in time. This problem might be potentially avoided with the recent development of senolytics, which include quercetin, fisetin, piperlongumine, and phloretin among natural bioactive compounds. These senolytics may be useful in the case cancer cells may use senescence as an escape strategy from therapy as well as to reduce side effects of therapy. Studies on senolytic compounds open the feasibility of a new therapeutic scheme with a single dose of the compound after radio- or chemotherapy without the needs for escalation doses and continuous treatments (Figure 1). Overall, combining therapies with natural compounds with the aims not only to induce senescence in cancer cells but also to clear off from the organism senescent cells appear as a promising strategy in this field.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The study has been partially funded through the revenues of the "5 per Mille" donations received by INRCA through the Italian Ministry of Health.

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 3705389, 13 pages
https://doi.org/10.1155/2018/3705389

Review Article

Anti-Inflamm-Ageing and/or Anti-Age-Related Disease Emerging Treatments: A Historical Alchemy or Revolutionary Effective Procedures?

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Received 29 September 2017; Accepted 13 December 2017; Published 8 February 2018

Academic Editor: Vera L. Petricevich

The "*long-life elixir*" has long represented for humans a dream, a vanity's sin for remaining young and to long survive. Today, because of *ageing population phenomenon*, the research of antiageing interventions appears to be more important than ever, for preserving health in old age and retarding/or delaying the onset of age-related diseases. A hope is given by experimental data, which evidence the possibility of retarding ageing in animal models. In addition, it has been also demonstrated in animal life-extending studies not only the possibility of increasing longevity but also the ability to retard the onset of age-related diseases. Interestingly, this recent evidence is leading to promise of obtaining the same effects in humans and resulting in benefits for their health in old ages. In order to achieve this goal, different approaches have been used ranging from pharmacological targeting of ageing, basic biological assays, and big data analysis to the recent use of young blood, stem cells, cellular, genetic, and epigenetic reprogramming, or other techniques of regenerative medicine. However, only a little fraction of these approaches has the features for being tested in clinical applications. Here, new emerging molecules, drugs, and procedures will be described, by evidencing potential benefits and limitations.

1. Introduction

The current goal of ageing's researchers is both to understand the biology of ageing and the *related inflamm-ageing* and to translate the potential scientific insights obtained into effective interventions, in order to improve the health in old ages and to retard and/or to delay the onset of age-related diseases (ARDs) [1]. Thus, antiageing and anti-inflamm-ageing interventions and the parallel health promotion for old people represent the very recent interests not only of the entire scientific community but also of all institutions/organizations from the Western countries and particularly from EU countries [2]. These last are recommending *health promotion programmes,* such as *EU Project Pro-Health* 65+, which engages ten EU member states [2]. Cotemporally, this growing research is becoming a very business for several biotechnological companies, which are promoting a growing number of therapeutic approaches, ranging from oxidant drugs, hormones, vitamins, and diet supplements to various aesthetic drugs and techniques [3]. In fact, these approaches are particularly costly for gullible patients in searching of well being and abused by a carefully organized marketing involving tacit complicity of doctors, laboratories, and companies [3]. However, the major number of these treatments until now constitute more a *palliative care* than a very "*long-life elixir*," which has long represented for humans a dream, a vanity's sin for remaining young and to long survive [3]. This leads to the extemporaneous consideration that the way, for developing effective antiageing and anti-inflamm-ageing treatments in humans, is distant. Despite this, there is among the ageing's researchers the optimistic hope in identifying successful treatments and approaches for humans too. It derives from the increase of lifespan observed in short-lived model organisms (i.e., yeast, worm, flies, mice, and rats) which undergone

to various genetic, dietary, and pharmacological interventions [4–7]. In addition, some of these treatments have also demonstrated to retard the onset of ARDs and, consequently, to extend the health-span (i.e., the length of time one lives in good health), as evidenced in 2014 by Kennedy and coworkers [8]. Thus, it is possible to affirm that this evidence in animal models leads to the promise to develop effective life-extending interventions in humans too. Accordingly, new treatments are emerging. Here, some of these interventions will be described and discussed, underlying the benefits and limitations. Before their dissertation, a brief clarification of some concepts will be pointed in the initial paragraphs. This will facilitate the understanding of arguments reported not only for the ageing's researchers but also for clinicians, since the author's message is to offer a concrete "*food for thought*" about ageing and new effective therapeutic strategies to counteract, to retard, or to delay ARDs, such as the cardiovascular diseases (CVDs), the first very problem of our populations. In fact, the concepts stressed, here, are fruit of considerations derived by expert opinion on the findings from author's studies on ageing, ARDs (particularly CVDs) and inflammation.

1.1. Aspects to Point about Antiageing Treatments: From Definition to Eventual Use and New Strategies for Their Identification. Today, the lack of a clear consensus on what exactly constitutes an *antiageing treatment* still exists. Initially, it imagined to define as antiageing treatment the approach or drug able to counteract the primary cause of ageing, which still remains to identify and may be consequently considered only a very abstraction and not a reality. Recently, Gems has stressed this concept and has suggested the necessity to propose a more pragmatic and realistic definition of *antiageing treatment* [9]. Precisely, he has proposed to define with the term *antiageing treatment* as any preventative approach able to reduce late-life disease, since new evidence considers the senescence (i.e., ageing) a disease's syndrome [10]. In fact, Gems's definition is essentially based on the emerging concept of ageing to consider it as set of pathologies, some of which provoke mortality [9]. Thus, Gems has suggested that the key to understanding ageing is both to better characterize the ARDs (including diabetes, CVDs, neurodegenerative disorders, and cancer) and discover their original causes. As a result, this definition would include all preventative approaches aimed for ARDs [9]. In addition, its use would help its translation, since it would change the weight of medical practice, and in the specific case, the opening to preventative approaches [9]. For examples, the cardiovascular polyp ill treatment, proposed as antiageing treatment for CVDs, could establish a medical practice that extends it to antiageing preventive interventions, such as dietary restriction mimetics [11].

The suggestion from Gems is the result of the symposium "*Interventions to Slow Aging in Humans: Are We Ready*?" held on October 8–13, 2013, in Erice (Sicily) and organized by Valter Longo, Luigi Fontana, and Donald Ingram [9]. In addition, it does not pretend to refute the initial definition of antiageing treatment, but it emphasizes the necessity of clearing the concept and stabilising a common consensus on a standardized definition of antiageing treatment and likely proposing novel strategies for achieving it. Accordingly, Saraswat and Rizvi [12] have recently proposed that one of the most promising approaches for a successful antiageing strategy includes the activation of *adenosine monophosphate-dependent protein kinase (AMPK)*. They have also suggested that another strategy may involve the activation of plasma membrane redox system (PMRS), and the consequent development of molecules able to activate or inhibit nutrient and energy-sensing molecular pathways, such as *mTOR*, *IGF-1*, *AMPK*, and *sirtuins* [12]. Other new antiageing (or precisely anti-ARD, that is, CVDs) strategies have been also proposed by our group. In particular, *very innovative frameworks* for developing therapeutic interventions for some ARDs, such as CVDs and neurodegenerative diseases, have been suggested by our group [13–19]. They are based on the relevant concept that each individual is the result of the sophisticated interplay between environmental factors and its genome, trascriptome, proteome, metabolome, microbiome, epigenome, and exposome. Thus, it is necessary to perform a more complex combination of investigations based on genetic, transcriptomic, proteomic, metabolomic, microbiomic, and epigenetic evaluations, for obtaining interesting data in the study of these diseases. In addition, computational investigations are also recommended, as well as collecting environmental and biometric data and medical/scientific/health care records [13–19]. Thus, we have suggested that the integration of all data, obtained in this large panel of investigations, and their elaboration might lead to the development of appropriate agonists, antagonists, and inhibitors of specific signaling disease pathways, which might be used in a near future as *personalized treatments* for these diseases, facilitating their management and outcome [13–19].

1.2. An Emerging Class of Antiageing Treatments, "the Pharmacological Targets of Ageing": A Brief Description. A plethora of genes, molecules, and pathways able to modulate the ageing or better the ARDs is emerging from several studies, in both animal models and humans, as recently stressed by our and other groups [4–6, 13–19]. They have been suggested as potential targets for antiageing (anti-ARD) treatments and are likely leading to development of that class of antiageing treatments defined as *pharmacological targets of ageing*, while the approach used has been defined as *pharmacological targeting of ageing* (of ARDs) (see Figures 1(a) and 1(b)) [4–6, 13–19]. Among the pathways detected as potential antiageing targets, it is possible to identify in first position *the immune inflammatory pathways*. Their relevant weight derives from several data, which have strongly suggested them as the promoters of ageing (senescence) and ARDs, as also evidenced in our studies [13–19]. This is in agreement to strong concept emerged by a very large number of ageing and ARD studies that *sterile inflammation* and the consequent systemic chronic inflammation at low rate (i.e., *inflamm-ageing*) [20–25], related to the release of the so-called *senescence-associated secretory phenotype* (SASP) [19, 26, 27], are the primary mechanisms of ageing and the pathophysiology of all ARDs [20–27]. Here, it reports a typical

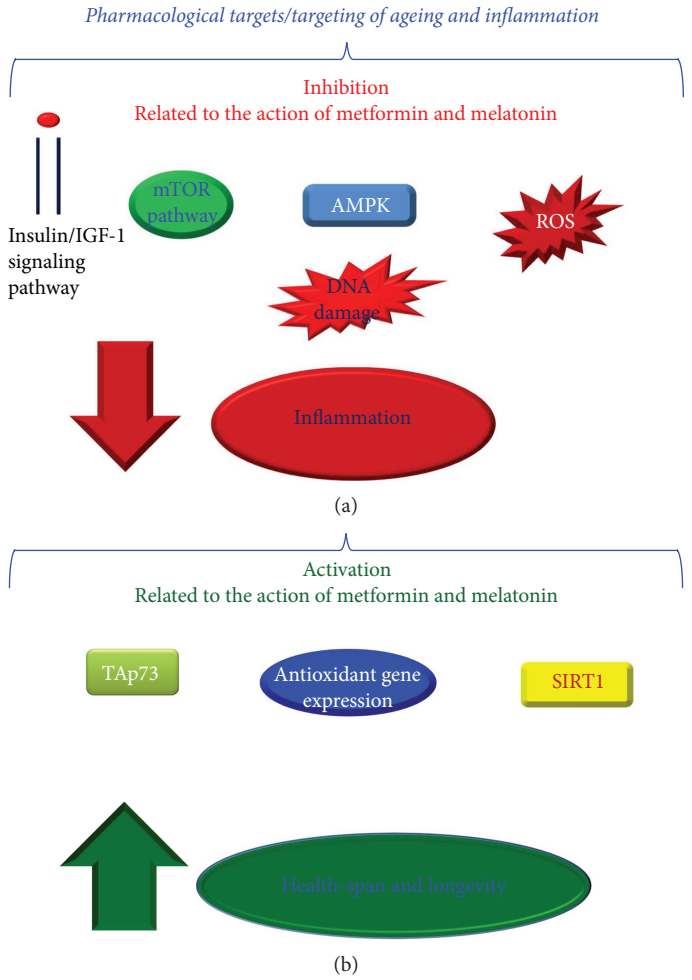

Figure 1: (a and b) Pharmacological targets/targeting of ageing and inflammation. In (a and b), examples of ageing and inflammatory target pathways. In particular, in (a), it describes the inhibitor actions of metformin and melatonin on ageing/inflammatory pathways. In (b), the activation of some ageing/inflammatory pathways for counteracting the ageing and inflammatory effects and for contributing to health-span and longevity.

example of these pathways, and precisely, the *Toll-like receptor-4 (TLR-4) signaling pathway* (see Figure 2), [13, 28], which the emerging evidence places in the center (constituting it a network with other evolutionally conserved pathways) of molecular mechanisms involved in senescence (ageing) of all cells, tissues, organs, and systems and the related ARDs [13–19, 25, 28–32], as well as in those associated with the host defence against Gram-negative bacteria, viruses, fungi, and mycoplasma [33]. Thus, TLR-4 pathway shows a double role in human tissues, which is modulated and influenced by the context of each tissue, organ, and system and particularly by the conditions of their microenvironment, as we have recently suggested in a model proposed for aorta homeostasis and disease [13]. The growing interest on TLR-4 pathway is leading some biotechnology companies to develop various drugs (i.e., agonists, antagonists, and inhibitors), ranging from proteins to metal ions, as pharmacological treatments to modulate ageing and ARDs, such as CVDs (see Figure 2). Their action has shown that both the TLR-4 activation and inhibition may have beneficial effects in various conditions, reducing tissue immune inflammatory responses (as amply quoted in [13, 19, 28]). However, the biological effects mediated by TLR-4 modulators seem to depend on several factors. They include the timing of administration during the various stages of ageing or disease onset and progression and the age and genetic background of animal models used during experimentation (as amply quoted in [13, 19, 28]). Regarding this consideration, we have stressed in a recent review [28] that there are age-related defects in TLR-4 function and expression, as reported in human studies. However, these studies did not lead to conclusive data, being limited by the heterogeneity of epidemiological and laboratory methods [28]. This might also be valid for the animals used as study's model. Furthermore, current evidence demonstrates that the TLR-4 function and expression are modulated not only by genetic variants and haplotypes [28] but also by environmental factors. Accordingly, diet (quoted in [13]), mite allergens (quoted in [13]), air pollution (quoted in [13]), and their cross-interaction with microbiota (quoted in [13]), which may remain in a healthy state or show alterations (i.e., dysbiosis and consequent endotoxemia associated with age or obesity) (quoted in

Molecules	Action
BCG	TLR-4 agonist
Eritoran	TLR-4 antagonist
Valsartan	TLR-4 inhibitor
Atorvastatin	TLR-4 inhibitor
Losartan	TLR-4 inhibitor
Fluvastatin	TLR-4 inhibitor
Cinnamic acid	TLR-4 inhibitor
Geldanamycin	TLR-4 inhibitor
Baicalin	TLR-4 inhibitor
Oxymatrine	TLR-4 inhibitor
Progesterone	TLR-4 inhibitor
Gynkgobiloba	TLR-4 inhibitor
Picroside 2	TLR-4 inhibitor
Oestrogen	TLR-4 inhibitor
Dexmedetomidine	TLR-4 inhibitor
Melatonin	TLR-4 inhibitor
Surforaphane Cinnamaldehyde Curcumin	TLR-4 inhibitors, by blocking oligomerization of the receptor
Epigallocatechin gallate (EGCG), resveratrol and certain flavonoids such as lutelolin, quercetin, chrysin, and eriodictyol	TLR-4 inhibitors have the ability to decrease kinase activity of TBK1 activating IRF
Xanthohumol (XN)	TLR-4 inhibitor XN is anti-inflammatory natural product from hops and beer; it can block the TLR-4 signaling by binding to MD-2 directly
Ligustrazine, a natural alkaloid compound	TLR-4 inhibitor

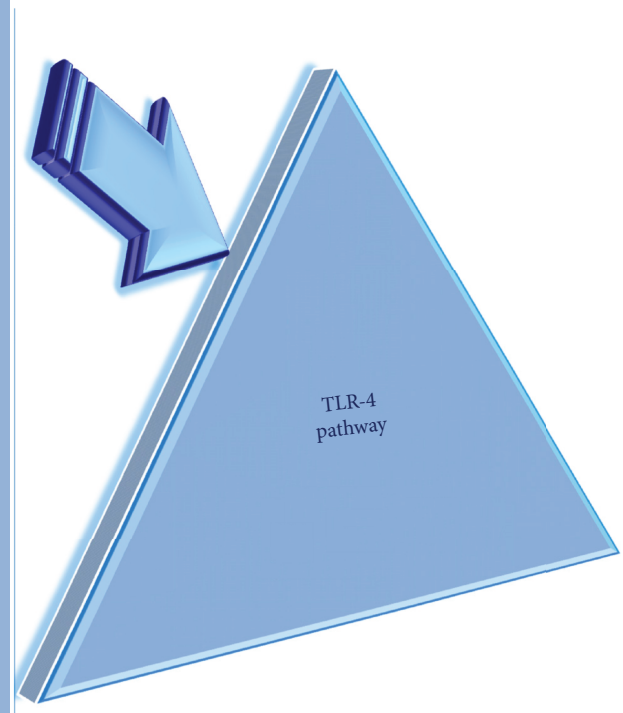

Figure 2: The TLR-4 pathway and a panel of agonist, antagonist, and inhibitor molecules.

[13]), and consequent epigenetic changes (quoted in [13]), have been significantly associated with TLR-4 functional alterations and modified tissue levels. Consequently, many limitations and concerns emerge. In addition, another relevant limitation derives from the type of investigation. In this specific case, TLR-4 agonists and antagonists have been mainly tested on cell cultures and animal models (quoted in [13]). Thus, at moment, only preclinical data exist, but not human trials. This leads to affirm that the encouraging data obtained until now in animal models may not be likely confirmed in humans. In fact, there are many concerns in this field. The first concern comes from the real action mediated by TLR-4 agonist and antagonist molecules, which is amply questioned. A large number of researchers claim that only a little subset of them is able to activate or inhibit directly the TLR-4 pathway. The limitation stays in their structural diversity that likely implies a promiscuity in their TLR-4 binding capacity, without specificity of action, and rather a pleiotropic effect on different receptors (quoted in [13]). Similar considerations may also be valid for small natural molecules, such as curcumin, cinnamaldehyde, and sulforaphane, which have been reported to mediate the anti-TLR-4 dimerization effects, as well as for resveratrol and some flavonoids (such as EGCG, lutelolin, quercetin, chrysin, and eriodictyol), reported to inhibit TBK1 kinase activity (a downstream TLR-4 signaling kinase), resulting in a decreased IRF3 activation (a TLR-4 signaling adaptator) and target gene expression (quoted in [13]). Thus, these observations support the idea that further studies are imperative for clinical implementations in the near future of these molecules. On the other hand, it has been suggested in the previous paragraph that the solution can be in putting together several types of investigations with the evaluations of TLR-4 modulators to facilitate the development of *personalized treatments* for ageing and ARDs, such as CVDs (as below reported).

2. Metformin: The "Miraculous" Drug for Counteracting Ageing and ARDs

Recently, *the metformin*, one drug normally used in diabetes therapy, is emerging as antiageing factor (quoted in [33, 34]). Studies on model organisms have, indeed, demonstrated that metformin has the ability to extend the lifespan (quoted in [33, 34]). The potential mechanisms used have been largely discussed and include the following: (a) the reduced insulin and IGF-1 signaling; (b) the inhibition of mTOR; (c) reducing the levels of reactive oxygen species (ROS); (d) lowering inflammation, (e) reducing DNA damage, and (f) the activation of AMPK (quoted in [33, 34]). Its effect on AMPK has attracted particular attention (see Figures 1(a) and 1(b)). It

also appears to modulate the intracellular mechanisms of caloric restriction, long recognized as the first intervention capable of extending the lifespans in animal models, but not feasible for widespread implementation in humans (quoted in [33, 34]). This, among other factors, has led to use metformin, being described as a caloric restriction mimetic (CRM) (quoted in [33, 34]). These encouraging results have attracted the attention of other groups to perform further investigations in a large panel of ARDs. The data obtained have demonstrated that metformin also shows antitumor, cardiovascular protective, and neuroprotective effects (quoted in [33–36]). Interestingly, this evidence has attracted the attention of the Campbell group in 2017 [35] that performed a meta-analysis for evaluating the effect of metformin on all-cause mortality or diseases of ageing relative to nondiabetic populations or diabetics receiving other therapies with adjustment for disease control achieved [35]. They obtained very promising data. Precisely, they observed that metformin is not only significantly able to reduce all-cause mortality in diabetic cases taking it than nondiabetics but also in did diabetics taking metformin compared to diabetics receiving nonmetformin therapies, such as insulin or sulphonylurea. In addition, they detected that metformin users also show a reduced risk for cancer and CVDs compared to nondiabetics or diabetics receiving nonmetformin therapies or insulin [35]. Thus, they concluded that metformin could represent an effective extending life and health-span drug, by acting as an antiageing factor [35]. This potentiality of metformin has encouraged the Wang group to propose potential models for explicating the mechanisms and the pathways involved [34].

However, many questions still remain unresolved. For example, it is not detected whether these potential indications of metformin can be also observed in nondiabetics and whether genetic factors can influence the effects of metformin in each individual. Thus, it is necessary to clarify these aspects, by performing not only further research basic investigations but also clinical trials.

3. Melatonin, the Molecule Regulating Human Physiological Rhythm as Antiageing Treatment

Another emerging antiageing molecule is the *melatonin (N-acetyl-5-methoxytryptamine)*, an indoleamine molecule highly and generally identified in the major number of plant and animal organisms, including human [37]. It is synthesized from the essential amino acid l-tryptophan thanks the action of four enzymes [37]. In vertebrates, including human, melatonin is recognized as a secretory product of the pineal gland. However, in these organisms, it and four enzymes also are the physiological cell components of other tissues, including the retina, skin, immune system, gastrointestinal tract, and reproductive tract (amply quoted in [38]). Here, melatonin is expressed at different levels, and it shows a higher density of expression within the membranes and the mitochondria, where it mediates several functions: interacts with lipids, stabilizes all cellular membranes, reduces lipid peroxidation, and increases ATP production as evidenced by García and coworkers in 2014 [39]. In addition, it evocates several beneficial effects: antioxidant and free radical scavenging capacity, that consent to melatonin to protect proteins and mtDNA from oxidative stress; capability of penetrating all morphophysiological barriers and entering all subcellular compartments due to its amphiphilic nature, that permit to melatonin to modulate a diverse number of physiological processes via different mechanisms; capacity to active a broad spectrum of molecular pathways, including particularly the sirtuins, such as SIRT1, through both receptor-dependent and receptor-independent signaling pathways; a powerful direct free radical scavenger ability against ROS and reactive nitrogen species (RNS); capacity to increase the activity of endogenous antioxidant enzymes; and anti-inflammatory properties (see Figures 1(a) and 1(b)) (amply quoted in [38–40]). This last action consents it to attenuate tissue damage under a variety of ARD conditions (amply quoted in [38–40]). Some of the anti-inflammatory properties are principally related to SIRT1 activation. Accordingly, melatonin has shown, in apolipoprotein E-deficient mice, the capacity to decrease the impairment of endothelial damage, the loss of SIRT1 and endothelial nitric oxide synthase, and the p53 and endothelin-1 expression [41]. In addition, melatonin has been also observed to confer a cardioprotective effect against myocardial ischemia-reperfusion injury, by reducing oxidative stress damage via activation of SIRT1 signaling in a receptor-dependent manner [42]. Melatonin treatment also seems to reduce cerebral ischemia-reperfusion injury in mice, by reducing ischemia-reperfusion-induced mitochondrial dysfunction through the activation of SIRT1 signaling and the attenuation of mitochondrial oxidative damage [43].

Recently, it has been also demonstrated that the production of melatonin within 24 hours is significantly higher in young animals than in old organisms, including humans. This has suggested a potential association between the loss of melatonin and the signs of ageing (amply quoted in [38]). In addition, in animal models, it has been observed that the rhythm of melatonin can be substantially preserved during ageing by restricting food intake (amply quoted in [38]). This has suggested that food restriction could mediate some of its beneficial physiological effects through its ability to sustain pineal activity in old age (amply quoted in [38]). Food restriction presumably conserves the melatonin rhythm in part, because it prevents the reduction in pineal-adrenergic receptors normally reported in old rats (amply quoted in [38]). The loss of melatonin in the elderly may lead to disorders of circadian rhythms that reflect in an increase of systemic and tissue inflammation and in the evocation of both senescence and ARDs (amply quoted in [38]).

The actions described above may elucidate, at least in part, its protective ability to delay the deleterious effects of ageing and a variety of ARDs, such as Parkinson's disease, Alzheimer's disease, ischemia-reperfusion, and sepsis (amply quoted in [36, 38, 40]). They are leading several companies to develop melatonin hybrids, as potent antiageing drugs. Furthermore, the use of foods rich of melatonin is recently recommended. Of course, both experimental basic investigations

and clinical trials are necessary for its future clinical applications (amply quoted in [36, 38, 40]).

4. Stem Cells as Therapeutic Agents for Ageing and ARDs?

Recently, the clinical application of stem cells, as therapeutic agents in ageing and ARDs, is acquiring a very consideration from the entire scientific community [44, 45]. Stem cell therapy might, therefore, constitute a very solution for both delaying/retarding ageing processes and the onset/progression of ARDs and permitting to expand the survival of individuals. The hope also is of applying it in a near future as preventive treatment in susceptible ARD individuals [44, 45].

Stem cells are defined as undifferentiated cells with the potential to renew themselves and to differentiate into any other specialized cell of the human body and, therefore (potentially and theoretically), to create any tissues or organs ([17, 46]). Under specific conditions, stem cells can, indeed, differentiate into diverse populations of mature and functionally specialized cellular types. To date, the following classes of stem cells are recognized: (a) *totipotent cells*, having the capacity to differentiate into embryonic and extra embryonic cell types, thereby generating the entire organisms, even if this capacity is limited to cells produced by the first few divisions after fertilization; (b) *pluripotent stem cell types*; and (c) *adult multipotent/unipotent stem cells*, which can only differentiate into several closely related cell types ([17, 46]). A variety of cellular types has been and is currently used in cell-based therapy, including *embryonic*, *multipotent stromal*, *induced pluripotent*, and *adult stem cells* ([17, 46]). By analysing the role of all stem cells as therapeutic antiageing (and particularly anti-ARD) agents, the enthusiasm of researchers has been reduced by diverse concerns related to the detection of various serious limitations (quoted in [17, 36, 46]). In fact, diverse undesirable factors have been evidenced, including firstly the teratogenic and tumorigenic potential of these cells on the recipient organisms and the immune reactivity, which consequently affect the safety of the treatments, but also their reduced success related to the inadequate dose administration, the imprecise phenotypic profile of cells used for the treatments, their biological age and senescent status, and the inappropriate administration ways and methods used (quoted in [17, 36, 46]). Thus, ulterior studies are certainly needed to convert the research data in clinical applications, from research basic investigations to preclinical and clinical studies.

5. Cellular Reprogramming and Young Blood as Potential Antiageing Therapies?

An optimistic help in the development of antiageing treatments might derive from *Regenerative Medicine* (RegMed), a branch of *translational medicine* (amply quoted in [36, 46, 47]). It arises from a necessity of eliminating the gaps existing between the increased risk of disease and the decreased capacity of the major number of human tissues and organs spontaneously to regenerate and to respond to insults and damages in the setting of ageing (amply quoted in [36, 46, 47]). RegMed is, indeed, as interdisciplinary field of research and clinical applications focused on the repair, replacement, or regeneration of cells, tissues, or organs to restore impaired function resulting from any causes, including congenital defects, diseases, trauma, and ageing (amply quoted in [36, 46, 47]). For this reasons, RegMed is suggested as the way "to improve the health and quality of life by restoring, maintaining, or enhancing functions of tissue and organs" (amply quoted in [36, 46, 47]). For achieving these objectives, RegMed uses a combination of several approaches, including (but not limited to) the use of soluble molecules, gene therapy, stem cell transplantation, tissue engineering, and reprogramming of cell and tissue types (amply quoted in [36, 46, 47]). These last approaches, that is, *cellular and tissue reprogramming*, are emerging as new and promising interventions [48]. They are based on de novo generation of cells, which may be obtained by converting adult somatic cells to pluripotent state termed *induced pluripotent stem* (iPS) cells (abovementioned) in vitro [48]. This cellular technology has been precisely defined *cellular reprogramming*, which has created new opportunities in understanding human disease, drug discovery, and regenerative medicine [49]. Today, the researchers are proposing a new generation or better a "second generation" of the cellular reprogramming [49]. It involves lineage-restricted transcription factors (as identified by Takahashi and Yamanaka in 2006 and defined Yamanaka factors [50]), or microRNAs able directly to reprogram one somatic cell to another (amply quoted in [51]). This "new version" of cellular reprogramming is based on the gene networks, which have been evidenced to be active during the development, and able to induce global shifts in the epigenetic landscape driving the cell fate decisions [49, 51]. The major advantage in the use of the direct reprogramming stays in controlling the resident support cells of adult stem cell niches inside eventual damaged organs or old tissues. This can facilitate the regeneration or repair of the lost or old tissue, by converting them into the desired cell type in situ [49, 51].

Interesting results on the use *in vivo* of reprogramming by forced expression of Yamanaka factors have been recently demonstrated in mice. Precisely, it has been observed that the induction of Oct4, Sox2, Klf4, and c-Myc transcription factors determined the detection of Nanog-positive cells (i.e., pluripotent cells) in diverse tissues, including the stomach, intestine, pancreas, and kidney [52]. Thus, reprogramming can be obtained *in vivo*. Furthermore, ulterior support derives from mouse bone marrow transplantation experiments and the detection of circulating iPSCs, suggesting that hematopoietic system may be reprogramed *in vivo* [52]. However, Abad and colleagues also demonstrated that Yamanaka factors induce a high grade of morality in mice [53]. The reasons prevalently are associated with the development of teratoma in multiple tissues, but they also hypothesize that the loss of cellular identified upon differentiation could impair organ or tissue functionality, resulting in organism death. Certainly, this may constitute a significant obstacle for applying it as regenerative or antiageing strategy. Alternatively, the partial reprogramming or the reprogramming of specific organ or tissue might be used. In these cases,

the duration of the partial reprogramming must be regulated and potentially adapted to a specific cell, tissue, or organ. Consequently, it is possible to affirm that de novo generation of human cells remains elusive. Thus, many questions remain to be resolved. However, these approaches may one day help to ameliorate ageing symptoms, reduce the associated diseases, and ultimately improve human health and lifespan (amply quoted in [46, 52]).

As an alternative approach, *the use of young blood* appears surprising. It is based on research into the effects of *parabiosis*, tested in experimental mouse studies. It consists in connecting the circulatory systems of two organisms of different ages. These *heterochronic parabiosis's experiments* have elegantly demonstrated that exposing old tissues and organs to a young circulatory environment can rejuvenate tissue-specific stem cells, leading to a youthful state, which is characterized by functional and regenerative improvements [52, 54]. These encouraging data have led researchers from Stanford University to develop and perform a spinout, *the Alkahest*, as treatment for Alzheimer's disease, where the Grifols company, the largest plasma manufacturer worldwide, has invested for developing *Alkahest*'s plasma-based products. In addition, *heterochronic parabiosis experiments* have recently demonstrated the revitalization of brain function in old mice [55].

6. Nutritional Recommendations as Another Innovative Antiageing Approach: The Opening to Precision Nutrition Science

The diet is emerging as another modifiable risk factor for ageing and ARDs. Precisely, a diet low in vegetables, seafood, grains, nuts/seeds, and milk and high in red meat represents a strong risk factor [56]. Its weight is confirmed by studies, where nutritional practises have shown to decrease the risk for CVDs by 60% [57] and to prevent cancer for 27–39% by improving diet, physical activity, and body composition [58]. Ulterior support may derive from population-based approaches that can favourably shift disease risk factors in the entire population (amply quoted in [59]). However, these approaches may reveal vain because of several determinant factors: (1) the population's knowledge about the risk is generally insufficient to change behaviour, even if the vast majority of people know what they should and should not eat; (2) in addition, people have a limited amount of mental energy required to resist temptation; (3) it is now becoming clear the concept on existence of an interindividual variability in the effects to diet interventions (amply quoted in [59]). Thus, some dietary interventions may ameliorate the health of some individuals or not of others, being dependent on their genotypes, phenotypes, general clinical conditions, and environment (amply quoted in [59]). Accordingly, the major number of large, randomized controlled trials have demonstrated a successful response to diet interventions only in 40% of individuals [60]. This has suggested that the biological effects of dietary interventions differ between individuals (amply quoted in [59]). The determinant factors able to influence them are as follows: sex, age, habitual dietary habits, genetics, epigenetic, and gut microbiota [61]. This last modulates the absorption, distribution, metabolism, and excretion of compounds and metabolites [62, 63]. Thus, gut microbiota may affect bioavailability and biological responsiveness, as demonstrated in some studies [62, 63].

These observations and relative considerations have pioneered the introduction of a new promising medicine branch, the *Precision Medicine*, in order to overcome the interindividual variability in response to therapies [64]. The opening to this new medicine's vision has led to plan some trials with the focus on individual response to therapies, such as dietary interventions, which are now recognized by regulatory agencies, such as the US Food and Drug Administration. Thus, the precision nutrition approaches may affect the way to perform dietary interventions in the future [59]. Other factors that might affect the success of the implementation of precision nutrition approaches and would be consequently determined and included in the future studies are the metabolomic factors and the role of nutrigenetics in the interindividual responses to food components and products [59]. In addition, the key role of gut microbiota in the interindividual responses is becoming to include the gut microbiota analysis of each individual as a method for personalized dietary recommendation [65].

In complex, these observations point out the necessity to apply precision nutrition approaches for personalizing ageing and disease risk and, in parallel, future dietary recommendations. Further developments in this area will depend on considering big data analysis and health informatics that can capture molecular and medical data, as suggested above. In addition, the triumph of precision nutrition approaches will depend on the robust application of appropriate study designs as the predictive role of clinical individual biomarkers cannot be definitively ascertained without randomly assigning subjects to some form of control treatment [59–61].

6.1. Microbiota and Microbiome: Friends and Enemies in Different Periods of Life, Likely with Antagonistic Pleiotropic Effects? As largely abovementioned, *microbiota and microbiome* are object of large interest in both ageing and ARD investigations, because of their crucial role in modulating diverse mechanisms and processes linked to both human health and diseases. For facilitating their understanding in the contest of this dissertation, it is well to define *microbiota and microbiome*. *Microbiota* represents an essential component of our body, constituted by a large panel of microorganisms (i.e., bacteria, virus, and mycetes) and in a number of about 3.8×10^{13} (amply quoted in [66]). It resides in different anatomical structures of the human body, organized in niches. However, it principally stays in the gut and precisely in the colon and consequently referred as *gut microbiota* (GM) [67, 68].

The term *microbiome* indicates the genome of microbiota, composed by a gene pool of about 150 times larger than that of the host (amply quoted in [66]).

GM creates a symbiotic relationship with the host (amply quoted in [66]). However, individual environmental and genetic factors can induce changes in its composition. In

the same time, the host physiology is influenced by GM, by adapting to its presence and status. For example, it is well documented its influence on the immune gut component, as well as on immune/inflammatory pathways expressed in all tissue and organs, such as Toll-like receptor pathways (amply quoted in [66]).

The GM composition is usually created in early childhood and is modulated by several factors, including geographical factors, type of delivery, breastfeeding, age of weaning, antibiotic exposure, and dietary regimens [67, 68]. Among these, the diet and geographical location have, however, a key role in modulating GM composition. In fact, they are responsible of the interindividual GM heterogeneity, as demonstrated by several studies [67, 68]. GM mature composition is precisely achieved at the age of three years. It is relatively stable during the lifespan of an individual. However, adverse events, such as moderate lifestyle changes, acute diseases, and antibiotic treatments, can induce several changes [67, 68].

As mentioned, the diet and geographical location are the principal factors, influencing the interindividual heterogeneity observed in GM composition [66–68]. For example, high-fat diets have been associated with adverse effects on GM. Precisely, they generally induce a reduction in the representation of *Bacteroidete*s and an overgrowth of *Firmicutes*, including a wide range of opportunistic pathogens. The modifications at the level of order/phylum are referred as *dysbiosis* [66–68].These changes improve gut mucosa permeability and support systemic inflammation, subclinical immune activation, and metabolic alterations towards insulin resistance [66–68]. In this context, the relative ratio between *Bacteroides* and *Prevotella* has been suggested as a biomarker of healthy and active ageing, diet, and lifestyle [51]. Differently, several studies have also recently demonstrated that *Mediterranean diet* is associated with beneficial effects on GM, including higher biodiversity, overrepresentation of *Prevotella*, and underrepresentation of opportunistic pathogens [66–68].

Dysbiosis is remarkable in old age, characterized by consequent and negative modifications in GM functionality and relative stability [66–68]. As mentioned, it is characterized by a lower *Firmicutes*/*Bacteroidetes* ratio in the elderly (aged 70–90 years) and age-related modifications in subdominant microbiota, particularly represented by an increase in facultative anaerobes, including *Streptococci*, *Staphylococci*, *Enterococci*, and *Enterobacteria* [66–68]. Antibiotic treatment, hospitalization, and *Clostridium* difficile related to diarrhea (CDAD) associated with old age also contribute to the increase of *Enterobacteria*. Furthermore, a commonly accepted ageing effect magnified by antibiotic treatment, hospitalization, and CDAD is also the decrease in *Bifidobacteria* in terms of both abundance and species diversity [66–68]. Remarkable discrepancies in the behavior of *Bifidobacteria* have, however, been evidenced with respect to ageing, recently explained by country-related differences as well as the remarkable temporal instability of the *Actinobacteria* (the phylum that includes the Bifidobacterium genus) population in the fecal microbiota of the elderly [66–68]. Age-related GM modifications seem to start after a subject-specific "threshold age" influenced, indeed, by individual characteristics, such as diet and geographical location. Age-related dysbiosis can determine not only several inflammatory gastrointestinal diseases (i.e., gastrointestinal bleeding, infections, gastric and colon cancers, and constipation) in elderly, but also it can contribute to the onset of several ARDs, such as obesity, metabolic syndrome, diabetes, CVD, Alzheimer's disease, infection, and cancer [66–68]. One of the major causes at the base of the onset of these ailments (all with a chronic inflammatory pathophysiology) is probably the lipopolysaccharide (LPS) that triggers the secretion of proinflammatory cytokines at both systemic and gut tract levels [66–68]. LPS is continuously produced in the gut through lysis of Gram-negative bacteria and absorbed into intestinal capillaries to be transported by lipoproteins. Changes in GM could, therefore, be responsible for increased endotoxemia, which in turn would trigger the development of several age-related inflammatory pathologies. Thus, dysbiosis itself represents a possible ARD cause, because it is a continuous source of antigenic stimulation (i.e., LPS), able to deregulate immunity, and contributes to immunosenescence, and consequently, it is responsible for frailty. At the same time, recent data underline that ageing is the principal cause of dysbiosis. Ageing seems relatively to have little effect on the overall gastrointestinal function but, due to the decreased adaptive capacity of gastrointestinal tract, elderly people may not recover from illnesses or injuries as quickly as young adults. Decreased adaptive capacity of gastrointestinal tract may also reduce tolerability of medications for elderly. Other age-related factors, such as changes in diet, lifestyle, and GALT immunosenescence, dramatically influence the human gut ecosystem [66–68].

The administration of probiotics and/or prebiotics to elderly is, hence, reported to induce changes in several immune and inflammatory parameters, demonstrating that manipulation of GM may result in modification of functionality of an aged immune system (amply quoted in [66]). Even though the possibility of keeping immunosenescence and inflamm-ageing under control by a simple supplementation and/or functional food is interesting, the concept of "*immuno-nutrition*" is still immature and needs to be better related to the health and immunological and nutritional status of the elderly, as well as their nationality and actual age (amply quoted in [66]). Furthermore, the eventual "improvement" in immune and inflammatory status of elderly involved in feeding trials needs to be better defined, in terms of a true health advantage. Indeed, actually only a shorter duration of common infectious diseases has been reported as a positive effect of a probiotic supplementation, but not a decrease in infection incidence. Furthermore, our considerations regarding the application of pro-/prebiotics in distinctive intestinal conditions of the elderly (i.e., constipation and CDAD) underscore the necessity to enhance the very limited clinical evidence confirming its efficacy in prevention and especially treatment of these pathologies (amply quoted in [66]).

7. Physical Activity as Another Beneficial Antiageing/Anti-Inflammation Intervention

It is well documented in literature that habitual physical activity has several advantageous effects on human health,

while its reduction has demonstrated to be one of the strong and independent factors associated with morbidity and mortality [69]. Among the beneficial effects, it is well recognized that exercise can positively modulate not only the life expectancy but also it can delay, retard, or prevent several ARDs, improving health-span for consenting likely to achieve a prolonged existence in health [70, 71]. In particular, physical activity has been demonstrated to reverse or attenuate the progression of tissue and organ ageing (i.e., the brain and cardiovascular system), since it induces positive vascular, structural, and neuromolecular changes. These last delay or retard insulin resistance, inflammation, and oxidative stress, all age-related conditions associated with vascular ageing and remodeling, CVDs, cognitive decline, and brain-related diseases [72, 73].

The beneficial or dangerous effect reciprocally mediated by active or reduced physical activity is the result of epigenetic factors [74], which modulate expression of genes codifying several pathways. For example, physical activity provokes insulin sensitivity and glucose disposal modulating the expression of pathways associated with inflammation and oxidative stress, which are high-risk factors for ageing and ARDs [75, 76]. In old individuals, independently by gender, the physical exercise reduces systemic inflammation, by decreasing the serum levels of typical inflammatory biomarkers, such as C-reactive protein, IL-6, and TNF-α (amply quoted in [77]). Accordingly, it has been demonstrated that in peripheral blood mononuclear cells from aged individuals, the exercise training reduces protein expression of TLR-2 and TLR-4 [78]. Similarly, physical exercise also seems to improve the circulating levels of endothelial progenitor cells (EPCs), cells related to both endothelial turnover, cardiovascular repair and regeneration, and adult angiogenesis [18]. Thus, physical exercise is associated with a good health of endothelium, an essential component of stroma of all tissues and organs [18]. Its dysfunction has been detected to contribute to the onset of the major number of ARDs, CVDs, and neurodegenerative diseases included, as amply described in our recent studies [13, 18].

Other accumulating data suggest that exercise is beneficial for human health in all periods of life only if it is of moderate intensity. In this case, it has health-promoting effects that are systemic and complex, undoubtedly involving regulation of redox homeostasis, inflammation, and related signaling, as described above [79]. In general, physical exercise causes an elevated generation of reactive oxygen species (ROS). ROS are important modulators of muscle contraction, antioxidant protection, and repair of damage from oxidation, all of which at moderate levels generate physiological responses. Several factors involved in mitochondrial biogenesis are modulated by exercise-associated redox changes. Certain endogenous thiol antioxidants, such as glutathione and thioredoxin, are modulated with exercise-related high oxygen consumption and ROS generation and control cellular function through redox-sensitive signaling and protein interactions. ROS may also play a role in exercise-induced angiogenesis, by inducing EPC mobilization. Also, exercise-related ROS production may be related to DNA methylation and histone modification, thereby creating heritable conditions controlled by epigenetics [79].

Based on these observations, it is possible to affirm that the regular physical activity of moderate intensity can be recommended as a beneficial antiageing/anti-inflammatory intervention. However, its effect appears to contribute to the maintenance of physiological conditions if synergically applied with nutritional interventions or other changes of lifestyle.

8. Considerations and Suggestions on the Treatments Described

Current evidence sustains a large grade of plasticity in cellular and organism ageing, and it is becoming to consider ageing as a set of pathologies. Accordingly, the manipulation of specific signaling pathways (e.g., insulin/IGF-1, mTOR, AMPK, and sirtuins) and particularly of immune inflammatory pathways, such as TLR-4, has demonstrated/is detecting the possibility to modulate both the ageing process and the onset/progression of ARDs in complex organisms (see Figures 1 and 2) [13–19]. In addition, heterochronic parabiosis experiments have elegantly demonstrated that exposing old tissues and organs to a young circulatory environment can rejuvenate tissue-specific stem cells, leading to a youthful state, which is characterized by functional and regenerative improvements [52, 54]. These encouraging data in animal models have given an optimistic hope to people in definitively achieving the *long life elixir*, considered a dream and a vanity's sin for a long time. Cotemporally, they are leading several ageing research groups to assure to obtain the same results in humans, developing several antiageing approaches, ranging from pharmacological targeting of ageing and basic biological assays to big data analysis, computational evaluations, the recent use of young blood, stem cells, cellular, genetic, and epigenetic reprogramming, or other techniques of regenerative medicine (see Figure 3) [1–8]. But, as evidenced in this brief overview on the emerging antiageing treatments, they are for the major number of more anti-inflamm-ageing and anti-ARD interventions than pure anti-ageing treatments, in agreement to current concept of ageing as disease's syndrome and the suggested Gems's definition of antiageing treatment, as stressed above [9, 10].

In addition, another crucial consideration emerges by their dissertation and their experimentation more in animal models than in humans. On the other hand, the major number of genes, molecules, and pathways associated with ageing and longevity has been detected in animal models and precisely in simple animal organisms [2–8]. As a consequence, only a small number of these genes, proteins, and pathways exist in human as homologs, but these are very rare, and consequently, our understanding on these molecules remains largely unknown [2–8]. This leads to insignificant discoveries. For example, pathways associated with longevity in simple animal models, such an IGF-1 pathway, often reveal irrelevant in human [2–8]. Consequently, the major open question is how antiageing interventions can reveal effective in humans. In order to achieve this goal, a possibility can derive from the use of big animals in the ageing experiments,

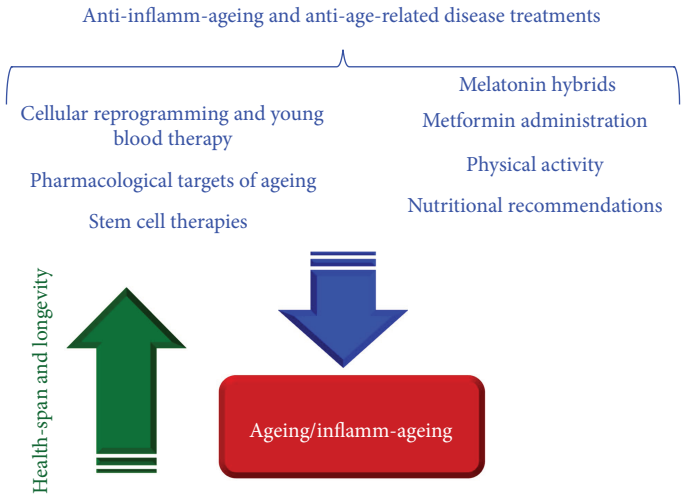

Figure 3: Anti-inflamm-ageing and anti-age-related disease treatments: described and discussed. They seem to have beneficial effects regarding the ageing/inflamm-ageing processes and reciprocally improving health-span and longevity.

including dogs [80] and primates [81]. But they show other problems for which the solutions are much less obvious. Firstly, large animal models are very, and sometimes, prohibitively expensive. In addition, they need a very intensive working setting, which requires a large and multidisciplinary staff and extended time cares. As a consequence, simple animal organisms appear until now the best animal model in ageing study, because they are relatively small, easy to handle, and not expensive. In addition, they can be genetically manipulated to mimic human diseases, by generating "knockout" or "knock-in" models and increasing their longevity in a rapidly progressing field (amply quoted in [46]).

Furthermore, most ageing-related genes and pathways have not yet been pharmacologically targeted. Computational studies might be developed, and they might help to identify and rank new modulator candidates and assemble biological insights obtained, as reviewed in our recent reviews and reported above. Such approaches are fundamental, as well as the integration of different types of investigations, as reported above. This reflects the relevant concept that each individual is the result of the sophisticated interplay between environmental factors and its genome, trascriptome, proteome, metabolome, microbiome, epigenome, and exposome [13–19]. Thus, it is necessary to perform a more complex combination of investigations based on genetic, transcriptomic, proteomic, metabolomic, microbiomic, and epigenetic evaluations, for obtaining interesting data in the study of ageing and ARDs. In addition, computational investigations are also recommended, as reported above, as well as collecting environmental and biometric data, medical/scientific/health care records. Thus, the integration of all data, obtained in this large panel of investigations, and their elaboration might lead to the development of appropriate agonists, antagonists, inhibitors of specific signaling ageing, and disease pathways, which might be used in the near future as personalized treatments for ageing and ARDs, facilitating their management and outcome [13–19]. On the other hand, this new vision of individual, as summary of environmental factors and its genome, trascriptome, proteome, metabolome, microbiome, epigenome, and exposome, also reflects the failure of the major number of antiageing interventions performed [13–19]. In fact, an interindividual variability of response among individuals, or better, among organisms enrolled in these investigations has been discovered. This, among other factors, has led to both the introduction of new branch of medicine, *the precision medicine* in order to overcome the interindividual variability in the response to therapies, and the goal to develop *personalized (tailored) therapies* [59, 64].

Thus, the capacity of scientific community to promote effective antiageing interventions in humans, which after are evaluated in clinical trials, until now remains reduced. This evidences another limitation, the long time required for the clinical validation needed to convert or translate the interventions proposed and analyzed in effective therapies and to obtain the regulatory approval. In fact, clinical trials are long and expensive, and the success of clinical trials performed until now remains very low. In line with these observations, interesting and promising might be the development of clinical trials having the goal to evaluate the effects of metformin in people without diabetes and at different ages or the consumption of foods rich in melatonin or melatonin hybrids, as well as to investigate the use of young blood therapies, senescent cell ablation, moderate physical activity, precision nutrition interventions, and nutraceuticals associated with the analysis of the composition and status of GM in everyone.

9. Concluding Remarks and Future Perspectives

Multidisciplinary approaches, as well as their integration and elaboration of big data, are fundamental in the vision to develop effective antiageing therapies and recommendations. In addition, the antiageing therapies developed and proposed until now by several biotechnology companies that appear very expensive *much lead us a large reflection*, as well as the *dishonourable tacit complicity* of doctors, laboratories, and companies in proposing palliative cares and not the desired long life elixir. The solution might be the goal of ageing and

ARD research in order to make new discoveries, although, the ways to execute still are long and difficult. On the other hand, Marcel Proust affirmed "*The real voyage of discovery consists not in seeking new landscapes, but in having new eyes.*"

Conflicts of Interest

The author declares no financial conflicts of interest.

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 2309034, 11 pages
https://doi.org/10.1155/2017/2309034

Review Article

Mitochondrial (Dys) Function in Inflammaging: Do MitomiRs Influence the Energetic, Oxidative, and Inflammatory Status of Senescent Cells?

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Received 29 September 2017; Accepted 20 November 2017; Published 27 December 2017

Academic Editor: Michal A. Rahat

A relevant feature of aging is chronic low-grade inflammation, termed inflammaging, a key process promoting the development of all major age-related diseases. Senescent cells can acquire the senescence-associated (SA) secretory phenotype (SASP), characterized by the secretion of proinflammatory factors fuelling inflammaging. Cellular senescence is also accompanied by a deep reshaping of microRNA expression and by the modulation of mitochondria activity, both master regulators of the SASP. Here, we synthesize novel findings regarding the role of mitochondria in the SASP and in the inflammaging process and propose a network linking nuclear-encoded SA-miRNAs to mitochondrial gene regulation and function in aging cells. In this conceptual structure, SA-miRNAs can translocate to mitochondria (SA-mitomiRs) and may affect the energetic, oxidative, and inflammatory status of senescent cells. We discuss the potential role of several of SA-mitomiRs (i.e., let-7b, miR-1, miR-130a-3p, miR-133a, miR-146a-5p, miR-181c-5p, and miR-378-5p), using miR-146a as a proof-of-principle model. Finally, we propose a comprehensive, metabolic, and epigenetic view of the senescence process, in order to amplify the range of possible approaches to target inflammaging, with the ultimate goal of decelerating the aging rate, postponing or blunting the development of age-related diseases.

1. Introduction

Aging constitutes the major risk factor for a variety of diseases with a high-incidence in the Western society. Type 2 diabetes (T2DM), metabolic syndrome, cardiovascular diseases (CVD), neurodegenerative diseases, and a plethora of common cancer types share aging as a common risk factor and therefore are often referred as aging-related diseases (ARDs). A pervasive feature of aging is a progressive and chronic state of systemic low-grade inflammation referred as inflammaging [1]. Major ARDs all share a common inflammatory background. The role of various inflammatory molecules—mainly tumor necrosis factor- (TNF-) α, interleukin- (IL-) 1b, IL-6, and transforming growth factor- (TGF-) β—in the promotion or exacerbation of a wide range of ARDs is increasingly emerging [1]. A number of mechanisms, pathways, and cell types have been shown to possibly contribute to inflammaging [2]. Initial hypothesis affirmed that inflammaging was mainly due to the long-lasting exposure to acute and chronic infections and the consequent life-long antigenic burden [3]. However, a long list of sterile potential sources of inflammatory molecules during both the cellular and the organismal aging process has been proposed [2].

The "old" free radical theory of aging (FRTA) states that organisms age because cells accumulate free radical-induced damage over time [4]. One interesting view that coupled FRTA with inflammaging is the oxi-inflammaging theory [5]. Accordingly, excessive or uncontrolled free radical production can induce an inflammatory response, and free radicals are themselves inflammation effectors. Oxidative metabolism at mitochondrial level is probably the major source of intracellular ROS production, which in turn represents a life-long-lasting stress. Aging-related ROS accumulation can contribute to telomere attrition [6], oxidative genomic damage [7], but can even act as signaling molecules in the development and maintenance of the senescent phenotype of the cell [8].

However, increasing evidence suggests oxidative stress is not per se a major determinant of aging; in fact, (i) deletion of many antioxidant enzymes increases rather than decreases life span and health span in various models of lower organisms and (ii) interventions with nonspecific antioxidant molecules do not reduce the incidence of ARDs [9–12]. On the contrary, (excessive) inflammation appears as a phenomenon that alone is sufficient to reduce both life span and fitness at old age [13, 14]. Thus, in aging cells, mitochondria-derived ROS and oxidative stress should be regarded for their role as proinflammatory triggers rather than damaging molecules that progressively disrupt cell components and cellular homeostasis alone.

MicroRNAs (miRNAs o miRs) are small noncoding RNAs involved in gene expression modulation, primarily silencing the mRNA target by binding its 3′-untranslated region (UTR) in the cytoplasm. However, increasing evidence proves that miRNAs can also exert posttranscriptional control when bound to a region outside of the 3′UTR and specifically within the 5′UTR and coding regions of the mRNA target. Moreover, in some cases, they activate transcription of a specific gene or stabilize the mRNA [15]. A single miRNA has the ability to regulate multiple targets and, in turn, a single mRNA can be targeted by several miRs [16]. Because of their regulatory functions, these small single strand RNAs are virtually implicated in all cellular processes. Over the years, a set of miRNAs with a well-recognized role in inflammaging, organismal aging, and development of ARDs has been defined [17–19]; examples are miR-146a-5p, miR-21, miR-126a-3p. Interestingly, recent studies suggest that a subset of these microRNAs can be found inside or indirectly affect the mitochondria (mitomiRs) [20]. They all have a nuclear encoding, but the existence of few small noncoding RNAs of mitochondrial origin is documented [21].

In this review, we explore the role of mitochondria in fostering "inappropriate" inflammatory responses during the aging process. We take advantage of cellular senescence as a fractal model to delineate the contribution of dysfunctional mitochondria to the inflammaging process since recent discoveries indicate that mitochondrial activities sustain or drive the inflammatory program of senescent cells. In addition, we explore mitochondria-linked mechanisms that can promote inflammaging independently of senescent cells. Finally, we explain the hypothesis that senescence-deregulated mitomiRs could directly affect mitochondrial function by targeting mtDNA, thus influencing the energetic, oxidative, and, in turn, inflammatory status of senescent cells, possibly playing a role in organismal aging.

2. Senescent Cells Fuel Inflammaging through the Senescence-Associated Secretory Phenotype

Recently, cellular senescence has been suggested as a relevant contributor to both the inflammaging and the aging process. This statement is mainly sustained by data on animal models showing that periodic clearance of senescent cells is accompanied by a mean life span and health span extension, coupled with a reduced inflammatory gene expression in multiple tissues, including kidney and the heart [22]. Senescent cells are characterized by a permanent cell cycle arrest, accompanied by morphological and gene expression changes [23]. They are usually identified by the high expression of proteins promoting cell cycle arrest, that is, p16, p21, and p53, the expression of DNA damage markers, that is, γH2AX phosphorylation, senescence-associated heterochromatin foci (SAHF), and telomere-associated DNA damage foci (TAF), and an increased activity of senescence-associated (SA) beta-galactosidase (β-Gal) [24]. Many stimuli can foster the acquisition of the senescent phenotype, for example, radiation, telomere erosion or damage, oncogenic stress, oxidative stress, and a number of DNA damaging agents [24–26]. In turn, senescent cells can acquire a proinflammatory phenotype named senescence-associated secretory phenotype (SASP). SASP consists in secretion of inflammatory cytokines, growth factors, and proteases [24]. Increased levels of SASP-related compounds have been reported in a number of human ARDs such as diabetes [27–29], atherosclerosis [30], and cancer [31]. More importantly, senescent cell accumulation in selected microenvironments—with a subsequent locally increased concentration of inflammatory cytokines—drives the pathogenesis of prototypical ARDs, that is, osteoarthritis and osteoporosis [32, 33].

A plethora of pathways and proteins has been implicated with inflammatory molecule secretion in senescent cells, for example, NF-κB, mTOR, and JAK [27, 34]. The classical SASP network relies on cell surface IL-1α as an essential cell-autonomous regulator of IL-6/IL-8 secretion [35]. However, recent findings indicate that an alternative inflammatory network is possible in senescent cells and that it is strictly dependent on mitochondrial function [36]. In addition, mitochondria appear as indispensable for the proinflammatory and the proaging features of senescent cells [37].

3. Senescent Cells Bear a Distinct Metabolic Phenotype, and Mitochondrial Dysfunction Is Partly Responsible for Their Proinflammatory Program

The senescence process is accompanied by metabolic changes within the cell. Many of the proteins important for the senescence process have a pivotal metabolic function

[38]. As a result, senescent cells show a distinct metabolic phenotype. They bear an active metabolic state, maybe due to the acquisition of their peculiar secretory profile, which implies a high transcriptional activity and possibly a high metabolic demand. Senescent cells increase their glucose consumption, a phenomenon not coupled by an increased energetic state. In fact, senescent cells present a strong reduction in the amount of ATP in favor of adenosine diphosphate (ADP) and adenosine monophosphate (AMP) [39]. The increases of AMP/ATP and ADP/ATP ratios are sensed by and activate AMP-activated protein kinase (AMPK). [40]. AMPK activation, in turn, can induce senescence via p53 phosphorylation [41], whose activation has been associated with the promotion of glycolysis and oxidative phosphorylation, but even to downmodulation of glycolysis [42]. Furthermore, mTOR, one of the master regulators of the SASP but even of the senescence process itself [43], is strictly linked to nutrient availability, in particular to amino acids and glucose [44]. The same nutrients promote the activation of the transcription factor NF-κB, which plays a pivotal role in the inflammatory program of both immune cells and senescent cells. NF-κB governs energy homeostasis and metabolic adaptation by controlling the balance between glycolysis and respiration for energy provision [45]. Inhibition of NF-κB diminishes oxygen consumption and causes reprogramming to aerobic glycolysis (i.e., the Warburg effect) in mouse embryonic fibroblasts (MEFs) under basal culture conditions and induced necrosis on glucose starvation [45]. This NF-κB-dependent modulation of oxidative phosphorylation system (OXPHOS) involves the p53-mediated upregulation of mitochondrial synthesis of cytochrome c oxidase 2 (SCO2) [46], a subunit of complex IV of the mitochondrial electron transport chain [46–48].

The OXPHOS is composed of complexes I-IV (the electron transport chain, ETC), located within the mitochondrial inner membrane, and generates a gradient of H^+ ions, which drives the ADP phosphorylation via the ATP synthase (F_oF_1 ATPase—complex V) [49, 50]. During aerobic respiration, a variable percentage of electrons leaks from the ETC, particularly from complexes I and III, prematurely reduces oxygen, and generates ROS [51]. This process is exacerbated in senescent cells and leads to an overproduction of ROS.

In "usual" senescence models, mitochondria do play a fundamental role. In fact, the absence of mitochondria reduced a spectrum of senescence effectors and phenotypes while preserving ATP production via enhanced glycolysis. A plethora of senescent-associated changes was dependent on mitochondria, particularly the proinflammatory phenotype. A DNA damage response (DDR) pathway converging on mTORC1 phosphorylation promoted PGC-1β-dependent mitochondrial biogenesis, contributing to ROS-mediated activation of the DDR and cell cycle arrest. Of note, the reduction in mitochondrial content *in vivo*, by either mTORC1 inhibition or PGC-1β deletion, prevented senescence in the aging mouse liver [37].

A recent paper clearly shows that mitochondrial dysfunction per se is sufficient to trigger a particular form of senescence accompanied by a peculiar proinflammatory program but independent from ROS and DNA damage. In this model, mitochondrial dysfunction-associated senescence (MiDAS) lacks an IL-1/NF-κB-dependent mechanism but would involve secretion of factors other than "classical" SASP factors. Major MiDAS-associated molecules are IL-10, TNF-α, and CCL27, which are secreted in a NF-κB-independent manner. This novel senescence phenotype would result from a reduced NAD^+/NADH ratio, which in turn may cause AMPK and p53 activation [36]. Of note, treatment with a NAD^+ precursor delays senescence of neural and melanocyte stem cells, increasing mouse life span [52].

4. Mitochondrial Dysfunction and Damage Promote Inflammaging through ROS- and PRR-Mediated Mechanisms

Life-long stimulation of the cells of innate immunity is still one of the more consistent hypotheses regarding the cellular origin of inflammaging. Beyond bacteria- and virus-derived products, a long list of endogenous molecules can promote this inflammatory program [2]. When misplaced, many "normal" cell components are recognized as damage-associated molecular patterns (DAMPs), which are recognized by pattern recognition receptors (PRRs), a large family of proteins that include toll-like receptors (TLRs) and NOD-like receptors (NLRs). During aging, PRRs are subjected to a prolonged and increased exposure to "stress" molecules that leads to chronic inflammatory responses.

Cell injury due to various stressors can trigger the release outside of the mitochondrial matrix of mitochondrial DNA (mtDNA), *N*-formyl peptides and lipids, such as cardiolipin, that can act as DAMPs by activating receptors of the innate immunity [2, 53] and the inflammasomes. In particular, there is a direct link between released oxidized mtDNA, activation of NLRP3, the most studied NLR, and caspase-1-mediated maturation of IL-1β and IL-18 [54, 55]. Accordingly, increasing evidence suggests an important role of inflammasomes in major ARDs [27]. For instance, the ablation of Nlrp3 gene in mice is able to attenuate inflammaging, thus increasing health span [13]. Once activated, inflammasome promotes the transmission of the senescence bystander cells via the release of inflammatory mediators [56]. In turn, mitochondria-derived ROS can activate the inflammasome, probably through the Trx/TXNIP complex, further linking mitochondrial function to low-grade inflammation [57]. Accordingly, ROS themselves can partly mediate the senescence "bystander effect", that is, the transition of the senescent phenotype from a SC to neighbor cells [58].

MtDNA can even bind TLR9, which senses DNA of bacterial and viral origin [59]. Because of the prokaryotic origin of mitochondria, mtDNA contains a significant number of unmethylated CpG DNA repeats, similarly to bacterial genomes. This is sufficient to trigger TLR9 activation, leading to NF-κB signaling and increased expression of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β [60]. Further, mtDNA can even activate the cytosolic DNA sensor cyclic GMP-AMP synthase (cGAS) [61]. During an infection, DNA of microbial origin by binding to cGAS activates the cGAS/stimulator of interferon gene (STING) pathway thus

triggering expression of inflammatory genes. Since DNA damage plays a prominent role in the development of the SASP [62], this pathway could be considered a mediator of inflammaging. Of note, even mitochondrial DNA (mtDNA) can bind the cytosolic cGAS, suggesting that mitochondria can play a major role in the senescence/SASP process in different manners [63, 64]. Notably, mtDNA can be released into extracellular fluids as a product of tissue damage and its plasma levels increase during aging and in selected ARDs [65], supporting the notion that circulating mtDNA is part of the systemic communicome initiating and propagating inflammaging [2].

Human mtDNA consists of circular, double-stranded, supercoiled molecules that are found in each cell in one up to 10,000 copies, depending on the bioenergetic requirements of the tissue and whose number declined with aging [66]. It encodes 13 subunits of respiratory chain complexes (in addition to 2 rRNAs and 22 tRNAs), which are transcribed and translated directly within the mitochondrion by its own gene expression system. These polypeptides include indispensable components of the complex I (7 subunits—ND1-ND6, ND4L), III (apocytochromeb), IV (3 subunits—COX I–III), and of F0/F1 ATP synthase (ATPase6 and ATPase8). The mitochondrial genome has a higher mutation rate than the nuclear genome; thus, its mutations accumulate during aging [67]. However, Greaves et al. have shown that selected pathogenic mutations increase and clonally expand during aging, but not the frequency of mutation [68]. Of note, mtDNA mutation accumulation in mouse tissues is influenced by the nuclear genetic background and correlates to neither cellular ROS content nor tissue senescence [69]. Interestingly, a number of genetic variants of the mtDNA, named haplogroups, have been identified. These, each containing functional single nucleotide polymorphism (SNPs), affect the oxidative metabolism of mitochondria, and several of them are associated to extreme longevity [70]. The interaction between nuclear genes and mtDNA has been proposed as a possible mechanism explaining a favorable/unfavorable inflammaging profile and consequently successful or unsuccessful aging [71]. Overall, these data suggest that any physiological SNP or pathogenic mutation in mtDNA could induce respiration alteration with a wide range of possible resulting (pathological) phenotype, affecting in turn inflammaging and ARD development [72, 73].

5. A Subgroup of MicroRNAs Targets mtDNA or Nuclear mRNA with Mitochondrial Function

The miRNA machinery is known to primarily act in the cytoplasm. miRNAs have also been detected in membrane-bound compartments, such as secreted vesicles and mitochondria [74]. Through different experimental approaches in different mammalian species, a number of studies have enabled identification of "signatures" of miRNAs located in the mitochondria. To date, they are summarized under a descriptive term: mitomiRs [75]. Indeed, this name includes nuclear-encoded miRNAs that translocate into the mitochondria organelle and target either mitochondrial or nuclear mRNA. Early studies on the subject are mostly descriptive to detect the presence of microRNAs within the mitochondrion. Kren and colleagues first detected 15 nuclear-encoded mitomiRs from rat livers that seemed to be involved in the modulation of genes associated with apoptosis, cell proliferation, and differentiation [76]. Bian et al. identified a pool of 20 miRs highly expressed in mitochondria of mice liver. Interestingly, mitochondria have a unique population of miRNAs, independent of the total cellular abundance of miRs and they may be involved in the regulation of mitochondria-specific and general cellular functions [77]. For the first time, in 2011, Barrey and coworkers showed the presence of pre-miRs inside mitochondria, postulating that some pre-miRNA sequences could be processed to mature miRNAs, which could be immediately active on the mitochondrial transcripts or exported in the cytosol in order to interfere with genomic mRNA [78]. Subsequently, other groups identified new mitomiRs from HeLa [79, 80], HEK293 [80], 143B cells [81], 206ρ° cells [82], and mouse heart [83] (Tables 1 and 2). The notion emerging is that mitochondria have a discrete and unique pool of mitomiRs; the association of miRs with mitochondria is species and cell type-specific [75].

6. MitomiRs Targeting Mitochondrial Genome Are Deregulated by Organismal Aging or Cellular Senescence

Mitochondria maintain and express their own genome that may be regulated by microRNAs. As mtDNA encodes for 13 subunits of the electron transport chain, their regulation by mitomiRs may have profound effects on ATP synthesis and ultimately may influence the whole mitochondrial function.

Bioinformatics analysis suggested that mitomiRs can target various mitochondrial transcripts: several potential mitochondrial targets for let-7b (ATP6, ATP8, COX2, and ND5) [78] as well as for miR-146a-5p and miR-181c-5p [82] have been described; miR-133a was predicted to target ND1 [78], whereas miR-130a-3p targets COX3 [76]. Using ingenuity pathway analysis (IPA) software to analyze mtDNA targets, Jagannathan et al. observed potential interactions between all 13 mitochondrial genome-encoded electron transport chain proteins with mitomiRs. The functional significance of a specific miRNA in heart-derived mitochondria was demonstrated for the first time by Das and colleagues: miR-181c-5p originates from the nuclear genome, is processed in the cytosol, and translates to the mitochondria, where it regulates mitochondrial energy metabolism by targeting mt-COX1 mRNA. Overexpression of miR-181c-5p results in a loss of mt-COX1 protein, resulting in an imbalance among the mitochondria-encoded subunits in complex IV, thus promoting ROS generation. Perturbations induced by miR-181c-5p could have important consequences in myocardial pathophysiology [84]. More recently, *in vivo* administration of miR-181c-5p in rats confirmed these data leading to complex IV dysfunction, altered mitochondrial metabolism, and ROS generation, ultimately promoting heart failure [85].

Table 1: List of miRs within human mitochondria.

Barrey et al. [78] Human skeletal/muscular cells		Bandiera et al. [20] HeLa cells	Sripada et al. [80] HeLa/HEK293	Zhang et al. [60] Skeletal muscle	Mercer et al. [81] 143B cells	Dasgupta et al. [82] 206 ρ° cells
let-7b	miR-193b	miR-328-5p	let-7b-5p	miR-1	miR-16	miR-181c
let-7g	miR-197	miR-494-3p	let-7g-5p		miR-146a	mR-146a
miR-19b	miR-199a-5p	miR-513a-5p	miR-107		miR-103	
miR-20a	miR-210	miR-638	miR-181a-5p			
miR-23a	miR-221	miR-1201	miR-221-5p			
miR-23b	miR-324-3p	miR-1246	miR-320a			
miR-24	miR-324-5p	miR-1275	miR-494-3p			
miR-34a	miR-365	miR-1908	miR-1275			
miR-92a	miR-423-3p	miR-1972	miR-1973			
miR-93	miR-484	miR-1973				
miR-103	miR-486-5p	miR-1974				
miR-106a	miR-490-3p	miR-1977				
miR-107	miR-503	miR-1798				
miR-125b	miR-501-3p					
miR-125a-5p	miR-532-3p					
miR-127-3p	miR-542-5p					
miR-133b	miR-574-3p					
miR-133a	miR-598					
miR-134	miR-720					
miR-149	miR-1974					
miR-151-5p	miR-1979					
miR-181a	miR-675*					

On the contrary, when miR-1 localizes into mitochondria, it promotes mitochondrial translation of ND1 and COX1 mtDNA-encoded transcripts while repressing its nuclear DNA-encoded targets in the cytoplasm. In this way, miR-1 enhances protein synthesis and ATP production, required for muscle cell differentiation [74]. This study suggests the possibility that the localization and relative abundance of a specific microRNA in the cytoplasm and mitochondria determines its different role in the regulation of specific targets and in the coordination of mitochondrial activities [86].

Another study showed that miR-1, together with other two mitomiRs, miR-133a and let-7b, is essential for adult skeletal muscle differentiation and maintenance; for this reason, they are called myomiRs [87, 88]. Muscles contain a high mitochondrial content, in order to provide massive demand of ATP for movement, postural maintenance, and respiration. During aging, skeletal muscle mitochondria revealed a progressive decline in respiratory chain function, a phenomenon associated with insulin resistance and type 2 diabetes mellitus [89]. These miRs seem to modulate some key proteins of OXPHOS complexes, so their deregulation may affect ETC functionality. Of note, some of these myomiRs are released in circulation after physical exercise [90]. Physical activity is associated to a plethora of beneficial effects and the underlying mechanisms are not fully understood. For instance, high-intensity aerobic interval exercise is associated with wide proteomic changes in the muscle of old subjects, with evident phenotypic gains in muscle mitochondrial function. Noticeable, mRNA expression changes do not overlap protein expression changes, suggesting that enhanced protein translation, but possibly even epigenetic mechanisms influence such positive adaptation [91].

Another remarkable study showed a redistribution of mitomiRs in the diabetic heart. Cardiac tissue is rich in mitochondria with spatially distinct subpopulations: subsarcolemmal mitochondria (SSM), located beneath the cell membrane, and interfibrillar mitochondria (IFM), situated between the myofibrils. MitomiRs were differentially regulated in the two mitochondrial subpopulations in diabetes relative to control. In addition, mitomiR-378-3p is highly expressed in diabetic IFM (versus IFM control) and targets mt-ATP6 with a concomitant reduction in the functionality of the ATP synthase. *In vivo*, miR-378-3p antagomir delivery led to the preservation of ATP6 protein levels in diabetic IFM, similar to nondiabetic control. Interestingly, miR-378-3p antagomir resulted in an increase of ATP synthase activity, which was significantly decreased in diabetic [83]. MiR-378-3p is located in the first intron of the peroxisome proliferator-activated receptor gamma (Ppargc1b) gene, which encodes for PGC-1β. PGC-1β is preferentially expressed in tissues with relatively high mitochondrial content, and miR-378-3p is coexpressed with its host gene and seems to counterbalance the metabolic actions of PGC-1β. Mice genetically lacking miR-378-3p exhibit enhanced

Table 2: List of miRs within mouse and rat mitochondria.

Bian et al. [77] Mouse liver	Jagannathan et al. [83] Mouse heart					Kren et al. [76] Rat liver	Das et al. [84] Rat cardiomyocytes
let-7f-5p	let-7b	miR-151-3p	miR-1934-3p	miR-130a	miR-574-5p	miR-130a-3p	miR-181c
miR-101-5p	let-7a	miR-203-3p	miR-211-3p	miR-497	miR-148a-3p	miR-130b-3p	
miR-122-5p	let-7c	miR-212-3p	miR-3072-3p	miR-188-5p	miR-200c-3p	miR-140-5p	
miR-181b-5p	let-7f	miR-5112	miR-320-3p	miR-3098-5p	miR-300-3p	miR-320-3p	
miR-181d-5p	miR-149-3p	miR-135a-1-3p	miR-1199-5p	miR-30c-1-3p	miR-181b-5p	miR-494-3p	
miR-188-5p	miR-149-5p	miR-721	miR-5108	miR-712	miR-5131	miR-671	
miR-29a-3p	miR-23b	miR-125a-3p	miR-375-3p	miR-3102-5p			
miR-29c-3p	miR-1	miR-1904	miR-203-3p	miR-877-3p			
miR-361-5p	miR-29a	miR-1894-3p	miR-126-3p	miR-3963			
miR-432	miR-125b-5p	miR-3102-5p	miR-26a	miR-341-3p			
miR-494-3p	miR-29b	miR-494	miR-23a	miR-342-3p			
miR-680	miR-709	miR-1939	miR-27b	miR-423-3p			
miR-689	miR-22	miR-3470a	miR-99a	miR-3081-5p			
miR-690	miR-24	miR-144	miR-139-3p	miR-1895			
miR-705	miR-680	miR-3107	miR-378	miR-720			
miR-711	miR-21	miR-451	miR-27a	miR-1897-5p			
miR-721	miR-133a-3p	miR-1224	miR-29c	miR-3085-3p			
miR-720	miR-133a-5p	miR-2861	miR-30a	miR-3092			
miR-762	miR-133b	miR-2137	miR-30d	miR-2145			
miR-805	miR-128-3p	miR-1937c	miR-30e	miR-652-5p			
miR-671-5p	miR-3095-3p	miR-466i-5p	miR-3082-5p	miR-1187			
miR-1982-5p	miR-1937b	miR-705	miR-483-5p	miR-466h-3p			

mitochondrial fatty acid metabolism and elevated oxidative capacity of insulin-target tissues [92], proving its key role in mitochondrial respiration.

According to their role in age-related diseases, for example, heart failure and diabetes [18–20, 74, 85, 93], many mitomiRs have been described to be deregulated during organismal and cellular aging (SA-miRs). Thus, it has been hypothesized that SA-miRs and mtDNA-targeting mitomiRs play a major role in the mitochondrial dysfunction observed in both cellular senescence and the inflammaging process [94]. Notably, among all mitomiRs targeting mitochondrial genome, a subset of miRs has been associated to inflammatory processes, that is, miR-130a-3p [95] or to both inflammation and aging, that is, let-7b [96, 97], miR-146a-5p [98], miR-181c-5p [99, 100], miR-133a [101, 102], and miR-1 [103, 104].

MiR-146a-5p is one of the best-characterized miRNAs involved in both SASP and inflammaging. NF-κB activation initiates the transcription of proinflammatory cytokines and of miR-146, which in turn directly targets IRAK1 and TRAF6, two key adaptor molecules in the TLR/NF-κB pathway, trying to switch off the proinflammatory signal at the end of inflammatory response. While this mechanism efficiently regulates immune system responses [105], in senescent cells, its increase is insufficient to ameliorate the SASP [93, 106, 107]. As each miRNA has multiple targets, sustained and chronic expression of miR-146a-5p in senescent cells could affect many pathways other than the proinflammatory ones. In fact, mir-146a-5p has the mitochondrial encoded proteins ND1, ND2, ND4, ND5, and ND6, as well as ATP8 as putative and potential targets, suggesting that senescence may affect epigenetically the expression and function of complexes I and V. Of note, complex I is the most relevant one in determining ROS production in dysfunctional mitochondria [108]. In addition, miR-146a-5p can even target superoxide dismutase- (SOD-) 2, a major mitochondrial antioxidant enzyme [109] and Bcl-2, a known determinant of mitochondrial dynamics, involved in the regulation of mitochondrial fusion and fission [94]. Thus, it is easy to speculate that senescence-regulated miRNAs, which are transcribed with the goal of ameliorating inflammation, could also influence mitochondrial behavior, with a still unknown positive or negative effect on mitochondrial oxidative and energetic functions.

7. Conclusions and Future Prospects

Recent discoveries suggest that mitochondria are major determinants of aging. Senescent cells carry dysfunctional mitochondria, which are partly responsible for their proinflammatory program. Aged, dysfunctional, or damaged mitochondria can promote inflammaging even through continuous immune system stimulation. In the complex picture of the dynamic interaction between nucleus and mitochondria, microRNAs should be considered as new, relevant players. Despite the few data available on this specific topic,

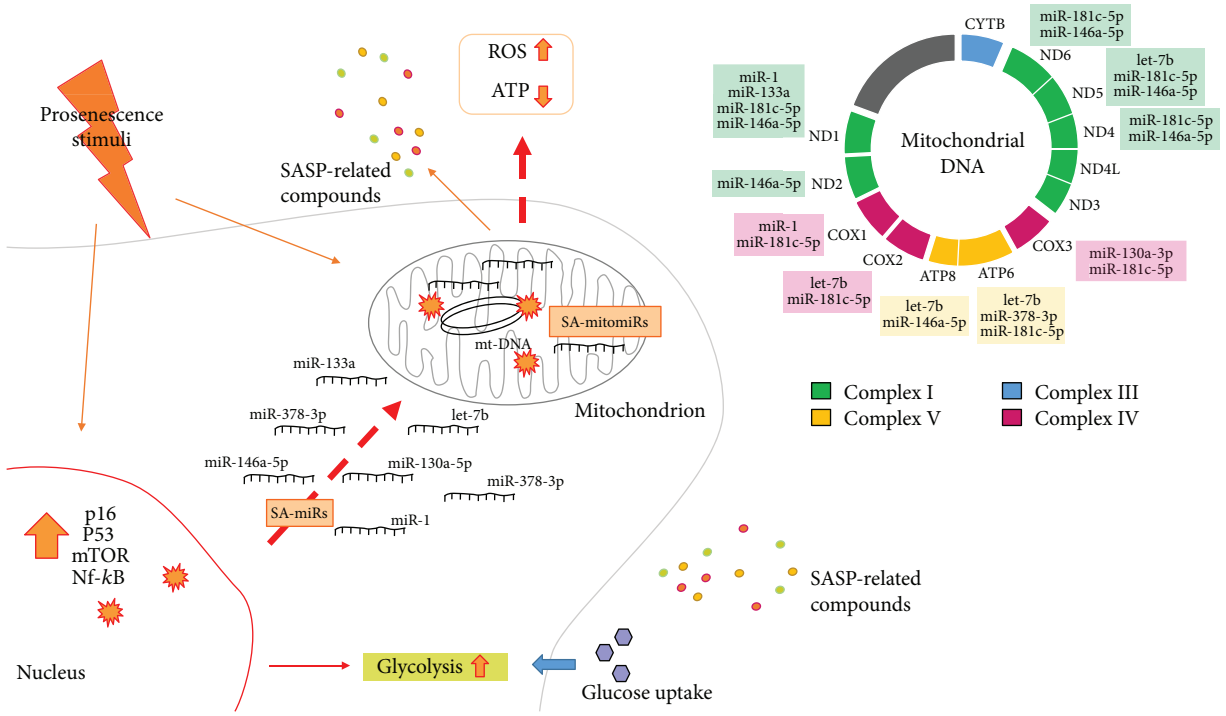

Figure 1: Potential effect of senescence-associated epigenetic rearrangement on mitochondrial function. Different damaging stimuli can induce senescence. Senescent cells bear peculiar metabolism and gene expression, which underlie a chronic proinflammatory program (SASP). This is accompanied by changes in the expression of a number of senescence-associated microRNAs (SA-miRNAs). Dysfunctional mitochondria play a major role in the promotion of the SASP. In turn, several SA-miRNAs can translocate to mitochondria (mitomiRs) and could target a plethora of mRNA with an important role within mitochondria, including transcripts derived from mitochondrial DNA (mtDNA). In this framework, we hypothesize a major role for SA-mitomiRs in determining the energetic, metabolic, and inflammatory status of senescent cells, mainly through their ability to regulate mtDNA-derived proteins.

emerging evidence and bioinformatics studies indicate that miRNAs of nuclear origin profoundly affect mitochondrial function. This phenomenon seems to be of particular importance for the aging process, considering that many senescence-associated miRNAs have got or may have targets within mtDNA. Overall, a comprehensive view of the epigenetic and nonepigenetic mechanisms triggered by the senescence process is of importance to understand the overall phenotype of aged cells, which behave differently from a transcriptional, metabolic, energetic, and oxidative point of view (Figure 1). Such holistic view could eventually extend the spectrum of possibilities to target senescent cells and, more in general, the aging process, especially considering that miRNA-based, epigenetic therapies are already in progress in order to functionally reprogram target cells to a desired phenotype.

Cutting-edge technologies such as ultradeep sequencing and single-cell RNAseq will help to disentangle the importance of SA-mitomiRs in the aging process at both cellular and population level thus accumulating knowledge to better define dynamics underlying the epigenetic regulation of mitochondrial function in healthy aging and major ARDs.

Conflicts of Interest

The authors have no conflicts of interests.

Authors' Contributions

All authors contributed significantly to the manuscript.

Acknowledgments

This work was supported by grants from UNIVPM to MRR and from Fundaciò La Maratò de TV3 to AC.

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 3868545, 12 pages
https://doi.org/10.1155/2017/3868545

Research Article

Gene Expression, Oxidative Stress, and Senescence of Primary Coronary Endothelial Cells Exposed to Postprandial Serum of Healthy Adult and Elderly Volunteers after Oven-Cooked Meat Meals

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Received 17 September 2017; Accepted 1 November 2017; Published 12 December 2017

Academic Editor: Francesco Prattichizzo

Epidemiological studies have linked high consumption of meat with major age-related diseases including cardiovascular diseases. Abnormal postprandial increases in plasma lipids after a meat meal have been hypothesized among the pathogenetic mechanisms. However, it is still unknown if the postprandial serum derived after a normal meat meal is able to affect endothelial function, and if the type of meat and the age of the donors are critical factors. Here, we show the effects of postprandial sera derived from healthy adults and elderly volunteers who consumed meat meals on human coronary artery endothelial cell (HCAEC) oxidative stress, gene expression, DNA damage, and cellular senescence. We observed that a single exposure to postprandial serum induces a slight increase in ROS that is associated with modulation of gene expression pathways related to oxidative stress response and metabolism. The postprandial-induced increase in ROS is not associated with a measurable DNA oxidative damage. However, repeated exposure to postprandial serum induces an acceleration of cellular senescence. Taking into account the deleterious role of cellular senescence in age-related vascular diseases, the results suggest a new mechanism by which excessive meat consumption and time spent in postprandial state may affect health status during aging.

1. Introduction

Many epidemiological studies have linked high consumption of processed meat, particularly processed red meat with cardiovascular disease (CVD) [1], stroke [2], diabetes [3], and colorectal cancer [4]. This is partly attributed to the generation of oxidation products during thermal processing of meat [5], as well as to the proinflammatory and prooxidative condition of the postprandial state [6]. Postprandial elevated triglycerides have been reported to impair endothelial function [7] and exert proapoptotic effects on endothelial cells [8]. Gene expression profiling of endothelial cells

exposed to hyperlipaemic postprandial sera from healthy volunteers after a high-fat challenge showed increased transcription of genes involved in growth arrest and apoptosis [9]. However, it is still unknown if the postprandial serum derived after a normal meat meal is able to affect endothelial function in a way similar to that shown after a high-fat challenge. Exposure of normal human coronary artery endothelial cells (HCAEC) to postprandial sera of healthy volunteers after a meat meal which includes commonly consumed drinks, side dishes, and spices [10] can provide a model to mimic the physiological impact of postprandial state on the endothelial cell [11]. Most importantly, the type and quality of the meat and other components of the meal as well as age and health status of the individual consuming the food can alter the characteristics of the postprandial response during a normal meal [12–14]. Even given that a normal meal may provide a lower physiological impact (e.g., postprandial lipid increase) compared to a fat challenge, the postprandial status may induce subcytotoxic stress that is detrimental on the long run. For example, models of repeated short exposure to subcytotoxic stressors (UV, hyperoxia, hydrogen peroxide, etc.) display cellular senescence as a common fate [15, 16]. Here, we report the effects of postprandial sera derived from healthy adult and elderly volunteers who consumed meals based on various type of meats on HCAEC oxidative stress, DNA damage, and cellular senescence while providing mechanistic insights by gene expression analysis.

2. Material and Methods

2.1. Study Design. The study group consisted of six adult (age range 26–51 years) and six elderly (age range 66–73 years), healthy normolipemic, nonsmoking males. All volunteers had normal physical examinations without any medical history of digestive, renal, cardiovascular, endocrine, or chronic diseases. The physical characteristics of the adult subjects were (mean ± SD) age (years) 39.3 ± 11.2, body weight (kg) 81.3 ± 4.9, and BMI (kg/m^2) 24.8 ± 2.4. The physical characteristics of the elderly subjects were (mean ± SD) age (years) 68.7 ± 3.4, body weight (kg) 72.7 ± 6.71, and BMI (kg/m^2) 22.3 ± 1.4. The purpose and potential risks of the study were explained to all subjects, and their written consent was obtained before participation. The study was carried out after approval by the ethics committee of INRCA.

Volunteers were instructed to consume each Tuesday and Thursday of the week a specific meal from 3 different types: (1) a pork meat- (PM-) based meal, (2) a rabbit meat- (RM-) based meal, and (3) a chicken meal- (CM-) based meal. All the food material was weighted and delivered to the home of each volunteer the day before consumption. Volunteers were also instructed to not change their weekly dietary habits for the whole length of the study (75 days, approximately 10.5 weeks were needed to complete all meals for all subjects) and to follow specific cooking instructions for the experimental meals. The meals were designed to accomplish the common Italian tradition to accompany oven-cooked meats with roasted potatoes and to use condiments such as rosemary, salt, and extra virgin olive oil. The main food material consisted of 200 g (all edible) of pork chop (PM meal) or a 250–350 g (approx. 60%–70% edible) of chicken quarter (CM meal) or 400 g (approx. 50% edible) of rabbit pieces (RM meal) obtained from local producers. The meal included also 5 ml (4 g) of extra virgin olive oil, 200 g of potatoes (carbohydrates 17.5%, protein 2%, and moisture 79%), 0.5 g of rosemary, and 2 g of salt (commercial products from a local market). All condiments were added to the meat and cooked in an oven at 180–200°C for 45–60 min, with the last 5–10 min of grill. The range of temperature and cooking time was established in order to meet the individual preferences of the volunteers. Drinks consisted of water ad libitum and a half glass of red wine (150 ml, 12.5% alcohol). Fresh pork chops showed the following proximate composition (g/100 g meat): protein 19.2, total fat 11.4, moisture 68.0, and ash 1.4. Rabbit pieces showed the following proximate composition: protein 22.0, total fat 2.4, moisture 74.3, and ash 1.3. Chicken meat showed the following mean composition: protein 23.6, total fat 2.5, moisture 72.6, and ash 1.3. The total caloric intake was 669, 521 and 536 Kcal for the pork, rabbit, and chicken meals, respectively.

2.2. Serum Collection. Fasting serum (FS) was obtained after an overnight fast of at least 10 h. Blood was withdrawn in the morning in order to obtain samples of fasting serum. Blood samples were collected into 7 ml Vacutainer serum tubes and placed 1 h at room temperature. After centrifugation, the serum was collected, divided into aliquots, and immediately frozen at −80°C. Fasting serum was pooled following the same strategy used for postprandial serum.

Postprandial serum was collected 4 hours after the meal from each volunteer. In order to ensure an adequate amount of postprandial serum for the experiments, adult volunteers consumed 5 meals of each type (for a total of 30 postprandial serum samples from each type of meal) and elderly volunteers consumed 2 meals of each type (for a total of 12 postprandial serum samples from each type of meal). After the establishment of individual serum lipid profiles, serum samples obtained from each meal were pooled (separately for adult and elderly volunteers) in order to dispose of 6 (elderly) or 10 (adult) pooled samples to be used in cell experiments (as described in Scheme 1 of the Supplementary Material).

2.3. Cell Culture and Proliferation. HCAECs (human coronary artery endothelial cells) were purchased from Clonetics Corporation (Lonza) and cultured in endothelial basal medium EBM, supplemented with EGM-2 or EGM-2/MV SingleQuots containing 0.1% rh-EGF (human recombinant epidermal growth factor), 0.04% hydrocortisone, 0.1% VEGF (vascular endothelial growth factor), 0.4% rh-FGF-B (human recombinant fibroblast growth factor), 0.1% rh-IGF-1 (insulin- like growth factor), 0.1% ascorbic acid, 0.1% heparin, 0.1% GA-1000 (gentamicin sulfate plus amphotericin B), and 10% fetal calf serum or 10% human fasting as well as postprandial serum.

HCAECs were plated at a seeding density of 2.500 cells/cm^2 in T25 flasks or in 12-well cell culture plate in the presence of a medium containing 10% fetal calf serum or in presence of 10% human serum (fasting or postprandial). Cell cultures in the flasks reached confluence after 6-7 days, as assessed by light microscopic examination, and they were passaged at weekly intervals. Cell cultures in the well culture plate reached confluence after 3-4 days and were passaged two times per week. After trypsinization and before replating, harvested cells were counted using a haemocytometer and the number of population doublings (PD) were calculated using the following formula: $(\log 10F - \log 10I) \times 3.32$ (where F indicates the number of cells at the end of the passage and I the number of cells when seeded) [17]. Endothelial cell senescence was studied by subjecting endothelial cells to subsequent passages until two consecutive population doublings equal or below 0 associated with senescence morphological changes revealed by microscopy examination. Cumulative population doubling (CPD) was calculated as the sum of all the changes in PD.

2.4. RNA Extraction. Total RNA was extracted from HCAECs subjected to the different treatments using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Subsequently, RNA concentrations were assessed by NanoDrop spectrophotometer (260/280 absorbance ratios) and RNA quality was determined by gel electrophoresis.

2.5. Cellular Senescence Biomarkers. Senescence-associated beta-galactosidase activity (SA-beta-gal) was performed by flow cytometry as previously described [18]. Briefly, C12FDG was added to the pH modulation/buffer. HCAECs were incubated with this solution for 1 h at 37°C at 5% CO_2. After incubation, HCAECs were trypsinized, washed with PBS, resuspended in 200 μl PBS, and analyzed using a Coulter Epics XL flow cytometer. Data were analyzed with the instrument software, and cell debris was excluded on basis of light scatter parameters. C_{12}FDG was measured on the FL1 median fluorescence intensity (MFI) of the HCAEC population.

For p16 expression, cDNA synthesis from total RNA was performed using the iScript reverse transcriptase (Bio-Rad, Hercules, CA) according to the manufacturer's guidelines. Messenger RNA for the housekeeping gene β-actin and for p16 was then measured by real-time PCR on a Bio-Rad iQ5 optical real-time thermal cycler (Bio-Rad, Hercules, CA) using 1 μg of cDNA in a total volume of 20 μl containing iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). β-Actin was used as the reference gene. Forward and reverse primers used in this model have been previously reported [19]. Any inefficiencies in RNA input or reverse transcription were corrected by normalization to the housekeeping gene. Relative amounts of p16 were calculated using the threshold cycle (Ct) values obtained from qPCR and applying the $\Delta\Delta$Ct method (ΔCt = Ct beta-actin − Ct p16 mRNA). A lower ΔCt value referred to lower expression of the p16 mRNA.

2.6. Determination of Total Reactive Oxygen Species Production. Total cellular reactive oxygen species (ROS) production in HCAECs was analyzed by flow cytometry after loading of cells with a highly sensitive fluorescent probe, [5 and 6 chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA)] (Molecular Probe, Life Technologies). One aliquot of 200, 000 cells was used as positive control and it was preincubated for 5 minutes with tert-butyl hydroperoxide solution (70%) diluted 1 : 100 in PBS then washed 2 times with PBS. All the other aliquots of HCAECs, including the positive control, were incubated at 37°C for 30 min in the dark with 2 μM of probe in PBS buffer. CM-H2DCFDA is cleaved by intracellular esterases and transformed into a fluorescent dye when oxidized. Cells were then washed 2 times with PBS and analyzed by flow cytometry (Coulter Epics XL). The mean fluorescence intensity of 5, 000 cells (corrected for autofluorescence) was taken as a measure for the total ROS load.

2.7. Measurements of Oxidative DNA Damage. HCAECs after 4 hours of exposure with postprandial serum pellets were trypsinized and one aliquot of 350.000 cells was frozen in liquid nitrogen then stored at −80°C. Another aliquot of 350.000 cells was plated in T25 flasks and incubated for 18 h at 37°C in the presence of a medium containing 10% fetal calf serum. The day after, the HCAECs were detached by trypsinization, then frozen in liquid nitrogen, and stored at −80°C until use. Genomic DNA was purified using the QIAamp DNA Micro Kit (Qiagen, Italy) following the manufacturer's protocol. Then, DNA was digested at 50° for 1 h with nuclease P1 (Sigma-Aldrich, Italy) following the manufacturer's instruction. After incubation with 1 unit of alkaline phosphatase at 37°C for 30 minutes, DNA samples were boiled for 10 minutes and placed in ice and subsequently stored at −20°C until use. 8-OHdG was measured using a competitive EIA assay (StressMarq Bioscience Inc.). The test utilizes an anti-mouse IgG-coated plate and a tracer consisting of an 8-OHdG-enzyme conjugate. This format has the advantage of providing low variability and increased sensitivity compared with assays that utilize an antigen-coated plate. Standard 8-OHdG was assayed over a concentration range of 0 to 3000 pg/ml in duplicates. Sample DNA assays were performed in duplicate, and the average concentration of 8-OHdG was expressed per nanogram of DNA.

2.8. Microarray Gene Expression Assays. The microarray procedure was performed according to the Affymetrix protocols (Santa Clara, CA, USA). In brief, 100 ng of total RNA was amplified and labeled using the GeneChip® 3′ IVT Plus kit (Affymetrix, Santa Clara, CA, USA). Hybridization cocktails containing fragmented, end-labeled single-stranded cDNA were prepared and hybridized to the GeneChip Human Genome U133 Plus 2.0 Array for 16 h at 45°C. (Affymetrix, Santa Clara, CA, USA). The signal intensities were measured using a GeneChip Scanner 3000 7G (Affymetrix) and converted to numerical data using the GeneChip Command Console® Software (AGCC). The microarray data analysis was performed by Partek® Genomics Suite 6.6 (Partek Inc.,

Table 1: Changes in the serum glucose, total cholesterol, HDL cholesterol, LDL cholesterol, triglyceride levels, and lipemic index after 4 h from the meat meal consumption. Data represent mean ± SEM.

	FS		CMS		RMS		PMS	
	Adult	Elderly	Adult	Elderly	Adult	Elderly	Adult	Elderly
Glucose (mg/dl)	91 ± 9	97 ± 9	87 ± 14	78 ± 5**	87 ± 8	83 ± 5*	90 ± 12	85 ± 3*
Lipemic index (mg/dl)	12 ± 5	10 ± 2	44 ± 17*	45 ± 15*	50 ± 20*#	74 ± 43**	98 ± 49**#	154 ± 64**
Triglycerides (mg/dl)	86 ± 25	82 ± 29	158 ± 27**	165 ± 19**	167 ± 29**	170 ± 39**	185 ± 63**	215 ± 47**
HDL cholesterol (mg/dl)	59 ± 14	68 ± 15	55 ± 11	60 ± 13	50 ± 11	64 ± 7	53 ± 10	62 ± 7
LDL cholesterol (mg/dl)	133 ± 31	106 ± 26	126 ± 36	105 ± 29	112 ± 32	87 ± 17	117 ± 25	84 ± 17
Total cholesterol (mg/dl)	204 ± 33	190 ± 27	199 ± 43	189 ± 22	176 ± 43	176 ± 13	196 ± 29	188 ± 16

*$p < 0.05$ versus the respective FS; **$p < 0.001$ versus the respective FS; #$p < 0.05$ versus elderly group. FS: fasting serum; CMS: chicken meal serum; RMS: rabbit meal serum; PMS: pork meat serum.

St Louis, MO, USA) using the default Partek normalization parameters. Affymetrix CEL files were imported and background correction and normalization were performed using GC-robust multiarray average (GC-RMA) algorithm. Comparisons among treatment groups were performed with the ANOVA tool implemented in the Partek software after removal of batch effects. In order to correct for multiple comparisons and reduce the number of false positives, a false discovery rate (FDR) adjusted p value <0.05 was used in combination with a fold change (FC) ≥ 2. Biofunctional analysis was performed using ConsensusPathDB (http://cpdb.molgen.mpg.de) [20] and Partek Pathway software (Partek, St Louis, MO, USA).

2.9. Statistical Analysis. Data were initially tested for normal distribution. Generalized linear models (for longitudinal data with normal distributed variables) or Kruskal-Wallis test (for nonnormal distributed variables) were used to compare data among experimental groups. Normal data were graphically represented as mean ± SEM while nonnormal data were represented as box plots. SPSS v. 23 was used as statistical software.

3. Results

3.1. Serum Triglyceride Levels in Postprandial Sera after Meat Meals. Baseline glucose, total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides were within the normal (70–110; <200; <100; >40; <150 for glucose, total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides) or borderline range (200–239; 130–159 for total and LDL cholesterol) in all subjects. As compared to fasting values, serum triglyceride levels and lipemic index increased 4 h after the meat meals ($p < 0.01$ versus baseline), while serum glucose was slightly decreased in sera from the elderly volunteers (Table 1). All the other variables remained unchanged. Lipemic index after the meal based on pork or rabbit was higher in elderly compared to adult volunteers ($p < 0.05$). The total amount of triglycerides was increased in the postprandial sera. The fatty acid composition of postprandial serum was only slightly different from the fasting serum composition. In fact, the postprandial serum contained few amount of some long chain fatty acids, docosatetraenoic (C22:4), lignoceric (C24:0), and nervonic (C24:1) acids, which were not detected in fasting serum (Supplementary Table 1).

3.2. Postprandial Sera Increase HCAEC ROS Production but Not Oxidative DNA Damage (8-OHdG) after a Few Hours of Exposure. Incubation (4 h) with serum collected 4 h after the pork meat lunch resulted in a significant increase in HCAEC ROS compared to incubation with preprandial (fasting) serum (Figure 1). Conversely, no difference in ROS was detected between HCAEC grown in their own media versus HCAEC incubated with FS (Figure 1). The levels of ROS induced by incubation with postprandial sera were consistently lower than those induced by treatment with t-But (Figure 1). We detected no significant changes in 8-OHdG in HCAEC exposed 4 h to the postprandial serum compared to fasting serum (Figure 2), neither there was any significant difference after replacement of HCAEC in their own medium for 18 h.

3.3. Transcriptional Profiling of HCAEC Cells Exposed to Fasting or Postprandial Serum from Adult and Old Donors. Global changes in gene expression were analyzed in HCAEC cells treated for 4 h with fasting or postprandial sera withdrawn from volunteers after the pork meat meals, which showed the highest prooxidative impact on HCAEC (Figure 1). A minimum of 4 independent replicates was performed for each condition. Two-way ANOVA identified 2877 genes significantly modulated by postprandial serum versus fasting serum ($p < 0.05$). Of these genes, 51 were significantly expressed ($p < 0.05$ and FDR < 0.05) with absolute fold change > 1.2. The top 20 genes with the greatest fold change showing the same direction in HCAEC exposed to elderly and adult postprandial serum are listed in Table 2. The most affected pathway was searched including the whole gene list in the gene set analysis tool available by ConsensusPathDB (http://cpdb.molgen.mpg.de) [20]. Significant pathways that were useful to the human endothelial cellular model are displayed in Table 3. The results confirm that the most important biological function affected by postprandial serum exposure include detoxification (chemical carcinogenesis, xenobiotic metabolism, and drug metabolism), oxidative stress (oxidative stress-induced senescence, MAPK signaling pathway, and oxidative stress), cellular senescence (oxidative stress-induced senescence and cellular senescence), and

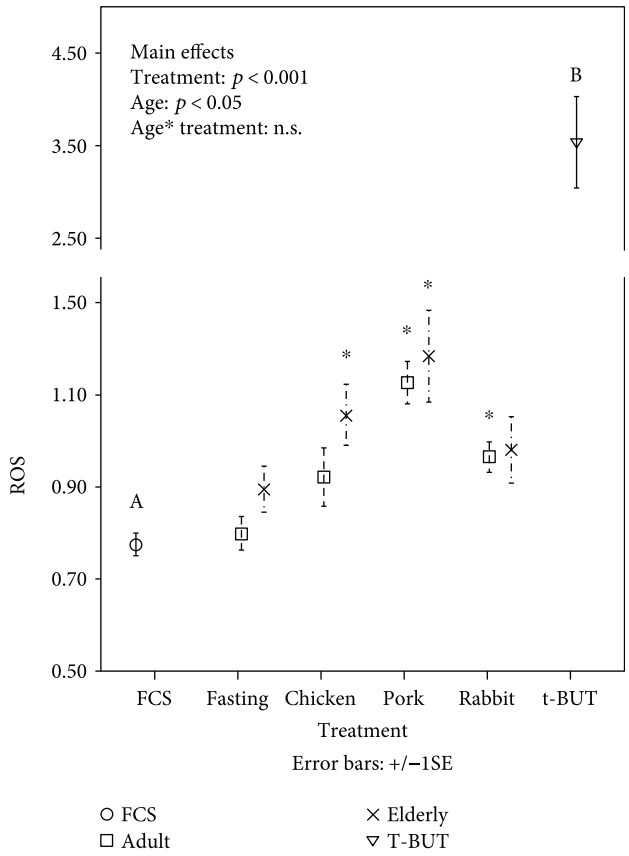

Figure 1: Effect of postprandial serum from adult (□) and elderly volunteers (×) after consumption of chicken, pork, or rabbit meat meals on HCAEC ROS production. Data are shown as mean ± SEM. All data are compared to fasting serum, fetal calf serum (FCS, marked by "○") and the strong oxidant tert-butyl hydroperoxide (t-BUT, marked by "▽"). In the left-high panel are reported the significance of the main effects: age, treatment (postprandial versus fasting), and their interaction analyzed by generalized linear models. All other comparisons are derived from posthoc by LSD: $^{*}p < 0.05$ versus the respective fasting serum, $^{A}p < 0.01$ versus all postprandial sera (chicken, pork, and rabbit), and $^{B}p < 0.01$ versus all other treatments. Pooled samples used for the experiments were $n = 6$ (elderly) and $n = 10$ (adult). Each sample was run as a technical triplicate. For control conditions, $n = 10$ (FCS) and $n = 6$ (t-BUT).

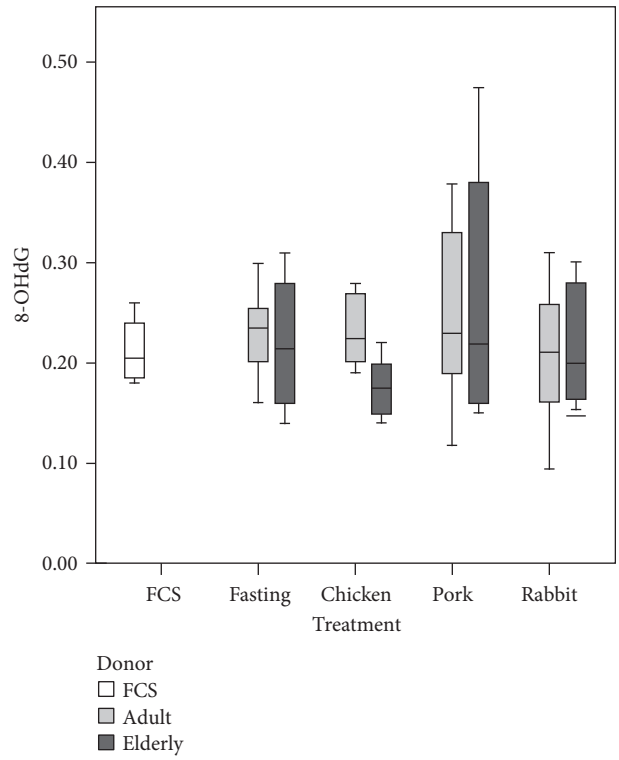

Figure 2: Effect of postprandial serum from adult and elderly volunteers after consumption of chicken, pork, or rabbit meat meals on HCAECs oxidative DNA damage (8-OHdG). Data are shown as box plots representing the median, interquartile range, and top and bottom whiskers (highest and lowest case within 1.5 times IQR). No significant differences were observed by the Kruskal-Wallis test or ANOVA even when data of postprandial sera are considered together and compared to fasting sera. Samples used for the experiments were $n = 6$ (elderly) and $n = 10$ (adult). Each sample was run as a technical duplicate. For control condition in fetal calf serum (FCS), $n = 4$.

mediators of inflammation (TNFR1-induced NF-κB signaling pathway and TNF signaling). Two representative pictures including those of the most important players involved in detoxification (aromatic hydrocarbons pathway) and oxidative stress-induced senescence (p53-pathway) were drawn by Partek Pathway (Figures 3 and 4). CYP1A1 (an important player in detoxification function) and MDM2 (an important player in p53-signalling and oxidative stress response) were the two genes represented in most pathways and were additionally characterized by marked fold changes (Figure 5). Additionally, CYP1A1 was significantly higher in cells exposed to elderly donors' serum (both fasting and postprandial) compared to adult donors' serum, whereas MDM2 was lower in cells exposed to elderly donors' postprandial serum compared to adult donors' postprandial serum.

3.4. Repeated Exposure to Postprandial Sera Slightly Accelerates Cellular Senescence in HCAEC Compared to Fasting Sera. HCAEC cultured in wells and in flasks in normal medium showed the phenotypic appearance of senescence after 4–6 weeks (10–12 CPD) and 6–8 weeks (18–20 CPD), respectively. After this period, the cells flattened became irregular in shape with an increased diameter, thus exhibiting the characteristic of senescent cells including beta-Gal staining. Although the "well system" was shown to accelerate the appearance of the senescence features and to reduce the proliferative potential in HCAEC, we were forced to adopt this system for the comparison of the effects of repeated exposure to fasting and postprandial sera due to the limited availability of the biomaterial (in particular, human postprandial serum). In the first set of experiments (performed in the "flask system"), we investigated if a single exposure (4 h) of early passage HCAEC to postprandial serum from different meat meals could have an impact on growth curve and senescence of the cells. In these experiments, it did not find any impact of postprandial serum on growth curves (measured as CPDs at each passage) of HCAEC (Supplementary Figure 1). In order to study the

Table 2: Representative genes with greatest fold differences in HCAEC exposed to fasting or postprandial* serum from adult and elderly donors.

Probeset ID	Gene symbol	Gene name	Fold-change postprandial versus fasting adult serum	p value	Fold-change postprandial versus fasting elderly serum	p value
(a) Upregulated genes						
205749_at	CYP1A1	Cytochrome P450, family 1, subfamily A	3.7992	<0.0001	2.4956	0.0008
228770_at	GPR146	G protein-coupled receptor 146	2.0300	0.0003	1.8857	0.0361
227652_at	FAM69B	Family with sequence similarity 69, member B	1.5366	0.0264	1.9603	0.0330
221565_s_at	CALHM2	Calcium homeostasis modulator 2	1.4354	0.0143	2.3629	0.0006
209830_s_at	SLC9A3R2	Solute carrier family 9, subfamily A	1.3971	0.0364	3.4436	<0.0001
219020_at	HS1BP3	HCLS1 binding protein 3	1.3794	0.0026	1.5682	0.0089
226488_at	RCCD1	RCC1 domain containing 1	1.3371	0.0127	1.7111	0.0054
211143_x_at	NR4A1	Nuclear receptor subfamily 4, group A, member 1	1.2800	0.0264	1.5553	0.0159
223415_at	RPP25	Ribonuclease P/MRP 25 kDa subunit	1.2674	0.0352	1.8069	0.0020
207978_s_at	NR4A3	Nuclear receptor subfamily 4, group A, member 3	1.2430	0.0310	1.4449	0.0261
(b) Downregulated genes						
210631_at	NF1	Neurofibromin 1	−1.6760	0.0017	−1.8129	0.0228
1569867_at	EME2	Essential meiotic structure-specific endonuclease	−1.4108	0.0338	−1.7198	0.0406
225160_x_at	MDM2	MDM2 protooncogene, E3 ubiquitin protein ligase	−1.4035	0.0112	−1.5440	0.0435
1553113_s_at	CDK8	Cyclin-dependent kinase 8	−1.3286	0.0020	−1.6654	0.0008
224712_x_at	SMIM7	Small integral membrane protein 7	−1.3229	<0.0001	−1.3019	0.0067
1552487_a_at	BNC1	Basonuclin 1	−1.3185	0.0470	−1.6384	0.0311
226881_at	GRPEL2	GrpE-like 2, mitochondrial (*E. coli*)	−1.3133	0.0129	−1.4588	0.0332
229027_at	PPM1A	Protein phosphatase, Mg2+/Mn2+ dependent, 1A	−1.3019	0.0236	−1.5777	0.0173
228397_at	TUG1	Taurine upregulated 1 (nonprotein coding)	−1.2723	0.0400	−1.5252	0.0285
221203_s_at	YEATS2	YEATS domain containing 2	−1.2717	<0.0001	−1.3036	0.0024

*The present analysis is restricted to the top 10 upregulated and downregulated genes expressed in HCAEC treated with postprandial sera withdrawn after pork meat meals versus fasting serum.

impact of repeated exposure to postprandial serum on replicative senescence of HCAEC, we used the "well system" and the postprandial serum obtained from adult volunteers after the pork meat meals, as this serum induced the highest oxidative stress after a single exposure in HCAECs (Figure 1). When fasting serum was added two times a day for 4 h, no distinct and reproducible differences in CPD or SA-beta-gal activity were observed between treated and untreated cultures (the medium of untreated cultures was replaced with the same timing and procedure for treated cells) during the whole growth curve (data not shown). Conversely, a reduction of CPDs compared to HCAEC treated with fasting serum was observed in HCAEC treated with postprandial serum added two times a day (for 4 h) from early passages up to the onset of replicative senescence (Figure 6). Moreover, when compared to the fasting serum, SA-beta-gal activity was higher in the late passages of HCAEC treated two times a day with postprandial serum starting from early passages up to the onset of replicative senescence (Figure 6). We observed also a loss of viability and an increased expression of p16 after passage 10 in cells exposed to postprandial serum compared to treatment with the fasting serum (Figure 6).

4. Discussion

This study aimed to answer the question whether acute and chronic exposure of HCAECs to postprandial serum withdrawn from volunteers after a normal meal composed of oven-cooked meat with seasoning and side dishes can have detrimental effects on endothelial cells and if these effects are influenced by the age of the donors. We found that exposure of HCAECs to postprandial serum, withdrawn independently from adult and elderly individuals, can induce a slight increase in ROS production. The postprandial serum-induced oxidative stress is also associated with the expression

Table 3: Enriched pathway-based sets* identified by ConsensusPathDB.

Pathway name	Set size	Candidates contained	p value	q value	Pathway source
Chemical carcinogenesis	82	18 (22.0%)	0.000842	0.203	KEGG
MAPK signaling pathway	168	30 (17.9%)	0.000889	0.203	WikiPathways
Xenobiotics metabolism	53	13 (25.5%)	0.00101	0.203	EHMN
Drug metabolism	46	12 (26.1%)	0.00126	0.203	KEGG
TNFR1-induced NF-κB signaling pathway	26	8 (30.8%)	0.00263	0.232	Reactome
Oxidative stress-induced senescence	129	23 (18%)	0.0031	0.242	Reactome
Ubiquitin-mediated proteolysis	137	24 (17.5%)	0.00359	0.243	KEGG
Cellular senescence	192	30 (15.7%)	0.00654	0.318	Reactome
Oxidative stress	30	8 (26.7)	0.00693	0.318	WikiPathways
The fatty acid cycling model	5	3 (60%)	0.00812	0.318	Reactome
TNF signaling	37	9 (24.3%)	0.00816	0.318	Reactome

*1243 genes (59.7%) from the input list (2877 genes) are present in at least one pathway.

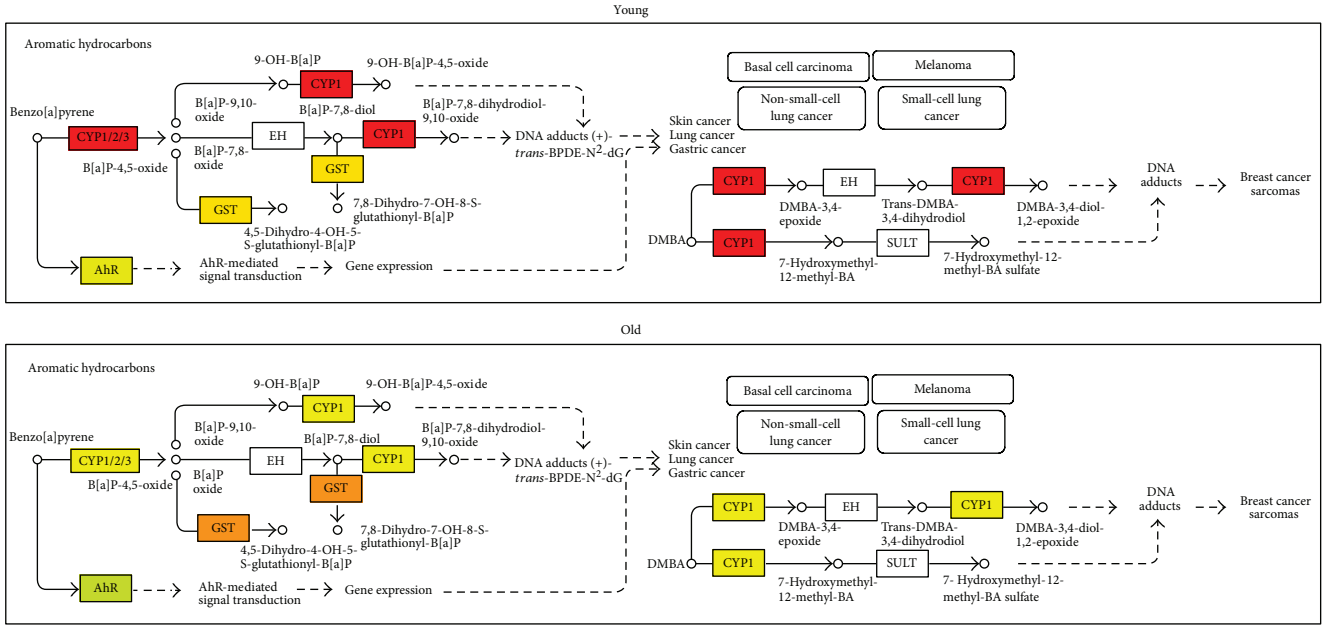

Figure 3: Aromatic hydrocarbon pathway and related significant changes observed in response to postprandial serum (red: strongly upregulated compared to fasting serum; orange: mildly upregulated compared to fasting serum; green: downregulated compared to fasting serum).

of genes involved in oxidative stress response, detoxification from chemical compounds, cellular senescence, and inflammation. However, we did not find any evidence of DNA damage; thus, suggesting that a single exposure of cells to postprandial serum is compatible with a mild stress event. Indeed, once the postprandial serum is removed and the cells are regrown in normal condition, the proliferative activity and the onset of replicative senescence is not affected. Conversely, repeated exposure of HCAEC to postprandial serum (2 times a day) over time affects the proliferative activity and accelerates the onset of replicative senescence *in vitro*. Taking into account that the accumulation of endothelial senescent cells is a common mechanism at the basis of atherosclerosis [21–23] and other age-related diseases [24], these data could form the rationale to explore a new mechanistic link among dietary habits and risk of age-related diseases.

Consistent with previous studies performed by administration of high-fat challenges in healthy men [9, 25, 26], we found that triglycerides and lipemic index increase in plasma 4 h after the oven-cooked meat meal (including potatoes on the side, seasoning, and half glass of wine). We additionally observed that lipemic index after the pork or rabbit meat meal were higher in elderly compared to adult volunteers. As documented previously, the magnitude of postprandial lipemia is increased with aging [27, 28], a phenomenon that might place elderly individuals at greater risk for cardiovascular disease compared with their adult counterparts. This phenomenon likely occurs as a consequence of a dysregulated secretion of hepatic triglycerides into the plasma during the postprandial period [28]. Postprandial lipemia is also associated with a general metabolic stress, characterized by the rise in oxidative stress and proinflammatory cytokines

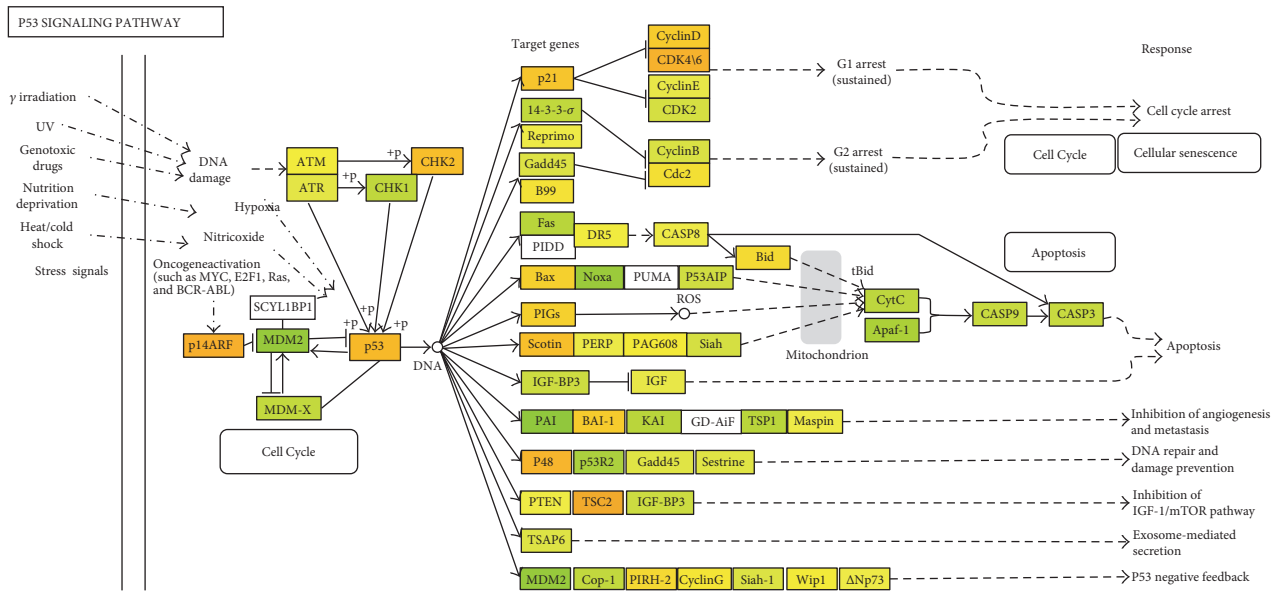

Figure 4: p53 signaling pathway and related significant changes observed in response to postprandial serum (orange: upregulated compared to fasting serum; green: downregulated compared to fasting serum).

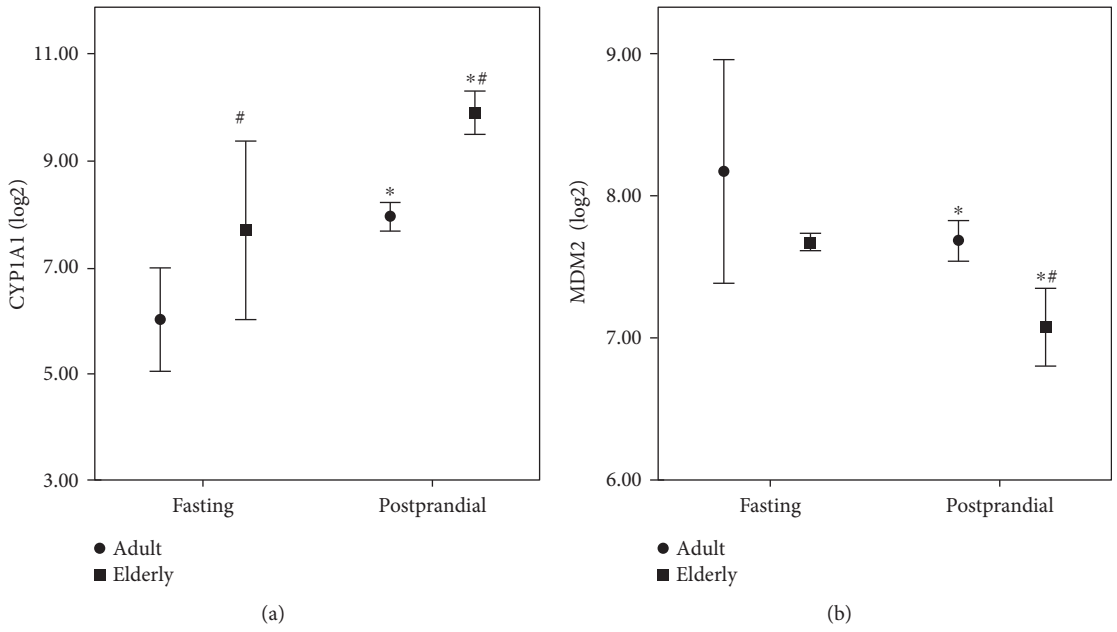

Figure 5: Log2 intensity of CYP1A1 and MDM2 genes in HCAEC exposed to fasting or postprandial serum from adult and elderly donors. $^{*}p < 0.01$ compared to fasting serum; $^{\#}p < 0.01$ compared to adult donors.

as well as endotoxemia which has been reported to be similar in younger adults and healthy older adults [29]. Indeed, in spite of the higher lipemic index in postprandial sera from the elderly, we observed that the increase in oxidative stress of HCAECs exposed to postprandial sera was comparable between sera derived both from elderly and adult volunteers, albeit the ROS were in general higher with sera derived from the former. In any case, it is possible that prooxidant mediators are already present in serum after meal consumption [29]. Alternatively, another hypothesis is that the high burden of lipids forces mitochondrial oxidation with consequent production of ROS in analogy to the phenomenon described in response to hyperglycemia [30]. This could eventually explain the proportionality of the results between the lipemic index with the various kinds of meals and the level of ROS.

It has been previously shown that human umbilical vein endothelial cells (HUVECs) exposed to sera withdrawn 4 h after a fat challenge decrease their proliferative activity [9]. However, evidence is increasing that HUVECs, derived from a vascular bed not present in the adult, differ phenotypically and epigenetically from adult cells, such as HCAECs [31]. Although our volunteers ingested less than half of the fat

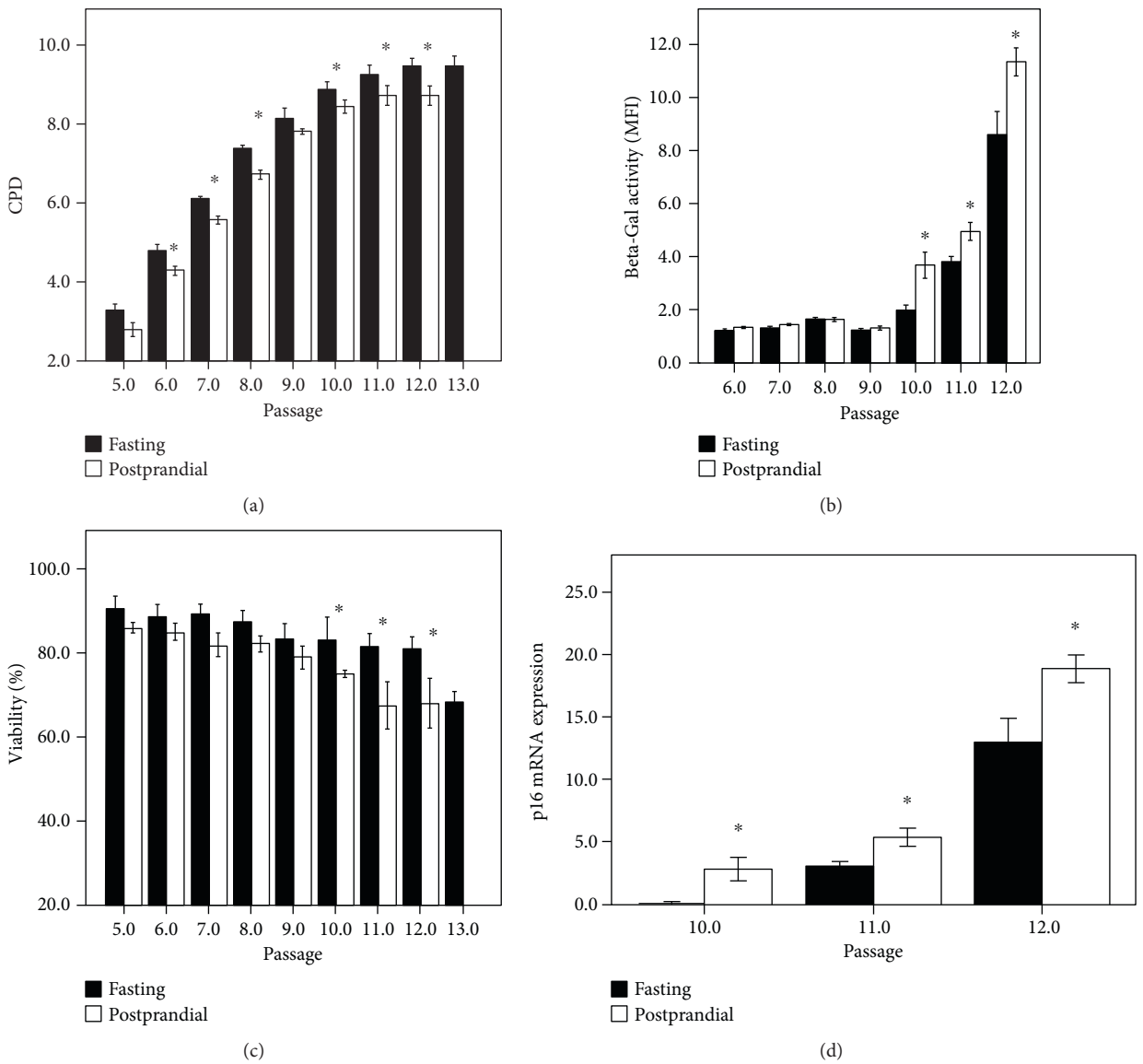

Figure 6: The effect of culture with postprandial serum (2 times per day for 4 h) compared to fasting serum (2 times per day for 4 h) on growth and senescence of HCAECs. Postprandial serum used for these experiments was obtained from adult volunteers after the pork meat meals. All data are mean ± SEM for $n = 4$–6 independent cell populations for each condition (fasting serum and postprandial). (a) Cumulative population doubling (CPD) was calculated using the formula $3.32^*(\log 10F - \log 10I)$ over consecutive population doublings. (b) Senescence-associated β-galactosidase (SA-β-gal) activity for cells grouped by passage number and treatment as indicated. (c) Viability for cells grouped by passage number and treatment as indicated. (d) p16 expression after passage 9 for cells grouped by passage number and treatment as indicated. Data are means with error bars showing SEM. $^*p < 0.05$ compared to fasting serum.

usually administered during a dietary fat challenge (approx. 50 g), we also detected a slight decrease in the spontaneous proliferation of the HCAECs repeatedly exposed to postprandial sera, which is consistent with the observed increase in oxidative stress. This decrease in proliferative activity might be interpreted as a compensatory and beneficial effect to avoid additional damage in the presence of stress. Indeed, a single exposure (4 h) of HCAECs to postprandial serum induces only a transient and modest increase in oxidative stress with the activation of several defensive pathways (detoxification pathways, oxidative stress response, and p53) that are reversed when cells are regrown in their normal medium (data not shown) without functional impairment (as demonstrated by the normal growth curve and replicative senescence timing). Conversely, when the exposure is repeated two times a day for the whole cellular lifespan, the impact of postprandial serum on the growth and senescence of the HCAEC is revealed. Subtoxic oxidative stress in cell culture is known to generate a transient adaptive response which may eventually produce a beneficial hormetic effect [32]. However, in the case of chronically repeated exposure to an excess of nutrients, the adaptive response might eventually culminate in a proliferative arrest and cellular senescence [33]. The process may be similar to the one reported for the acceleration of senescence induced in cultured human cells by high concentrations of glucose in the medium [34, 35].

Our transcriptomic results and pathway analysis showed an additional hypothesis. Indeed, postprandial serum promotes the activation of pathways involved in the detoxification by chemical carcinogens, in particular, the aromatic hydrocarbons detoxification pathway. This is well represented by the postprandial-induced expression of CYP1A1 (Figures 3 and 5). This gene (a member of the superfamily of enzymes of cytochrome P450) is not only involved in metabolizing a multitude of polycyclic aromatic hydrocarbons and other chemical compounds (including carcinogens) that arise from meat cooking [36] but also in the conversion of polyunsaturated fatty acids into signaling molecules that have physiological as well as pathological activities. Dietary fatty acids can mediate P450 induction [37] and are agonists of Toll-like receptors, which can explain the modulation of inflammatory signature after exposure to postprandial sera and the altered expression of CYP1A1 [38]. Interestingly, the basal levels of CYP1A1 were upregulated in the cells exposed to the elderly's postprandial serum compared to the adult one, which may be consistent in the increase in serum free fatty acids with aging [39]. Regarding the production of potential carcinogens, we know that red and white meats cooked at high temperature may have high levels of meat mutagens, including benzo[a]pyrene [40], one of the most studied polycyclic aromatic hydrocarbons derived from cooked meat. Since exposure to polycyclic aromatic hydrocarbons has been implicated not only in colorectal cancer [41] but also in cardiovascular diseases [42], it might formed a speculative hypothesis on the possible influence of these compounds on endothelial senescence. By the way, benzo[a]-pyrene was found to restrain cell cycle in murine brachial epithelial cells [43], to increase the mutation frequencies and carcinogenesis *in vivo* in the lacZ transgenic (mutation reporter) mouse [39] and to induce p53 protein accumulation, a mediator of cellular senescence, in various cellular models [44, 45]. This last result is consistent with our finding of an upregulation of cellular senescence and p53 pathway in the HCAEC model exposed to postprandial serum. Our analysis additionally suggests that NF1 downregulation is induced in HCAEC exposed to postprandial serum. This could be relevant in the acceleration of replicative senescence as NF1 loss is known to induce senescence in human melanocytes [46] and fibroblasts [47] as well as altered vascular morphogenesis in human endothelial cells [48]. Last but not least, our pathway analysis reported that TNF and NF-κB signaling are affected by exposure to postprandial serum. While we have not been able to predict if this modulation can result in a pro- or anti-inflammatory response, the results are in agreement with the studies that have demonstrated that the postprandial phase is characterized by mediators of inflammation able to modulate NF-κB and tissue expression of TNF-alpha during the postprandial phase [49, 50]. By the way, it has been recently demonstrated that treatment of peripheral blood mononuclear cells with postprandial VLDL lipolysis products increased expression of TNFα, IL-1β, and IL-8 over controls, with concurrent activation of NF-κB [51].

At our knowledge, our study addresses for the first time the potential consequences of a repeated exposure to this specific environment. Unfortunately, it is not possible to disentangle the effects of the inflammatory mediators from oxidative stress as well from the potential exposure to chemicals arising from the cooking process. However, it is likely that all of them act synergically in the establishment of the functional outcome (reduced proliferative activity and acceleration of senescence following repeated exposure) of postprandial serum on endothelial cells. Interestingly, a very similar *in vitro* technique, where cell lines have been exposed to the serum collected from humans or animals on calorie-restricted diets, has been proposed as a tool to predict the potential of dietary manipulations to affect markers of longevity [52, 53]. Indeed, cell cultures exposed to the postprandial serum from subjects at the end of a caloric restriction trial showed greater resistance to oxidative stress, upregulation of longevity genes, an increase of Sirt1, a proliferation reduction, and an increase in thermal shock resistance [52]. Considering that these experiments were developed on hepatic cancer cells that do not suffer from replicative senescence, our model of exposure by using human primary endothelial cells might be a useful advance. One of the limits of our model is that we cannot take into account the physiological fluctuation of the postprandial period depending on hormonal excretions and other factors that characterize the *in vivo* condition. Moreover, the clinical markers of the elderly enrolled in this study were consistent with an exceptional health status. This fact may eventually hamper the generalization of the findings to the elderly population.

On the basis of these results, it is possible to conclude that treating *in vitro* endothelial cells with postprandial sera could be a useful experimental tool to monitor the potential health impact of dietary intervention and challenges. This experimental tool could be useful in the design of experiments aimed to study the impact of processed foods on health, especially in the context of Western societies, where a significant part of the day is spent in the postprandial state [6].

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

This study was supported by a grant from the Italian Ministry of Economy and Finance "Industria 2015—Bando Made in Italy—Programma n. MI01_00148."

Supplementary Materials

Scheme 1: Design of the pooling strategy adopted with the withdrawal obtained after each meal in order to get the samples used for the experiments with the HCAECs "in vitro." Supplementary Table 1: Fatty acid composition* of pork meat, fasting serum and postprandial serum after consumption of a meal based on pork meat. Supplementary Figure 1. Growth curves of HCAECs cultured in flasks after a single exposure (4H) to various postprandial sera. No difference was reported in comparison to treatment with fasting serum and to normal culture conditions with fetal calf serum (not shown in this figure). (*Supplementary materials*)

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 8102170, 5 pages
https://doi.org/10.1155/2017/8102170

Research Article

Mouse Thyroid Gland Changes in Aging: Implication of Galectin-3 and Sphingomyelinase

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Received 29 August 2017; Accepted 12 November 2017; Published 11 December 2017

Academic Editor: Fabiola Olivieri

Prevalence of thyroid dysfunction and its impact on cognition in older people has been demonstrated, but many points remain unclarified. In order to study the effect of aging on the thyroid gland, we compared the thyroid gland of very old mice with that of younger ones. We have first investigated the changes of thyroid microstructure and the possibility that molecules involved in thyroid function might be associated with structural changes. Results from this study indicate changes in the height of the thyrocytes and in the amplitude of interfollicular spaces, anomalous expression/localization of thyrotropin, thyrotropin receptor, and thyroglobulin aging. Thyrotropin and thyrotropin receptor are upregulated and are distributed inside the colloid while thyroglobulin fills the interfollicular spaces. In an approach aimed at defining the behavior of molecules that change in different physiopathological conditions of thyroid, such as galectin-3 and sphingomyelinase, we then wondered what was their behavior in the thyroid gland in aging. Importantly, in comparison with the thyroid of young animals, we have found a higher expression of galectin-3 and a delocalization of neutral sphingomyelinase in the thyroid of old animals. A possible relationship between galectin-3, neutral sphingomyelinase, and aging has been discussed.

1. Introduction

Galectins are a family of proteins with specific domains of 130 amino acids able to bind β-galactosides [1]. 15 mammalian galectins are classified into three groups: prototype galectins, tandem galectins, and chimera-type group, of which galectin-3 (Gal-3) is the only member [2]. Gal-3 is widely distributed in a large number of tissues, and at the cellular level, it can be located in the membranes, cytoplasm, and nucleus [3]. Gal-3 is known to modulate many immune reactions [4]. It can be released extracellularly under different inflammatory stimuli like lipopolysaccharide, known to induce upregulation of Gal-3 expression [5]. Moreover, Gal-3 plays a role in leukocyte recruitment to the inflamed microcirculation [6]. Recent evidence shows that Gal-3 plays a role in numerous pathologic conditions such as inflammation [7], metabolic disorders [8], and cancer [9]. In humans, Gal-3 is upregulated in malignant thyroid neoplasms as compared to benign neoplasms and in particular in papillary thyroid carcinoma (PTC), the most prevalent type of malignant tumor of the endocrine system [10]. In rats, the microgravity induces the upregulation of Gal-3 in thyrocytes as well as its release in colloid [11].

Increasing studies demonstrate that neutral sphingomyelinase (nSMase), also known as sphingomyelin phosphodiesterase, an enzyme that uses sphingomyelin (SM)

as a substrate to produce ceramide and phosphocholine, may regulate many cell physiopathology processes [12]. Recently, it has been demonstrated that nSMase is upregulated in aging [13]. nSMase is present in the thyrocytes of the thyroid gland, and it is more expressed in the right than in the left lobe [14]. In thyrocytes in culture, the nSMase activity depends on their physiological state by influencing, together with sphingomyelin synthase (SM-synthase), the ceramide/diacylglycerol balance [15]. Thyroid nSMase activity is stimulated during spaceflight and after ionizing and nonionizing ray treatment [16].

At the moment, nothing is known about Gal-3 and nSMase behavior in the thyroid gland during aging and, therefore, the aim of the present study was to investigate this point.

2. Methods

2.1. Animals. Three CD-1 male mice six weeks old and three CD-1 male mice eighteen months old (Harlan Laboratories Srl, Correzzana D'Adda, Milan, Italy) were used. Mice were kept under a 12-hour light/dark cycle and housed under controlled conditions as reported in Traina et al. [17]. Mice had free access to pelleted food and water. The weight was 26 ± 3 g and 47 ± 2 g for young and old animals, respectively.

2.2. Ethical Approval. The experimental protocol was approved by the Ethical Committee for Animal Experimentation at the University of Perugia, Italy. Animal care was in compliance with Italian regulations (Ministerial Declaration 04.03.2014 n°26), as well as with European Economic Community regulations (O.J. of European Commission 2010/63/UE).

2.3. Reagents. Anti-TSH receptor (TSHR), anti-nSMase, and fluorescein isothiocyanate- (FITC-) conjugated secondary antibody were obtained from Santa Cruz Biotechnology Inc. (California, USA). TSH, thyroglobulin, and Gal-3 antibodies were from Leica Biosystems (Newcastle Ltd., UK).

2.4. Thyroid Tissue Treatment. The thyroid tissue was fixed in 4% neutral phosphate-buffered formaldehyde solution for 24 h, as previously reported [14]. 4 μm thick sections were prepared and mounted on silane-coated glass slides, two for a slide at a distance equal to 140 μm. Between 7 and 24 pairs, sections were sampled excluding the first and the last; sections 7, 13, and 19 were used for hematoxylin-eosin staining; 8, 14, and 20 for TSH detection; 9, 15, and 21 for thyroglobulin detection; 10, 16, and 22 for TSHR detection; 11, 17, and 23 for Gal-3 detection; and 12, 18, and 24 for nSMase detection. Tissue sections were deparaffinized and rehydrated with a series of xylene and ethanol washes.

2.5. Morphological Analysis. The sections were stained by the hematoxylin-eosin (Chroma-Gesellschaft, Germany) staining method and investigated by using an inverted microscope, EUROMEX FE 2935 (ED Amhem, The Netherlands), equipped with a CMEX 5000 camera system (40x magnification), as previously reported [14]. The morphometric analysis was performed by using ImageFocus software.

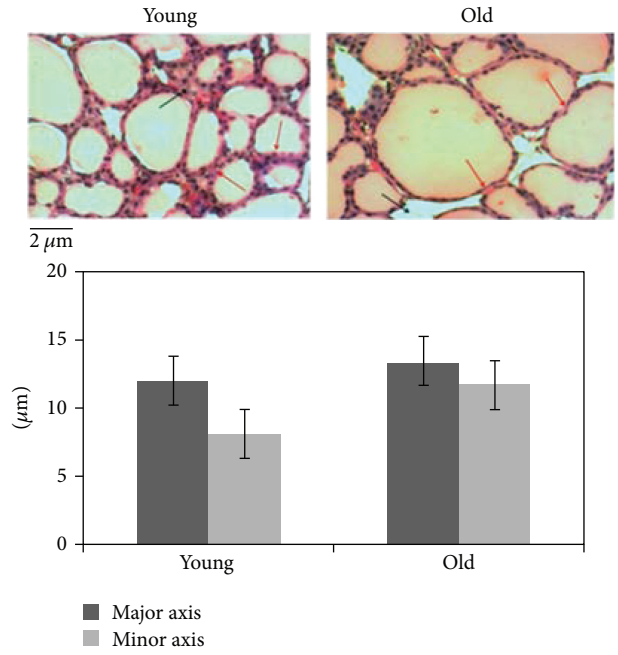

Figure 1: Thyroid morphology in aging. The analysis was performed with hematoxylin-eosin staining. Although some thyroid follicles of old animals appear wider than those of young mouse thyroid, the size of the follicles is not at all homogeneous. The morphometric analysis was performed by using ImageFocus software. Data are reported as mean ± S.D. of three independent experiments performed in duplicate. The results are not statistically significant, as reported in the results. Notably, the structure of thyrocytes and interfollicular spaces appears different in young and old thyroid mice. In particular, thyrocytes are less tall (red arrows) and interfollicular spaces are smaller (black arrows) in old than in young animals. 40x magnification.

2.6. Immunohistochemical Analysis. To remove paraffin from tissue sections before rehydration and immunostaining on the Bond automated system (Leica Biosystems Newcastle Ltd., UK), Bond Dewax solution was used as previously reported [14]. Immunostaining detection was performed by using TSH, thyroglobulin, and Gal-3 antibodies. Bond Polymer Refine Detection was from Leica Biosystems (Newcastle Ltd., UK) [14]. The images were investigated by using microscopy as reported for morphological analysis at 40x magnification.

2.7. Immunofluorescence Analysis. After 3 washes with phosphate-buffered saline (PBS), sections were incubated with 2 μg/ml anti-TSHR or anti-nSMase primary antibodies diluted in a 0.5% solution of bovine serum albumin (BSA) in PBS overnight at 4°C. The slides were washed 3 times with PBS and incubated with fluorochrome-conjugated secondary antibodies for 1 hour at room temperature. Then, after 3 washes with PBS, the slides were mounted with glycerol and coverslips. The samples were examined under a fluorescence microscope (OLYMPUS IX 51) equipped with an OLYMPUS DP 50 camera system and analyzed at 40x magnification.

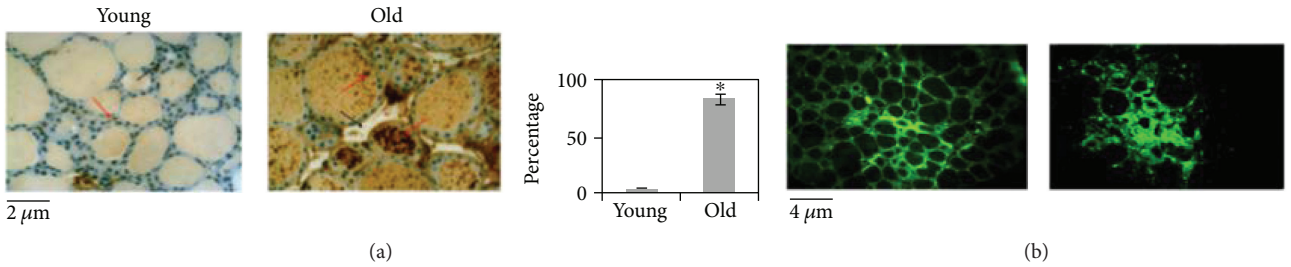

Figure 2: Thyrotropin (TSH) and thyrotropin receptor (TSHR) in the thyroid gland from young and old mice. (a) Immunohistochemical expression of TSH. Red arrows indicate the staining in thyrocytes, and black arrows indicate the staining in interfollicular spaces. In the thyroid of young mice, a low positivity is evident in thyrocytes and it is absent in interfollicular spaces. Strong staining is seen in the majority of follicles either in thyrocytes or in colloid of old mice. The positivity of staining was measured as the percentage of total area (follicles and interfollicular spaces). Data represent the mean ± S.D. of three independent experiments performed in duplicate. Significance, $^{*}P < 0.001$, versus young mice. 40x magnification. (b) Fluorescence immunostaining of thyrotropin TSHR. The intensity of florescence is higher in old animal than in young animals. Moreover, the fluorescence has a disordered localization in the thyrocytes, within the follicles, and in the interfollicular spaces as shown by arrows. 20x magnification.

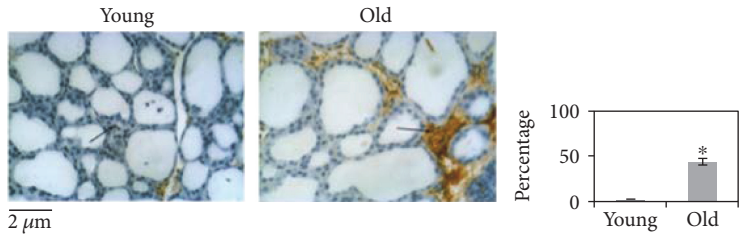

Figure 3: Thyroglobulin in the thyroid gland from young and old mice. Immunohistochemical expression of thyroglobulin. Black arrows indicate staining in interfollicular spaces. In the thyroid of young mice, a low positivity is evident in interfollicular spaces. Strong staining is seen in the interfollicular spaces of old animals. The positivity of staining was measured as the percentage of total area (follicles and interfollicular spaces). Data represent the mean ± S.D. of three independent experiments performed in duplicate. Significance, $^{*}P < 0.001$, versus young sample. 40x magnification.

2.8. Statistical Analysis. Three experiments were performed for each analysis. Data are expressed as mean ± SD, and *t*-test was used for the comparison between young and old mice.

3. Results and Discussion

3.1. Results. In order to study the effect of aging on the thyroid gland, we have first investigated the changes of the microstructure. Morphometric analysis, performed on the thyroid of young and old mice subjected to hematoxylin-eosin staining, shows differences in length of the major axis and of the minor axis of follicles. Since the size of the follicles is not at all homogeneous, the results are not statistically significant (Figure 1). Changes in thyrocyte height and in interfollicular space amplitude are evident. In particular, thyrocytes are less tall and consequently the thyroid epithelium versus colloid volumetric ratio is reduced. Interfollicular cells are poorer in old than in young animals (Figure 1). Then, we have tested the possibility that molecules involved in thyroid function might be associated with structural changes. Figure 2(a) shows a high content of TSH in old animals accompanied by its abnormal localization in colloid. We next asked whether increasing TSH content in old animals might be associated with modifications of TSHR. To address this question, the immunofluorescence analysis was performed by using specific antibodies against TSHR. Our results demonstrated that the aging induces an increase of TSHR with disordered localization in the thyrocytes, within the follicles, and in the interfollicular spaces (Figure 2(b)). In Figure 3, a strong labeling of thyroglobulin in an interfollicular space is evident in old animals. Since the Gal-3 has been shown to change in the pathological conditions [18], we wondered if this also occurs in aging. To this end, we applied the immunohistochemistry analysis to measure the content and distribution of Gal-3 in the thyroid tissue. We found that the level of Gal-3 is increased in old animals and especially it is abundant in colloid (Figure 4). Following the above finding, we investigated whether nSMase was also changed during the aging process. As shown in Figure 5, nSMase is present in thyrocytes and accumulates in the colloid of young animals; in aging, the nSMase fluorescence is weaker in colloid and increases in thyrocytes.

3.2. Discussion. In the human thyroid gland, there is an age-dependent variation of follicular size and expression of iodine transporters [19]. Sorrenti et al. have recently reported an increase of nodular thyroid disease in the elderly [20]. Pasqualett and colleagues [21] reviewed the international scientific literature showing an increase of TSH associated with a reset of the hypothalamus-pituitary-thyroid (HPT) axis. A prompt diagnosis and treatment of HPT axis hypofunction are strongly recommended in elderly patients

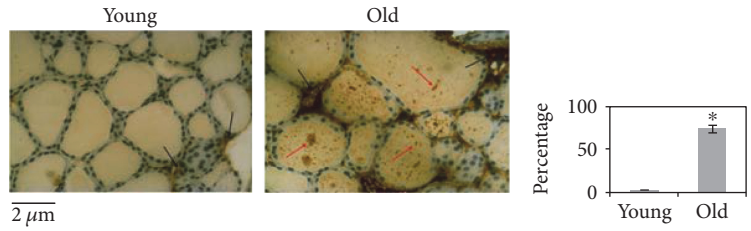

Figure 4: Immunohistochemical expression of galectin-3. Strong staining is seen in the majority of follicles and in interfollicular spaces of old mice, as shown by arrows. The positivity of staining was measured as the percentage of total area (follicles and interfollicular spaces). Data represent the mean ± S.D. of three independent experiments performed in duplicate. 40x magnification. Significance, $^{*}P < 0.05$, versus young sample.

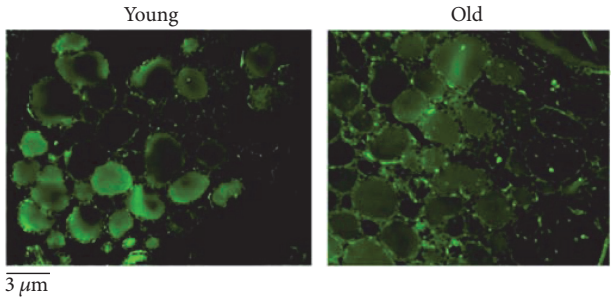

Figure 5: Fluorescence immunostaining of neutral sphingomyelinase (nSMase), by using anti-nSMase antibody in thyroid tissues of young and old mice. The high level of fluorescence is found in the thyrocytes and colloid of young animals. With aging, the fluorescence localization increases in thyrocytes and reduces in colloid. 20x magnification.

[22]. Interestingly, our study showed an abnormal distribution of TSH and TSHR inside the follicles. TSH is present in great quantity in the colloid. It is possible that the accumulation of TSH is due to a lack of hormone receptor response. We showed that the TSHR is overexpressed in aging but distributed in a totally disordered manner. Moreover, the thyroglobulin fills the interfollicular spaces which have been increased in volume with aging. The possibility that thyroglobulin is released in the interfollicular spaces because it cannot be used for the synthesis of thyroid hormones cannot be excluded. In this way, our findings might support previous results showing the reduction of thyroid hormone in the blood [19–22]. Prevalence of thyroid dysfunction and its impact on cognition in older adults has been described [23].

However, what molecule is involved in the structure/function alteration of thyroid in aging remains yet unclear. In this study, we provided strong evidence that Gal-3, known to be overexpressed in thyroid cancer [10] by leading to the attenuation of apoptosis [18], is upregulated and moves into colloid during aging, an effect similar to that shown in thyroid damage induced by microgravity conditions [11]. Our results suggest that some thyroid changes occurring during aging parallel modifications are associated with the onset of thyroid gland cancer. Importantly, there are instances in which nSMase is known to mediate alterations in different organs in response to aging, via ceramide production [24]. Thus, we studied the localization of nSMase in the thyroid from young and old animals. The changes of nSMase that result from our study could indicate its possible role in thyroid gland disorders induced by aging. The involvement of nSMase in thyroid damage has been widely described. nSMase in thyroid cells is so important that the enzyme was considered a marker for damage induced during space flights [11]. The exact role of Gal-3 and nSMase is not clear, but the expression levels of both molecules are known to directly stimulate thyroid disorders, as it occurs in microgravity [11]. Our data merely suggest that during aging, not only the HPT axis, the follicular structure, and the synthesis of thyroid hormones are altered as reported in the literature, but also the thyroid gland takes damage similar to cancer as well as damage similar to those induced by microgravity and radiation.

4. Conclusions

Our data show an increase in the volume of follicles not statistically significant, an increase in the TSH expression that fills the colloid, and in thyroglobulin that diffuses in the interfollicular spaces. Moreover, TSHR also appears upregulated. Notably, Gal-3 and nSMase are upregulated and delocalized in comparison with control mice. Taken together, these data suggest that not only the follicle structure and molecules involved in the synthesis of thyroid hormones change in aging, by supporting previous data [19–22], but also molecules involved in the thyroid damages, such as Gal-3 [11, 18] and nSMase [11–24]. To our knowledge, this is the first study correlating thyroid changes in aging and markers of thyroid damages including cancer. Here, we found higher expression of Gal-3 and a redistribution of nSMase in the thyroid of old animals in comparison with those of young animals. This body of work suggests that Gal-3 and nSMase, by inducing thyroid changes, might be the cause of the increased expression and altered distribution of TSH, thyroglobulin, and TSHR or vice-versa. Future studies will clarify this point.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Giovanna Traina and Samuela Cataldi contributed equally to this work.

Acknowledgments

The authors thank the University of Udine and University of Perugia for the realization of the work.

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 6825493, 16 pages
https://doi.org/10.1155/2017/6825493

Review Article

HIV as a Cause of Immune Activation and Immunosenescence

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Received 30 June 2017; Revised 9 October 2017; Accepted 11 October 2017; Published 25 October 2017

Academic Editor: Carmela R. Balistreri

Systemic immune activation has emerged as an essential component of the immunopathogenesis of HIV. It not only leads to faster disease progression, but also to accelerated decline of overall immune competence. HIV-associated immune activation is characterized by an increase in proinflammatory mediators, dysfunctional T regulatory cells, and a pattern of T-cell-senescent phenotypes similar to those seen in the elderly. These changes predispose HIV-infected persons to comorbid conditions that have been linked to immunosenescence and inflamm-ageing, such as atherosclerosis and cardiovascular disease, neurodegeneration, and cancer. In the antiretroviral treatment era, development of such non-AIDS-defining, age-related comorbidities is a major cause of morbidity and mortality. Treatment strategies aimed at curtailing persistent immune activation and inflammation may help prevent the development of these conditions. At present, the most effective strategy appears to be early antiretroviral treatment initiation. No other treatment interventions have been found effective in large-scale clinical trials, and no adjunctive treatment is currently recommended in international HIV treatment guidelines. This article reviews the role of systemic immune activation in the immunopathogenesis of HIV infection, its causes and the clinical implications linked to immunosenescence in adults, and the therapeutic interventions that have been investigated.

1. Introduction

More than 3 decades following the discovery that the human immunodeficiency virus (HIV) causes the acquired immune deficiency syndrome (AIDS), there is an increasing evidence that systemic immune activation plays a significant role in the disease pathogenesis [1]. High levels of systemic immune activation and inflammation not only promote viral replication and $CD4^+$ T-cell apoptosis, but also may lead to more rapid decline of immune function and competence. This resembles the phenomenon of immunosenescence that has been associated with ageing [2]. While combination antiretroviral therapy (cART) has improved the quality of life and reduced mortality and morbidity in HIV-infected persons, long-term virally suppressive treatment has not been successful in normalizing elevated markers of systemic immune activation [3]. HIV-infected individuals remain at a high risk of developing degenerative, dysfunctional, or malignant non-AIDS-defining diseases; many of which have been linked to immunosenescence and inflamm-ageing [4].

An ageing immune profile is characterized by decreased production of naïve T-cells and an increase in the proportion of memory T-cells with oligoclonal expansion [5]. The senescent T-cell phenotype is marked by a lack of CD28 expression, decreased homing receptors (e.g., CD62L and CCR7), and increased expression of the senescence marker, CD57 [6]. In addition, senescent cells are characterized by decreased proliferative capacity as indicated by shortened telomere length (TL), cell cycle arrest, increased β-galactosidase activity, limited proliferation in response to antigen stimulation, and activation of proinflammatory secretory pathways [6]. Several immunological changes seen in HIV-1-infected people are comparable to those observed in the aged. Proinflammatory cytokines, which are increased in HIV infection, including tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, are known to play a role in

ageing [7, 8]. Increased secretion of interferon (IFN)-α and reduced production of IL-2 are observed in both HIV infection and ageing [9]. Similarities in T-cell differentiation also exist, such as a reduction in the longevity of $CD4^+$ and $CD8^+$ T-cells, reduced production of naïve $CD4^+$ T-cells, increased numbers of late-differentiated $CD4^+$ and $CD8^+$ T-cells, and shortened TL [9].

In HIV-infected persons, systemic immune activation and $CD4^+$ T-cell function are inextricably linked to immunosenescence, in what appears to be a self-perpetuating cycle. The changes in immune and cytokine release resulting from HIV-induced immune activation increase susceptibility to activation-induced cell death [10–13]; consequent immune exhaustion results in senescence and programmed $CD4^+$ T-cell death, which further drive immune activation [14–17]. In both the aged and in HIV, immunosenescence has been associated with negative immune outcomes, such as thymic involution, reduction in the overall T-cell repertoire, autoimmunity, and poor antigen responsiveness [6]. Immunosenescence seems to be of particular importance in the pathogenesis of conditions where inflammation represents a significant risk factor, such as atherosclerosis and cardiovascular disease (CVD), neurodegeneration, and cancer [6]. Indeed, in the ART era, development of non-AIDS-defining, age-related comorbidities, such as osteoporosis, atherosclerosis, and neurocognitive decline, is a major cause of morbidity and mortality in HIV-infected persons [18]. The Strategies for Management of Antiretroviral Therapy (SMART) study demonstrated that deaths were mostly due to non-AIDS-defining malignancies (19%) and CVD (13%), while opportunistic diseases only accounted for 8% [19].

This study reviews the role of systemic immune activation in the immunopathogenesis of HIV infection and the causes of systemic immune activation and inflammation. We also review the clinical implications of accelerated ageing and age-related morbidity in adults and therapeutic interventions investigated to date. Data for this review were identified through searches of publicly available databases, for example, Medline and Pubmed, and in the references of studies found through these searches. Particular attention was paid to biologically *mechanistic* studies and review articles focused on systemic immune activation in HIV-infected persons. Preference was given to recent studies, that is, published in the last decade, but earlier studies that were relevant were also included.

2. Systemic Immune Activation in the Immunopathogenesis of HIV Infection

Introduction of HIV into host cells activates a complex network of protective responses originating from both the innate and adaptive immune systems [20]. These responses are either insufficient or too late to eliminate the virus. This enables life-long viral latency and chronic infection, which drives ongoing immune activation and progressive immunodeficiency, characterized by high cell turnover, apoptosis, and activation-induced death of immune cells [21].

Studies of pathogenic and nonpathogenic models of simian immunodeficiency virus (SIV) infection have provided insights into the role of systemic immune activation in the progression to AIDS [22]. The natural hosts of SIV, such as the African green monkey and sooty mangabey, are able to live normally with the virus and do not progress to immunodeficiency, regardless of high levels of viral replication. On the other hand, inoculating other nonhuman primates, such as rhesus macaques and Asian pigtailed macaques, with SIV results in immunodeficiency and progression to AIDS similar to that in HIV-infected humans [23–26]. During both pathogenic SIV (pSIV) and nonpathogenic SIV (npSIV) infection, robust viral replication and early antiviral responses occur during the acute phase of infection. However, it appears that the natural hosts have devised an evolutionary strategy to maintain an effective response, which enables symbiotic coexistence [27, 28]. This adaptive response appears to be associated with early resolution of acute T-cell activation, rather than an improved viral control.

It is thought that differences in immune response determine whether pSIV or npSIV infection develops. pSIV studies have demonstrated substantial loss of mucosal T-helper (Th) 17 cells, with subsequent microbial translocation as evidenced by high levels of plasma lipopolysaccharide (LPS) and soluble CD14 (sCD14) [28]. pSIV is associated with dysregulation of cell cycle and T regulatory cell (Treg) loss. This indicates a failure to the control of T-cell activation/proliferation and contributes to poor outcome [28]. Other characteristics distinguishing natural from unnatural hosts include superior cell homeostasis, higher numbers of $CD4^+$ T-cells, the presence of anti-inflammatory mechanisms such as attenuated IFN signalling, maintenance of progenitor cell regeneration, and more limited immune activation, and T-cell apoptosis [27, 28].

In humans, elite controllers are a unique yet heterogeneous group of people that maintain adequate control of viral replication even in the absence of cART [22, 29]. Unlike in npSIV, elite controllers are able to downregulate viral replication in lymphoid tissue. They also have powerful and durable anti-HIV immune responses, with significantly higher activation of T-cells compared to uninfected individuals. However, this is relatively less than that seen in HIV-infected persons who are not elite controllers [29, 30]. Many elite controllers do eventually experience immune-mediated depletion of $CD4^+$ T-cells and develop AIDS-defining diseases. It has been shown that basal levels of immune activation determine this progression [31].

3. Causes of Systemic Immune Activation in HIV

3.1. Direct Effects of Virions and/or Viral Proteins. HIV gene products, such as gp120 and Nef, directly stimulate the activation of lymphocytes and macrophages, resulting in the secretion of proinflammatory cytokines and chemokines [32]. Certain HIV proteins imitate and/or enhance TNF-receptor signalling, causing persistent HIV replication in infected cells through activation of nuclear factor (NF)-κB, a prototypical proinflammatory signalling pathway [33], and apoptosis of uninfected bystander T-cells [34].

3.2. Viral Coinfections. Coinfection with other viruses, such as cytomegalovirus (CMV), Epstein-Barr virus (EBV), and hepatitis B virus (HBV) and hepatitis C virus (HCV), is common in HIV-infected individuals. Pathogenic gene products enhance the replication of HIV by transactivation of HIV long terminal repeats (LTRs) [35]. HIV-induced immunodeficiency and replicative senescence, which result in the loss of $CD8^+$ T-cell populations that control viral replication, may, in turn, reactivate other pre-existing viruses or exacerbate infection by increasing viral load (VL) and consequent viral persistence [2]. This accelerates disease progression and contributes to systemic immune activation [36, 37]. CMV accounts for approximately 10% of the circulating memory T-cell repertoire in healthy, asymptomatic, HIV-uninfected CMV-seropositive individuals. The vast majority of HIV-infected individuals, between 75% and 90%, elicit significant CMV-specific T-cell responses [37, 38]. Chronic coinfection with CMV has been associated with immunological senescence, that is, gradual age-related deterioration of the immune system, homeostatic changes, and low $CD4^+$ T-cell counts. It is noteworthy that the latter is particular for naïve T-cell counts, possibly due to decreased T-cell renewal capacity and thymic involution, which lead to inadequate T-cell reconstitution [39].

HIV-1-infected individuals normally have a higher content of EBV in their lymphoid tissues, or a larger number of EBV-infected cells in their peripheral blood mononuclear cells (PBMCs), than HIV-uninfected individuals. It is thought that the expansion of EBV-positive B-cells may be caused by chronic B-cell stimulation driven by HIV proteins and impaired immune surveillance against EBV secondary to immunodeficiency [40]. A strong association has been found between HIV viremia, markers of immune activation, and EBV DNA load in PBMCs [41].

Hepatocytes and Kupffer cells, the latter of which are liver macrophages, are derived from blood monocytes, phagocytose, and clear particles draining through the portal system. Decreased Kupffer and $CD4^+$ T-cell counts have been found in individuals coinfected with HIV and HCV [42–44]. This cell loss may be due to the direct cytotoxic effects of HIV, specifically induced programmed cell death due to soluble viral or host factors, and altered Kupffer cell trafficking to target sites [44]. In coinfected people, elevated levels of sCD14 and LPS are found in the blood, due to a decrease in the clearance of particles and microbial products following diminished Kupffer cell numbers [42–44]. The reduction in $CD4^+$ T-cells occurring during HIV-1 infection may also lead to inadequate viral control, thereby permitting reactivation of HCV, which perpetuates the cycle of viral replication and immune activation [32].

3.3. Persistent Elevation of Type I and II Interferons (IFNs). IFNs I and II are produced by the innate immune system during HIV infection. IFN I plays an important role in mediating continuous inflammation. It is produced by plasmacytoid dendritic cells (pDCs) following direct activation of toll-like receptor (TLR)-7 and toll-like receptor (TLR)-8 by HIV RNA [45–47]. IFN I levels increase with plasma HIV-1 RNA levels and decrease with $CD4^+$ T-cell counts [48]. IFN I leads to the synthesis and recruitment of more target cells for HIV by upregulating the HIV coreceptor, C-C chemokine receptor type 5 (CCR5), and inducing pDC production of CCR5 ligands. IFN I also suppresses thymic output, limits $CD4^+$ T-cell recovery, induces $CD4^+$ T-cell apoptosis, and limits antigen-specific $CD4^+$ and $CD8^+$ T-cell responses [49]. IFN I further stimulates expression of the immunosuppressive enzyme, indoleamine 2,3-dioxygenase (IDO), leading to dysfunctional and immunosuppressive Tregs [48]. The elevated production of IFN-α leads to upregulation of proapoptotic molecules [50]. The administration of IFN II to HIV-infected individuals reduces the number of $CD4^+$ T-cells [49]. There is a close association between the elevation of types I and II IFN, IL-12, monocyte- and DC-derived inflammatory cytokines, and T-cell activation in HIV-infected individuals on ART [51]. The inadequate regulation of IFN responses drives chronic immune activation [52, 53].

3.4. Microbial Translocation. In the early stages of infection, HIV preferentially infects and depletes CCR5-expressing $CD4^+$ T-cells in the gastrointestinal tract (GIT) [54–58]. The accumulation of inflammatory cells, such as pDCs, neutrophils, and monocytes, and a concomitant decrease in cells that regulate epithelial homeostasis, such as IL-17 and IL-22-producing $CD4^+$ T-cells, progressively compromise mucosal integrity [59–64]. In turn, this inflammatory environment may lead to alterations in tight junction protein expression, decreased expression of claudins, upregulation of channel-forming claudins (e.g., claudin 2), and possibly increased epithelial and enterocyte apoptosis [65–69]. Dysfunction of the epithelial barrier in the GIT then allows translocation of microbial products from the intestinal lumen into the systemic circulation [70].

Pattern recognition receptors, such as nucleotide-binding oligomerization domains (NODs) and TLRs, detect microbial-associated molecular patterns (MAMPs), such as peptidoglycan, LPS, flagellin, and CpG DNA. The binding of microbial products to these receptors on cells of the innate immune system, most notably monocytes, macrophages, and DCs, activates a signalling cascade resulting in the production of proinflammatory cytokines, such as IL-1β, IL-6, TNF-α, and type-1 IFNs, such as IFN-α and IFN-β [43, 71]. For example, when TLR-4 recognises LPS, peripheral macrophages and DCs are directly stimulated to secrete proinflammatory cytokines [32]. This results in local and systemic immune activation and inflammation [65, 72–74].

Elevated levels of intestinal fatty acid-binding protein (I-FABP), originating from enterocytes, are found in the bloodstream of HIV-infected individuals [75]. I-FABP is a marker of enterocyte damage, which is associated with impaired intestinal function and microbial translocation. Enterocyte loss may be due to their reduced glucose uptake and increased expression of proinflammatory markers, such as TNF-α [43]. In response to the interaction between cell surface TLR-4 and monocyte activation, sCD14 is secreted into the blood [76–78]. sCD14 is a marker of LPS bioactivity and monocyte activation and is an independent predictor of mortality in HIV infection [75]. It may consequently be a

clinically useful surrogate marker of immune activation [51]. The interaction between LPS and LPS binding protein (LBP) leads to activation of NF-κB and increased cytokine expression. LPS-induced monocyte activation may also trigger the coagulation cascade through increased production of procoagulant tissue factor (TF), which correlates with increased levels of sCD14, D-dimer, and LBP [79]. Microbial translocation correlates with poor $CD4^+$ T-cell recovery, HIV disease progression, and susceptibility to non-AIDS conditions such as CVD and neurocognitive impairment [80].

4. The Detrimental Consequences of Systemic Immune Activation

The detrimental consequences of systemic immune activation are multifaceted. While some are particular to HIV, for instance immune system dysregulation, many are similar to the human ageing process and affect multiple organ systems.

4.1. Immune System Dysregulation. Immune dysregulation is characterized by a shift in leukocyte activity and an imbalance in cytokine levels. Derangement of both the innate and adaptive immune systems is associated with increased apoptosis of $CD4^+$ T- and B-cells, immunoparalysis of monocytes, and endotoxemia following microbial translocation [81]. In addition, continuous viral replication leads to a loss of T-cell homeostasis, characterized by increased T-cell turnover, an increase in the differentiation of naïve to antigen-exposed cells, T-cell replicative exhaustion, and apoptosis.

Immune activation also leads to depletion of T-cells and memory T-cell pools by other mechanisms, such as a decrease in the overall half-lives of $CD4^+$ and $CD8^+$ T-cells, irregular T-cell trafficking within T-cell subsets, and selective T-cell clonal exhaustion [21, 57]. A reduction in $CD4^+$ T-cells compromises the host's ability to combat pathogens and results in frequent and recurrent opportunistic and nonopportunistic infections. Inhibition of the normal functions of B-cells, NK, and other antigen-presenting cells also results in suboptimal viral control, further contributing to continuous activation of the immune system [82]. T-cells reach a state of persistent replicative senescence and immune exhaustion with the loss of antigen specificity in the immune system [83].

Cytokines play a vital role in coordinating host inflammatory response and are consequently useful markers of inflammation and systemic immune activation. Excessive production of either proinflammatory, for example, IL-1β, IL-2, IL-6, IL-8, and TNF-α, or anti-inflammatory cytokines, for example, IL-4, IL-10, and IL-13, imbalances immune responses [84]. Activation of T-, B-, and NK cells by HIV antigens and their components may increase the secretion of proinflammatory cytokines, chemotactic cytokines, for example, macrophage inflammatory protein (MIP)-1α, and adhesion molecules associated with inflammation, such as intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) [85–87]. Activation of monocytes, pDCs, and myeloid DCs may increase secretion of CXCL9, (monokine induced by gamma interferon (MIG)), CXCL10 (IFN gamma-induced protein 10 (IP-10)), CCL2 (monocyte chemoattractant protein-1 (MCP-1)), and TNF-α [51]. This culminates in T-cell activation and cytokine-driven T-cell apoptosis [88]. Increased proinflammatory cytokine levels increase susceptibility to inflammation-related conditions, such as osteoporosis, arteriosclerosis, cardiovascular conditions, and cancers [32].

Infection of pDCs by HIV may also increase immunosuppressive IDO and transforming growth factor (TGF)-β1, which impact immune dysregulation and T-cell homeostasis. The predominant origin of TGF-β1 is likely to be Tregs, but platelets, macrophages of the M2 phenotype, and immunoregulatory $CD8^+$ T-cells may also produce it [88]. Activation of TGF-β1 signalling in fibroblasts triggers increased procollagen and chitinase 3-like-1 production. This leads to collagen deposition, tissue fibrosis, and fibroblastic reticular cell network loss within the parafollicular T-cell zone of lymph nodes [89–91]. The interaction between mucosal intestinal myofibroblasts (IMFs) and LPS also leads to an increase in the soluble mediators of fibrogenesis (IL-6 and TGF-β1), which directly increase collagen deposition by IMFs [92]. This may contribute to the disproportionate depletion of $CD4^+$ T-cells in the GIT [90]. The ratio of Th17 to Tregs remains diminished during ART [93]. Such an imbalance may drive elevated IDO production by DCs, with subsequent impaired production of IL-17 and IL-22, leading to compromised antimicrobial immunity and tissue repair at barrier surfaces, with sustained immune activation [94, 95].

4.2. Thymic Function Alteration. During successful HIV suppression, $CD4^+$ and $CD8^+$ T-cell numbers are replenished, either through de novo thymic production, or through the proliferation of existing cells. As thymic output diminishes with age, naïve cells are mainly created through the latter process [96]. HIV infection can induce thymic damage through direct infection and killing of thymocytes, apoptosis, or disruption of the thymic stromal architecture, resulting in defective thymopoiesis and apoptosis of $CD4^+$ T-cells [97]. These changes mimic those induced by ageing, characterized by a decrease in the size and compartments of the thymus, and reduced thymopoiesis [5]. Thymic involution is associated with immunosenescence, with dysfunction of the immune system secondary to alterations in T-cell composition, most notably a shift from naïve to terminally differentiated cells [5, 98]. Thymic recovery may occur in some patients on ART; however, extensive thymic damage generally hampers immune reconstitution.

Systemic immune activation, independent of $CD4^+$ T-cell count and HIV VL, also results in inflammatory damage to the thymus [99]. In this case, thymic dysfunction through suboptimal production of naïve T-cells and greater differentiation of naïve into effector/memory cells occurs [100]. Immune reconstitution in HIV-infected individuals has been directly associated with thymic cellularity and volume, with the efficacy of reconstitution inversely correlated with age [101–103].

4.3. Systemic Inflammation. The proinflammatory state is associated with the development of major degenerative

diseases in the elderly [104]. In HIV-associated immune activation, there is an increase in proinflammatory mediators, TNF-α, IFN-α, IL-2, and IL-8, and dysfunctional Tregs, which lead to such an inflammatory state. HIV-infected individuals are predisposed to chronic inflammatory conditions leading to a host of progressive age-related diseases, so-called "Inflamm-ageing" [18]. This includes inflammatory bowel disease, osteoarthritis, heart disease, kidney and liver diseases, metabolic syndrome, dementia, cancer, and frailty [105, 106]. Inflammatory biomarkers, such as C-reactive protein (CRP), IL-6, and D-dimer, are elevated in HIV-infected persons compared to HIV-uninfected persons. Randomized clinical trials have demonstrated correlations between these biomarkers, disease progression, and mortality [18, 107].

4.4. Development of Non-AIDS-Associated Disease. The most significant consequence of systemic immune activation, especially in terms of long-term morbidity and mortality, is the development of non-AIDS-associated diseases. In fact, increased inflammatory biomarkers are predictive of the development of non-AIDS conditions, independent of $CD4^+$ T-cell count and HIV VL [32]. Many of these are also associated with ageing and have been linked to immunosenescence. The most common non-AIDS conditions related to immune activation include the following.

4.4.1. Cardiovascular Disease. Individuals in the chronic phase of HIV disease have a greater risk of endothelial dysfunction and subclinical atherosclerosis than HIV-uninfected persons [108]. Endothelial dysfunction is characterized by elevated levels of endothelial lesion biomarkers and endothelial cell adhesion molecules, such as ICAM-1, VCAM-1, E-selectin, P-selectin, thrombomodulin, class 1 tissue plasminogen activator, and class 1 tissue plasminogen activator inhibitor (PAI-1) [109]. When HIV infects endothelial cells, endothelial dysfunction may result from the release of cytokines by activated monocytes or directly by gp120 and transactivator of transcription (Tat) HIV proteins altering cell signalling pathways [110, 111].

Both HIV and its treatment have been associated with vasculopathy and hypercoaguability with subsequent thrombosis [112]. *In vitro* studies have demonstrated that HIV may affect the storage and secretion of proteins that affect homeostasis, such as von Willebrand factor. HIV may also affect the fibrinolytic system through the release of TNF-α, which in turn increases the expression of PAI-1 in endothelial cells, a known risk factor for thrombosis. HIV proteins, specifically gp120, activate arterial smooth muscle cells to release TF, triggering coagulation through the extrinsic pathway. Conversely, HIV infection is also associated with reduced levels of anticoagulant proteins C and S and antithrombin III [113]. Thrombosis, often in the context of the metabolic syndrome, has also been linked to the protease inhibitor (PI) class of HIV treatment [114]. High levels of TNF-α and PAI-1, and increased expression of the scavenger receptor, CD36, with subsequent increased absorption of cholesterol, have been found in PI-treated individuals [115, 116].

A key component of atherogenesis in both HIV and ageing is the formation of lipid-laden macrophages (i.e., foam cells), secondary to unregulated uptake of modified lipoproteins, especially oxidized low-density lipoprotein (oxLDL), under the influence of CD36 [117]. HIV-infected persons have been shown to have increased levels of oxLDL and higher expression of CD36 and TLR-4 in monocytes [118]. OxLDL levels correlate with markers of monocyte activation, for example, sCD14, and TF expression on inflammatory monocytes [118]. Oxidized lipids are thought to play a role in atherosclerosis through alteration of nitric oxide (NO) signalling, initiation of endothelial activation, and expression of adhesion molecules that promote leukocyte homing [119]. The ensuing inflammatory process releases downstream biomarkers, such as IL-6, VCAM-1, selectins, fibrinogen, D-dimer, CRP, and TF, that predispose the patient to accelerated coronary atherosclerosis and arteriosclerosis and subsequent CVD including myocardial infarction, heart failure, stroke, and sudden cardiac death [120–123]. A recent mouse model has shown that the pathological process is driven by macrophages in the subendothelial space expressing senescence markers, namely elevated senescence-associated β-galactosidase activity, $p16^{Ink4a}$, p53, and p21. This increases expression of key atherogenic and inflammatory cytokines and chemokines and promotes plaque instability by elevating metalloprotease production [124].

4.4.2. Renal Disease. Individuals living with HIV are at an increased risk of renal diseases such as acute tubular necrosis, HIV-associated nephropathy (HIVAN) [125], HIV immune complex kidney disease, hypertensive and atherosclerotic renal diseases, and toxicity secondary to potentially nephrotoxic medication, such as tenofovir disoproxil fumarate (TDF) [126]. HIVAN is one of the major risk factors of end-stage renal disease and is histologically defined as a collapsing form of focal segmental glomerulosclerosis (FSGS), microcystic tubular dilation, tubointerstitial inflammation, and fibrosis [127]. FSGS is similar to atherosclerosis and involves the buildup of cholesterol, activation of monocytes, release of lipid-laden macrophages, and fibrosis, suggesting that similar inflammatory processes may be involved [128]. The pathogenesis of HIVAN is not entirely understood; however, it has been suggested that it is triggered by direct viral infection of renal epithelial cells, Nef-induced podocyte dysfunction, renal tubular epithelial cell apoptosis induced by Vpr, and upregulation of proinflammatory mediators, especially those induced by NF-κB [127].

In ageing, senescent cells are important sources of inflammation and increased levels of biomarkers of inflammation, coagulation, and endothelial dysfunction, such as TNF-α, IL-6, MCP-1, CRP, and PAI-1, are commonly seen in this population [128]. Recruitment of T-cells into the renal tubulointerstitial compartment has been implicated in many renal inflammatory diseases and is an important mediator of tubular injury leading to progressive renal failure in HIVAN [129, 130]. Interactions between primary renal tubule epithelial cells (RTECs) and HIV-infected T-cells induce potent inflammatory gene responses. The consequent release of cytokines/chemokines from RTECs may then attract additional T-cells. Resident proximal tubular epithelial cells also

participate in the inflammatory process by enhancing cytokine/chemokine communication with interstitial immune cells [131]. Activation of RTECs by infiltrating T-cells perpetuate local inflammatory responses through upregulation of proinflammatory chemokine/cytokine production mediated by soluble factors or by direct cell-to-cell contact [132]. The HIV-upregulated cytokines/chemokines in the RTECs include inflammatory cytokines CCL20, IL-6-, and the IL-8 related chemokines: CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL8 (IL-8). The receptors to these chemokines are expressed on certain populations of T-cells (reviewed in [133]) and, thus, may also be involved in promoting the mononuclear infiltration observed in HIVAN. The infiltration of HIV-infected cells into the kidney and activation of chemokines have been implicated in reduced survival of kidney allografts after transplantation, despite undetectable viremia [134] and the high prevalence of interstitial nephritis found in kidney biopsies in HIV-infected patients [135].

4.4.3. Cognitive Impairment. HIV-infected individuals manifest a spectrum of cognitive, motor, and psychological dysfunctions similar to that found in ageing, ranging from asymptomatic neurocognitive impairment to HIV dementia. Following infection, HIV is believed to enter the central nervous system (CNS) in infected mature $CD14^+CD16^+$ monocytes that traffic to the CNS as part of the turnover of perivascular macrophages [136]. Once inside the CNS, the virus infects microglia and may remain dormant for an extended period of time. HIV does not directly destroy cells of the CNS in large quantities; instead, it triggers a cascade of deleterious inflammatory changes affecting cellular signalling and resulting in oxidative stress [137]. Proinflammatory cytokines may damage neurons, while high levels of reactive oxygen species (ROS) may damage DNA and RNA [138]. The HIV VL in the brain does not determine the extent of the inflammatory response. In individuals on ART, minuscule amounts of residual virus may be sufficient to maintain a self-perpetuating inflammatory response [137]. High levels of macrophage activation markers, such as sCD163, sCD14, and CCL2 in cerebrospinal fluid and blood, together with inflammatory biomarkers, such as CRP, IL-6, TNF-α, IP-10, and neopterin, have been implicated in the development of HIV-associated neurocognitive disorders (HAND) [139, 140]. This is similar to what has been observed in the elderly, where inflammatory markers, particularly IL-6 and CRP, have been linked to cognitive decline and an increased risk of dementia [141].

The CNS and microglial cells may potentially serve as anatomical and cellular reservoirs, respectively, where HIV-1 may persist during chronic infection despite successful cART. The persistence of HIV in the CNS and microglia may result in immune activation with consequent microglia senescence [142]. Brain imaging of HIV-1-infected patients on cART using positron emission tomography imaging and ^{11}C-PK11195 as an *in vivo* marker of microglia activation reveals activation of microglia even in the absence of neurological symptoms [143]. The CSF from HIV-1 patients also contains increased levels of inflammatory cytokines including TNF-α, β2-microglobulin and neopterin, IL-1α, and S100β [144]. The latter, an intraneuronal calcium-inducing cytokine, could further contribute to neuronal degeneration [145]. Microglia have been demonstrated to undergo telomere shortening, which is a characteristic of senescence, in an animal model [146]. Emerging evidence from *in vitro* models also suggests that microglia could potentially develop a senescence-like phenotype characterized by reduced phagocytic and migratory capacities of microglia [147]. A dystrophic microglial phenotype has been observed to increase with ageing and has been detected in neuropathological conditions, such as Alzheimer's disease [148]. Although the progression and exact nature of microglial "ageing" remains unclear, activation and senescence appear to be integral parts of the process. Moreover, HIV-1 infection or bystander effects of HIV-1 infection seem to disrupt the delicate balance of cell survival, cell cycle progression, and apoptosis, which could contribute to the development of HAND [142].

4.4.4. Osteoporosis. HIV-infected persons have an increased prevalence of osteoporotic fractures compared to age-matched, HIV-uninfected individuals [149]. In addition to traditional risk factors, such as smoking, alcohol, low body weight, and vitamin D deficiency, HIV-infected patients have additional risk factors brought about by the virus' direct and inflammatory effects on bone resorption [150], as well as the effects of ART, especially TDF [151]. The major inflammatory pathways that have been identified involved cytokines that have also been shown to be elevated during senescence [152]. For example, TNF-α increases the expression of the receptor activator of NF-κB (RANKL), which accelerates osteoclastic bone resorption [150]. In addition, TNF-α and IL-1 inhibit osteoblast function and stimulate osteoblast apoptosis through activation of the inflammatory mediator, NO [152].

4.4.5. Cancer. Due to immune deficiency, HIV-infected persons are at an increased risk of developing non-AIDS-defining malignancies, such as Hodgkin's lymphoma, head and neck, lung, liver, kidney, skin, and anal cancers [153, 154]. Factors contributing to the development of non-AIDs defining cancers include the virus itself, tobacco exposure, and possibly ART [154]. HIV may activate proto-oncogenes, alter the regulation of the cell cycle, inhibit tumour suppressor genes, or cause endothelial abnormalities, such as proangiogenesis signalling that may facilitate tumour growth and metastasis [154]. Other persistent viral coinfections commonly found in HIV-infected persons, such as HBV, HCV, human papillomavirus, and EBV, also play a role. Elevated levels of EBV-positive B-cells, which express latent membrane protein 1, a key viral protein in EBV-mediated transformation of B-cells, correlate with an increased long-term risk for such individuals to develop Hodgkin's lymphoma [40].

The risk of cancer increases with lower $CD4^+$ T-cell counts; however, there appears to be an added risk even among infected people with well-preserved immune systems. $CD8^+$ T-cells and NK cells maintain surveillance of the body

and kill cells showing signs of anomalous growth or malignant modification. However, in HIV infection, the signalling cascades that control cell development and tissue restoration may be disrupted, leading to uncontrolled cell proliferation [155].

In HIV-infected and uninfected persons, inflammation contributes to cancer development, primarily by causing oxidative stress and DNA damage. ROS and proinflammatory cytokines, such as TNF-α, activate NF-κB, which induces the expression of genes involved in cell proliferation, apoptosis, and carcinogenesis. This leads to further production of proinflammatory cytokines [156]. Macrophages, platelets, fibroblasts, and tumour cells are all sources of inflammatory angiogenic mediators, for example, basic fibroblast growth factor, vascular endothelial growth factor, and prostaglandin-E_1 and E_2 that increase the production of ROS. Additionally, many oncogenes inhibit apoptosis and, in doing so, facilitate survival of preneoplastic and malignant cells [156]. This combination of DNA damage and unchecked proliferation contribute to an increased risk of cancer.

IL-7 is important in T-cell homeostasis as it maintains the survival of the naïve T-cell pool during HIV infection [157]. Increased IL-7 leads to abnormal B-cell differentiation [158] and the upregulation of both programmed cell death protein (PD-1) and its ligands [159]. Under physiological conditions, PD-1, a negative costimulatory molecule, prevents excessive T-cell activation and assists in peripheral tolerance through promotion of Tregs [160]. The expression of PD-1, together with its cognate ligand PD-L1, is upregulated during chronic HIV infection. This is caused by the HIV Nef protein via a p38 MAPK-dependent mechanism, the cytokine-rich microenvironment, T-cell receptor-independent stimulation, and persistent activation of the innate immune system [161]. Persistently elevated levels of PD-1 expression have been observed on exhausted $CD8^+$ T-cells. The PD-1/PD-L1 signalling pathway is critical in tumour immune surveillance. Tumours may escape host immune surveillance by expressing PD-L1 [162]. PD-1 signal inhibitors have emerged as a useful therapeutic strategy in the treatment of many cancers. They are also being investigated as approaches to reverse HIV latency and facilitate eradication [160, 162].

5. Immune Activation and Early Initiation of ART

Owing to improved ART access, the prognosis of HIV-infected patients has improved, although increased morbidity and mortality persist. This is caused by clinical events such as CVD, malignancy, and inflammatory conditions exacerbated by incomplete immune recovery and residual immune activation [29, 163]. The timing of ART initiation is thought to play an important role in immune activation [53]. Data indicate that an immunologic activation set point develops in the acute phase of HIV infection, which determines the rate at which $CD4^+$ T-cells are lost over time [164]. Early ART initiation may protect and preserve lymphoid gut homeostasis and reduce microbial translocation through maintenance of epithelial integrity, maturation of mucosal DCs, and conservation of intestinal lymphoid structures [165]. Other long-term benefits include conservation of HIV-specific $CD4^+$ T-cells, reduction of the turnover rate and activation of $CD4^+$ and $CD8^+$ T-cells, and in prevention of viral evolution [166–171].

6. Therapeutic Interventions

A number of therapeutic measures have been explored with the aim of reducing systemic immune activation in HIV-infected persons. To date, most studies have been observational in nature, making it impossible to rule out confounding factors, and to our knowledge, no human trials have used markers of immunosenescence as the primary outcome. Prospective interventional studies have rather focused on the causes of immunosenescence, such as immune activation and inflammation, linked with specific outcomes [6]. Unfortunately, there is no consensus regarding the optimal combination of biomarkers for measuring either immune activation or treatment success. No single strategy has been found effective in large-scale clinical trials, and no adjunctive treatment is currently recommended in international HIV treatment guidelines.

6.1. ART Intensification and Strengthening. Intensification with the integrase strand transfer inhibitor, raltegravir, in virally suppressed persons on ART has been found to lead to a rapid increase in 2-LTR circles with a significant decrease in levels of D-dimer [172]. Most studies have not shown any significant change in $CD8^+$ T-cell activation with this strategy [173–176]. Intensification with maraviroc, a selective, reversible CCR5-receptor antagonist that inhibits the binding and signalling of CCR5 ligands, produced no effect on $CD4^+$ or $CD8^+$ T-cell counts and actually increased LPS and sCD14 levels [177, 178].

6.2. Gastrointestinal Repair Strategy. The use of prebiotics and probiotics to modify the imbalance in the bacterial profile in the GIT of HIV-infected persons has been explored. Prebiotic use showed a significant reduction in levels of sCD14 and improved the functional capability of $CD4^+$ T-cells [179–181]. Supplementation with probiotics in infected macaques demonstrated reduced IDO-1 activity, indicating improved ability to maintain mucosal homeostasis [182, 183]. Other studies have shown increased $CD4^+$ T-cell counts and lower levels of IL-6 and LBP with probiotic use [180, 181]. Administering bovine colostrum containing LPS-specific antibodies/immunoglobulin did not yield any significant change in LPS, sCD14 levels, or $CD4^+$ T-cell counts [173, 184].

Recently, it has been reported that elite controllers, who spontaneously maintain sustained control of HIV, possess a microbiota that is richer and differs in predicted functionality from treatment naïve HIV progressors, resembling the micobiota of HIV-uninfected persons [185]. Therapeutic interventions that modulate gut microbiota richness, not only composition, are important in reducing HIV-related inflammation [185]. In addition to bacterial composition, other factors such as stability, resistance, resilience, and

redundancy contribute to the functional properties of the microbiota [186]. Confirmation of microbiota-related control of HIV infection in elite controllers by metabolomic studies may result in new intervention strategies, such as faecal transplants, to control HIV [185, 187].

6.3. Treatment of Coinfections. Treatment of CMV seropositive patients with valganciclovir has demonstrated significant decreases in CMV DNA expression and activation of $CD8^+$ T-cell, but had no effect on CRP, IL-6, and sCD14 [188]. The treatment of HCV with IFN-α and ribavirin did, however, correlate with a significant decrease in TNF receptor-1 and endothelial dysfunction markers, for example, soluble E-selectin and sVCAM-1 [189].

6.4. Interleukins. The coadministration of IL-21 and probiotics to SIV-infected animals was found to increase the production of polyfunctional Th17 and reduce pathobiont translocation [190]. Administering IL-7 to patients on ART restored functionality of $CD4^+$ and $CD8^+$ T-cells, enhanced $CD4^+$ T-cell production, and restored intestinal Th17 and Th22 populations [191]. In addition, IL-7 significantly decreased the viral reservoir by activating latent virus replication [192]. Reconstitution of the immune system with excitatory cytokines such as IL-2 or IL-15 has improved $CD4^+$ T-cell counts and HIV-specific T-cell responses [9, 193].

6.5. Immune Suppressive Agents. Administering cyclosporine A as a conjunctive therapy increases average $CD4^+$ T-cell counts, possibly through the inhibition of T-cell activation and proliferation [194].

6.6. Reducing Activation of Plasmacytoid Dendritic Cells. Chloroquine and hydroxychloroquine prevent the endosomal acidification and fusion in pDCs and also inhibit IDO, a regulator of T-cell responses [195]. There is some controversy regarding the effect of chloroquine and hydroxychloroquine in HIV-infected people. Studies on chloroquine report a substantial reduction in VL in newly ART-treated patients [196, 197], a reduction in memory $CD8^+$ T-cell activation and $CD4^+$ and $CD8^+$ T-cell proliferation [195, 198]. Additional beneficial effects, such as reduced levels of LPS, IFN-α, IL-6, and TNF-α and an increase in $CD4^+$ T-cell counts, have also been demonstrated [195, 198, 199]. On the other hand, there have also been reports of no significant changes in $CD4^+$ and $CD8^+$ T-cell activation and proliferation [200]. An increase in VL and a reduction, or no change, in $CD4^+$ T-cell counts have also been found [196, 197, 201].

6.7. Immune Modulators. Administering 3-hydroxy-3-methyl-glutharyl-coenzyme A (HMG-CoA) reductase inhibitors was found to reduce D-dimer and CRP [202–207]. A study of atorvastatin demonstrated a significant reduction in $CD8^+$ T-cells compared to the control group [202]. Another study observed that the addition of statins to ART correlates with a decline in the occurrence of non-AIDS-associated cancer, non-Hodgkin's lymphoma, and a decreased mortality rate [206]. Selective cyclooxygenase type 2 (COX-2) inhibitors have been found to reduce $CD8^+$ T-cell activation and immune activation levels [208]. The active metabolite of leflunomide, a disease-modifying antirheumatic drug, reduced activated T-cell proliferation in an *in vitro* study while no significant change was observed in HIV VL or $CD4^+$ and $CD8^+$ T-cell counts in patients treated with leflunomide in a randomised clinical trial [209–212]. Studies administering rapamycin and mycophenolate as a supplementary therapy with ART have shown to lower activation and proliferation of T-cells [213, 214].

6.8. Senolytics. Senescent cells are known to accumulate in various tissues during the aging process [215], and even a small number of these cells can cause adverse age- and disease-related phenotypes due to their "proinflammatory senescence-associated secretory phenotype" [216]. Senolytics are drugs that selectively promote apoptosis of senescent cells by temporarily disabling prosurvival signalling pathways, for example, those involving "PI3K/AKT, p53/p21/serpines, dependence receptor/tyrosine kinases, and BCL-2/BCL-X_L." This has delayed or alleviated the appearance of age- and disease-related phenotypes in several animal models [216]. These drugs consequently hold promise in attenuating the appearance of age-related cell phenotypes and chronic diseases, such as diabetes, pulmonary fibrosis, osteoporosis, cardiovascular disease, and cancers [216, 217].

Various drug candidates have been identified, for example, the tyrosine kinase inhibitor, dasatinib; the naturally occurring flavonoids and related compounds, such as quercetin, fisetin, and piperlongumine; drugs that target components of the BCL-2 pathway, for example, navitoclax; and the specific BCL-X_L inhibitors, A1331852 and A1155463 [215–219]. However, none of these drugs have demonstrated efficacy on all senescent cell types, significant side effects have been observed, none have yet successfully completed preclinical studies, and concerns exist regarding toxicity following long-term use. Fisetin, A1331852, and A1155463 appear to have more favorable side effect profiles and are potentially better candidates for use in humans [215, 216].

7. Conclusion

Systemic immune activation has become a focus of research into the immunopathogenesis of HIV. This immune activation is characterized by an increase in proinflammatory mediators, dysfunctional Tregs, and a pattern of T-cell-senescent phenotypes similar to those observed in the elderly. These changes predispose HIV-infected persons to comorbid conditions that have been linked to immunosenescence and inflamm-ageing. Treatment strategies aimed at curtailing persistent immune activation may help prevent the development of these conditions. At present, early ART initiation appears to be the most effective strategy although there is difficulty in achieving this in many settings. More studies of supplementary strategies are required. Consensus should also be reached regarding the optimal combination of biomarkers for measuring systemic immune activation and its successful treatment.

Disclosure

Opinions expressed and conclusions arrived at are those of the authors and are not necessarily attributed to NRF or SACEMA.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

This work is based on the research supported in part by the National Research Foundation of South Africa, Unique Grant no. 93944, and DST/NRF Centre of Excellence in Epidemiological Modelling and Analysis (SACEMA).

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 7953486, 13 pages
https://doi.org/10.1155/2017/7953486

Review Article

The Light and Shadow of Senescence and Inflammation in Cardiovascular Pathology and Regenerative Medicine

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Received 20 July 2017; Accepted 12 September 2017; Published 8 October 2017

Academic Editor: Carmela R. Balistreri

Recent epidemiologic studies evidence a dramatic increase of cardiovascular diseases, especially associated with the aging of the world population. During aging, the progressive impairment of the cardiovascular functions results from the compromised tissue abilities to protect the heart against stress. At the molecular level, in fact, a gradual weakening of the cellular processes regulating cardiovascular homeostasis occurs in aging cells. Atherosclerosis and heart failure are particularly correlated with aging-related cardiovascular senescence, that is, the inability of cells to progress in the mitotic program until completion of cytokinesis. In this review, we explore the intrinsic and extrinsic causes of cellular senescence and their role in the onset of these cardiovascular pathologies. Additionally, we dissect the effects of aging on the cardiac endogenous and exogenous reservoirs of stem cells. Finally, we offer an overview on the strategies of regenerative medicine that have been advanced in the quest for heart rejuvenation.

1. Introduction

Cardiovascular diseases affect millions of patients worldwide and are the primary cause of mortality and comorbidities. Thanks to the incredible technological progress realized in the post second war period (1945–1964) in the Western countries, a whole generation of people started to benefit increased wealth and ameliorated life quality and expectancy [1].

For this generation and those to come, the prolonged lifespan is associated with a dramatic increase of cardiovascular diseases, mainly related to aging. This is at least partly due to a progressive impairment of the cellular processes regulating cardiac and vascular homeostasis, finally leading to the development of cardiovascular pathologies. In fact, the molecular mechanisms that protect the heart against stress are downregulated by aging, making the myocardium more susceptible to injury. Pathologies, as atherosclerosis, cardiac fibrosis, and cardiomyopathy, are often linked to the failure of cardiovascular tissue cells to reenter the cell cycle, namely, senescence, due to endogenous or exogenous causes.

This review will focus on the cardiovascular pathologies correlated to senescence, the effect of aging on the cardiac endogenous resources of stem cells, and the potential strategies of regenerative medicine to be applied to maintain the heart younger and healthier.

2. Positive and Negative Effects of Senescence on Cardiovascular Disease Onset and Progression

Cellular senescence has been for long time an underestimated biological process, even after its discovery in 1961 by Hayflick and Moorhead [2]. As demonstrated by the growing body of

literature in the latest years, senescence is an important function involved in the maintenance of tissue homeostasis, as well as the more extensively studied apoptosis.

Mitotic cells might undergo senescence by failing to replicate. Unlike the quiescence state, in which cells are reversibly dormant, senescence takes place in cells with an active metabolism that had entered the cell cycle but got stopped at G1 phase by the action of specific inhibitors (as reviewed in [3]).

While it is commonly accepted as an aging-related phenomenon, senescence might happen also during the embryonic development with the biological meaning of replacing transient structures or specific cell types with other ones [3–5]. This is partly conflicting with the hypothesis of antagonistic pleiotropy as an evolutionary justification for senescence. In fact, it was George J. Williams to observe as first that it could be described as an adaptive event during evolution, since some genetic traits demonstrated to be beneficial in the initial life stages, but disadvantageous in the elderlies [6].

Senescence in adult tissues can be classified in two main subcategories, based on the underlying molecular mechanism: replicative (or intrinsic), caused by telomere shortening, and stress-induced, in response to reactive oxygen species (ROS) and/or oncogenes [7–9].

Activation of the cellular senescence genetic program prompts a series of molecular changes, mostly affecting cell cycle, extracellular matrix (ECM), secretion of growth factors, and inflammatory mediators.

A more detailed description of the triggering stimuli and molecular pathways involved in the pathogenesis of several cardiovascular diseases will be provided below.

Senescent cells might be easily recognized in culture by typical morphological features, that is, flatness, enlargement, vacuolization, multinucleation, and progressively reduced proliferation.

Hallmarks of cellular senescence are represented by lysosomal immunodetection of β-galactosidase activity (also known as senescence-associated β-galactosidase activity), lipofuscin rendered evident after Sudan black B staining, downregulation of proliferation markers, for example, phosphohistone 3 and Ki-67, reduced incorporation of 5-bromodeoxyuridin, identification of heterochromatin foci, and increased expression of senescence mediators (e.g., p15, p16, p21, p27, and ARF) and metalloproteinases. As previously mentioned, a further phenotypic characteristic of senescent cells is their secretion of several specific proinflammatory cytokines and growth factors (e.g., IL-6, IL-8, and TGF-β) [10]. Endowed with this senescence-associated secretory phenotype (SASP), cells exert a paracrine effect on neighboring cells, as well as an autocrine conditioning [11].

Consequently, the role of senescence in a variety of pathologies has been increasingly studied in the last years. Among cardiovascular diseases, atherosclerosis and heart failure are those whose progression has been shown to be most deeply associated with cellular senescence.

2.1. Atherosclerotic Lesions. Senescence-associated alterations are commonly observed in vascular cells harvested from atherosclerotic regions [12–16]. Atherosclerosis is a chronic inflammatory vascular disease that evolves from an initial dysfunction of the intimal endothelial layer to a progressive development of advanced atherogenic plaques [17].

High blood cholesterol levels, inflammatory cytokines, growth factors, angiotensin II, and hyperglycemia, with its associated advanced glycation end products (AGEs), are powerful extrinsic stimuli for vascular cell senescence [17]. Telomere shortening and activation of the Ras pathway are regarded as intrinsic vascular senescence triggers [17].

In addition, mitochondria have been revealed to play an important role in the development of atherosclerotic lesions. In fact, mitochondrial dysfunction, mtDNA mutations, and/or release of mitochondria-specific ROS in both senescent endothelial and smooth muscle cells are considered as further putative causes of atherogenesis [18].

As recently reviewed [19, 20], the molecular basis for aging of vascular cells involves several genes (as Klotho) and proteins (e.g., Sirtuins, progerin, JunD, $p66^{shc}$, and β-amyloid peptides).

Replicative senescence in vascular cells activates molecular pathways similar to those triggered after DNA damages caused by external agents. The stress-induced typology is also frequently found in endothelial and smooth muscle cells from atherosclerotic tissues. A variety of pathways are interested in vascular DNA damage-induced cellular senescence, most converging on the activation of cyclin-dependent kinase inhibitors (p15, p16, p21, p27, and/or p53). The mediators of cell cycle progression are conversely downregulated, for example, derepressing the gene encoding for cyclin-dependent kinase inhibitor 2A. This prevents cyclin-dependent kinases (1, 2, 4, and 6) and cyclins (A, E, and D) to downregulate tumor suppressor RB. Consequently, the vascular cell is arrested at the Gap 1 phase of the mitotic replication cycle (as reviewed in [3]).

In addition to mentioned DNA damage-related pathways, activation of nuclear factor NF-κB and CCAAT/enhancer-binding protein-β pathways is required by the senescent vascular cells to activate SASP program, which includes the secretion of IL-6, IL-8, chemokines, and activators of macrophages and monocytes (MCP, MIP, TNF-α, TGF-β, and GM-CSF) as well as ECM proteases [3, 21]. Interestingly, smooth muscle cells secrete also procalcific factors, such as RUNX-2, alkaline phosphatase, collagen I, matrix GLA protein, and BMP-2 [12, 22].

The proteolytic secretome of vascular smooth muscle cells, together with ROS and the enzymes released by recruited macrophages, contributes to undermining plaque stability in the atherosclerotic cap region through ECM remodeling [23].

Moreover, also endothelial nitric oxide synthase (eNOS) and prostacyclin pathways result impaired in the senescent endothelial cells from atherogenic regions [14, 24–26].

Growing evidence suggests a crosstalk between nitric oxide (NO) signaling and the occurrence of oxidative stress in the onset and progression of age-related vascular diseases, such as hypertension, heart failure, ischemia, and stroke. Consequently, NO is being considered as an emerging molecular target for the development of new therapeutic

Table 1: Senescence in atherosclerotic lesions.

Extrinsic vascular senescence triggers	
(i) High blood cholesterol levels	
(ii) Inflammatory cytokines and growth factors	[17]
(iii) Angiotensin II	
(iv) Hyperglycemia and associated AGEs	
Intrinsic vascular senescence triggers	
(i) Telomere shortening	[17]
(ii) Activation of Ras pathway	
(iii) Mytochondrial dysfunction, mtDNA mutations, and/or release of mitochondria-specific ROS	[18]
Activated (↑) and inactivated (↓) molecular pathways and functions	
(i) Involvement of several genes (e.g., Klotho) and proteins (e.g., Sirtuins, progerin, JunD, $p66^{shc}$, and β-amyloid peptides)	
(ii) ↑ DNA damage signaling	
(a) ↑ cyclin-dependent kinase inhibitors (p15, p16, p21, p27, and/or p53)	[3]
(b) ↓ mediators of cell cycle progression (cyclin-dependent kinase inhibitor 2A; cyclin-dependent kinases (1, 2, 4, and 6) and cyclins (A, E, and D))	
(c) ↓ tumor suppressor RB	
(iii) ↑ nuclear factor NF-κB and CCAAT/enhancer-binding protein-β pathways	[3, 21]
(iv) ↑ SASP program, which includes the secretion of IL-6, IL-8, chemokines, and activators of macrophages and monocytes (MCP, MIP, TNF-α, TGF-β, and GM-CSF), as well as ECM proteases	[3, 21]
(v) ↑ procalcific factors (RUNX-2, alkaline phosphatase, collagen I, matrix GLA protein, and BMP-2) in SMCs	[12, 22]
(vi) Impairment of eNOS and prostacyclin pathways in senescent ECs	[14, 24–26]
(vii) Association between decreased levels of CDKN1 and CDKNA2 and increased propensity to develop atherosclerosis	[27, 28]
(viii) Link between CDKN1 and CDKNB2 polymorphisms and aortic aneurism	[29]
(ix) ↑ ICAM-1, PAI-1, and IL-1α and ↓ lipid metabolism ability in ECs	[30–33]
(x) ↑ SASP secretion of annexins and BMPs and $\uparrow Ca^{2+}$ in ECs favors calcification onset	[34]

AGEs: advanced glycosylation end products; SMCs: smooth muscle cells; ECs: endothelial cells.

strategies for cardio- and cerebrovascular pathologies, particularly in aged patients. Accordingly, nowadays, several natural-derived compounds or pharmacological inhibitors are being proposed as modulators of NO-mediated pathways in this population. Curiously, vascular senescence is thought to have beneficial effects in the evolution of atherosclerosis. In particular, polymorphism analyses in humans and in atherosclerotic murine models revealed that lower levels of cyclin-dependent kinase inhibitors, especially CDKN1 and CDKNA2, are associated with increased propensity to develop atherogenic lesions [27, 28]. In other genome-wide associated studies, a link between aortic aneurysms and the polymorphisms for CDKNA2 and CDKNB2 has been shown [29].

On the other hand, other studies are supportive of a view of pathologic deterioration induced by senescent vascular cells. In fact, endothelial cells with an activated senescence program secrete high levels of ICAM-1, PAI-1, and IL-1α and show a reduced ability to metabolize lipids [30–33], which is likely to aggravate atherosclerosis.

It has been recently demonstrated that endothelial SASP, and in particular its microvesicular component, is able to stimulate the onset of calcification through the overexpression of bone-related proteins, for example, annexins and BMPs, and the increase of Ca^{2+} content too [34].

Typical hallmarks, triggers, and effects of vascular senescence in atherosclerosis have been summarized in Table 1.

2.2. Heart Failure. A potentially predictive biomarker of cardiovascular senescence is leukocyte telomere length. Remarkably, its shortening is associated with several cardiovascular diseases, among which are atherosclerosis, aortic valve stenosis, and thrombogenesis. Moreover, it is also linked to the main risk factors for these pathologies, such as hypercholesterolemia and hypertension. Leukocyte telomere length correlates inversely with plaque progression, but also heart failure (HF), a severe chronic condition characterized by dilation and decreased thickness of the ventricular wall. HF is the final stage of all cardiovascular diseases and results in a progressive weakening of the global cardiac function, which is related to the dysfunctional hypertrophic and apoptotic state of the terminally differentiated cardiac myocytes [33, 35–40].

Besides apoptosis, autophagy is as well a crucial mechanism for maintaining cellular homeostasis during aging, since it ensures the removal of dysfunctional organelles and misfolded proteins that dramatically increase in aged organs. Generally, elderly patients have reduced levels of autophagy, probably due to the elevation of oxidative stress. In this regard, it has been demonstrated that cardiac-specific deletion of Atg5 in mice associates with early signs of senescence, accumulation of dysfunctional mitochondria, disorganization of sarcomere structure, and age-related cardiomyopathy [41]. Similarly, mice deficient in Parkin gradually showed a decrease in cardiac function and survival [42]. Coherently,

experimental evidence suggests that the modulation of autophagy may slow down or prevent cardiac aging. For example, stimulation of mitophagy (i.e., autophagy of mitochondria) with spermidine, a natural polyamine, was found to preserve cardiac function in old mice [43, 44]. Similarly, inhibition of miR-22, whose expression was reported to be elevated in the aged myocardium in parallel to decreased autophagic activity, rescues autophagy and improves cardiac function in old mice [45, 46].

Alterations of the inflammatory, endothelial, and myogenic phenotype of cardiac cells are also observed in senescence events occurring in the failing heart. In particular, most of these changes are related to the aging-dependent manifestation of mutations in genes involved in the calcium cycling and signaling. The activity of sarcoplasmic reticulum calcium adenosine triphosphatase, that is, SERCA2, and calsequestrin is drastically decreased during aging [47, 48], due to the lower level of expression of these proteins. Similarly, also Ica, that is, the L-type calcium current, is reduced and often completely inactivated. In this condition, the sarcoplasmic reticulum has a considerably diminished calcium concentration and, consequently, calcium transient amplitude and propagation result were impaired.

Nevertheless, also other pathways involved in heart rate modulation might be compromised by aging, for example, as cardiac sympathetic innervation. In fact, the catecholamine uptake is reduced in senescent cardiac neuronal cells as well as it is the response to isoproterenol [49]. In particular, the decreased epinephrine reuptake is physiologically compensated by the reduction of the arterial baroreflex response [49]. Moreover, noradrenalin transporter is downregulated in aging, causing an impairment in the neurotransmitter reuptake and a consequent reduction of its positive inotropic effect [50].

The human K^+ channel ether-à-go-go gene (hERG) might also be interested by age-dependent mutations causing rhythm instabilities, as well as disorganization of the structure of the sarcomeric structure and myofibrillary proteins, as evidenced in a mouse model of a mutated hERG homolog [51].

However, all these aging-related impairments increase the vulnerability of the single cells and hence of the whole myocardium to develop arrhythmic events. In fact, these occurrences are typical of elderly patients, which are therefore submitted to ICD implantation.

In addition to cardiac myocytes and neuronal cells, senescence affects also the vascular compartment, for example, compromising coronary microcirculation.

Cardiomyopathy-induced HF has very similar signs to those described for atherosclerosis. Furthermore, it has been shown that aging downregulates the mitochondrial nicotinamide adenine nucleotide histone deacetylase (SIRT) 3, which is associated with pericyte loss and endothelial dysfunction, further exacerbated in the case of concomitant diabetic condition [52].

In the presence of risk factors, such as obesity, aging-related microvascular rarefaction is correlated to adipose tissue activation of the ADAM 17/TACE gene, encoding for a metalloproteinase that is able to cleave out the active TNF-α polypeptide from pro-TNF-α. ADAM-17/TACE overexpression is also facilitated by the loss of negative regulation by caveolin-1, analogously decreased in aged obese patients [53].

In acute settings of cardiac ischemia, the activation of the senescence-associated genetic program in recruited fibroblasts can be considered a protective mechanism from endothelin-1-mediated cardiac fibrosis [54, 55].

Table 2 offers a schematic overview on the classic signs, causes, and consequences of senescence in heart failure settings.

A detailed description of age-related events that irreversibly compromise heart functionality is provided in these suggested review articles [3, 20, 56].

Moreover, other cardiovascular pathologies, as aneurysms and peripheral artery disease, have a strong correlation with the onset of senescence in the cellular and ECM components of interested tissues and might be implicated in the genesis of heart failure [57–62].

3. Tissue Development, Regeneration, and Aging in the Heart

During ontogenesis, the heart originates from the mesodermal tissue of the embryonic cardiogenic plate. From this, the primitive cardiac tube develops, loops and twists, and finally forms the atria, ventricles, and outflow tracts [63].

More accurately, two heart fields in strict vicinity and relation constitute the primitive cardiac tissue. In particular, the cellular elements of the cardiac crescent, or anterior heart field, will mainly develop the left ventricle and the atria, while the ones of the second heart field will mainly colonize the right ventricle and the outflow tract, with small contribution to the atria [64]. Indeed, the cells contributing to the development of the heart originate from several sources. As recently demonstrated, Isl-1 progenitors will give rise to the atria and outflow tract by following different specializations. The expression in Isl-1 progenitors of the cardiac-specific homeobox Nkx2.5 and of the patterning gene Wnt-2 is associated with the differentiation towards the atrial phenotype. For the development of the outflow tract and right ventricle, the same progenitors acquire a different phenotype, expressing Nkx2.5, the myocyte-specific gene Mef2-c, the transcription factor gene T-box 1, and the fibroblast growth factor genes Fgf-8/10. Other Isl-1 cells, coexpressing the early endothelial marker Flk-1, are more involved in the formation of the endothelial lining in the endocardial layer or in the vessels. In the immediate postnatal life, Isl-1 cells and cardioblasts progressively differentiate into mature cardiomyocytes [64, 65].

Additional cell types with immature and plastic characteristics have been identified in the heart. In particular, cardiac stem cells and progenitors have been detected and isolated from pediatric and adult heart tissues. These cells express a typical hematopoietic marker, that is, c-kit, in combination with other proteins and transcription factors, for example, the hematopoietic Sca1, CD34, and/or the more cardiac-committed Mef2-c and GATA-4 or the endothelial

Table 2: Senescence in heart failure.

Extrinsic heart senescence triggers	
(i) Hypercholesterolemia (ii) Hypertension	[33, 36–40]
Intrinsic heart senescence triggers	
(i) Leukocyte telomere length shortening	[33, 36–40]
Upregulated (↑) and downregulated (↓) molecular pathways and functions	
(i) ↓ autophagy, mediated by ↑ oxidative stress	
(a) Early signs of senescence, accumulation of dysfunctional mitochondria, disorganization of sarcomere structure, and age-related cardiomyopathy in Atg5-deficient mice	[41]
(b) ↓ cardiac function and survival in Parkin-deficient mice	[42]
(c) Stimulation of mitophagy with spermidine, a natural polyamine, preserves cardiac function in old mice	[43, 44]
(d) Inhibition of miR-22 rescues autophagy and improves cardiac function in old mice	[45, 46]
(ii) Alterations of the inflammatory, endothelial, and myogenic phenotype of cardiac cells are also observed, with most of changes related to the aging-dependent manifestation of mutations in genes involved in the calcium cycling and signaling (↓ activity of SERCA2 and calsequestrin and Ica)	[47, 48]
(iii) ↓ cardiac sympathetic innervation	
(a) ↓ catecholamine uptake, ↓ isoproterenol response, ↓ epinephrine reuptake, and ↓ noradrenalin transport in senescent cardiac neuronal cells	[49, 50]
(iv) Age-dependent hERG mutations (a) Disorganization of the structure of the sarcomeric structure and myofibrillary proteins	[51]
(v) ↓ SIRT 3 (a) Pericyte loss and endothelial dysfunction, further exacerbated in the case of concomitant diabetic condition	[52]
(vi) ↑ ADAM/TACE overexpression in adipose tissue, mediated by ↓ negative regulation by caveolin-1 (a) ↑ TNF-α activity	[53]
(vii) ↑ senescence-associated genetic program in recruited fibroblasts of cardiac ischemia can be considered a protective mechanism from endothelin-1-mediated cardiac fibrosis	[54, 55]

SERCA2: sarcoplasmic reticulum calcium adenosine triphosphatase; Ica: L-type calcium current; hERG: human K^+ channel ether-à-go-go; SIRT3: mitochondrial nicotinamide adenine nucleotide histone deacetylase.

CD31. Cardiac stem cells have been shown to possibly originate all the lineages of the heart [66–70].

Further cells of particular interest in the heart tissues are the epicardial stem cells. As by their name, these cells can be retrieved in the epicardium and are identified by the expression of the Wilms tumor protein, codified by the gene Wt1 [71].

Exogenous cells with plastic features can participate in the organ development also in other phases. Neural crest cellular elements contribute to the construction of the distal outflow tract, working myocardium, glia, and nervous tissue during late gestation [72]. Additionally, endothelial progenitor cells are involved in the formation of new vessels in adult life [73].

Despite the existence of several cardiac stem cells and progenitors, the heart is not able to undergo a sustained self-renewal, especially after extensive damage. This property is related to its primary function, that is, pumping the blood throughout the whole body, which consumes massive amount of energy resources.

In the final stages of gestation, neonatal cardiomyocytes withdraw from reentering the cell cycle. Indeed, in the immediate postnatal period, cells undergo an incomplete mitosis and can be seen in the myocardium having dual or multiple nuclei, due to the inability to progress in karyo- or cytokinesis [74, 75]. In fact, they modify their growth modality from hyperplastic to hypertrophic and will not reenter the cell cycle. Actually, Ahuja et al. demonstrated that a preparatory dedifferentiation process, consisting of cycles of myofibrillary disassembly and reassembly, is necessary for a cardiomyocyte to divide [76]. However, a study of genetic cell fate mapping by Zhang et al.'s group revealed that mouse mature cardiomyocytes are able *in vitro* to undergo dedifferentiation-proliferation and show multipotent capacities [68].

Cardiac stem cells maintain their plastic ability during adult life, but remain quiescent in special microenvironments, namely, the cardiac niches, and are reactivated to replace the physiological loss of cardiomyocytes. However, the reactivation of the cell cycle only in cardiac stem cells is not sufficient to face the large loss of cardiac myocytes that occurs after an ischemic attack.

The sustained metabolic conversion of the heart is emblematic of mammalian species, but not of other vertebrates. Salamanders, axolotls, and zebrafish respond to heart injury inducing a blastema tissue, as part of an epimorphic regeneration [77]. This program is realized through the cell-cycle activation in mature cardiomyocytes, the degradation of the ECM, and a relatively low inflammatory response, mediated by a deficient adaptive immunity [78], in a tissue

particularly poor of fibroblasts [79]. Conversely, mammalians have a higher fibroblast/cardiomyocyte ratio; thus, the upregulation of genes involved in ECM synthesis and a strong immunoinflammatory response dictate the outcome of the damage response towards a scar formation.

4. Senescence Events in the Endogenous Stem Reservoirs of the Heart and in Cardiac Cell Therapy

From their discovery, cardiac stem cells have been studied intensively to develop cardiac cell therapies for treating organ failure. Cardiac stem cells can be cultured *in vitro* through the generation of cardiospheres (CS) and then expanded serially as cardiosphere-derived cells (CDCs) for clinical application [66–69]. As with the other differentiated cells of the heart, also cardiac stem cells might undergo senescence, showing reduced telomere length and telomerase activity, expression of $p16^{INKA}$ and $p21^{CIP}$, and an IL-6- and IGFBP7-enriched SASP [80].

The molecular mechanism for the onset of senescence in these cells is still not completely elucidated. The cardiac niche is rich in fibronectin and thus should represent a protective environment, which, evidently, does not exclude stem cell conditioning by aging-inducing triggers. In addition, also the niche ECM is a potential target of aging.

Independently from their localization, senescent stem cells tend to accumulate during aging. Nakamura et al. have shown that an increased expression of the Wnt inhibitor Sfrp1, together with higher levels of p16 and a peculiar SASP, is typical of human CDCs of old subjects (65–83 years), in comparison to those derived from younger ones (2–65 years) [81]. A recent study by Piegari et al. evidenced that the antitumoral drug doxorubicin exerts its cardiotoxicity also in cardiac stem cells by inducing cardiac stem cell senescence. In fact, telomere shortening, impaired migration, and differentiation were demonstrated in the anthracycline-treated human cells [82]. In addition, their number, self-renewing, and clonogenicity abilities decrease during age-related cardiovascular pathologies, compromising heart function [35, 80, 83–85]. Interestingly, Wu et al. recently demonstrated in a mouse model that although aged animals in physiological settings possess a remarkable number of cardiac stem cells, these ones result dysfunctional in several biological activities, as the metabolism of vitamins and tyrosine, the circadian rhythm, and the complement and coagulation cascades. Consequently, cell proliferation, multipotency, and differentiation abilities are impaired [86].

Besides anthracycline-induced damage, a strong body of evidence suggests that other epigenetic modifications are introduced in cardiac stem cells undergoing senescence [87].

Apart from resident stem cells of the heart, senescence can affect also exogenous reservoirs, which are used for physiological and pathological heart remodeling and have been studied to develop new stem cell-based therapies. Hematopoietic stem cells (HSC) have, in fact, been widely applied for this aim, since they are relatively easy to select through a consolidated profile of surface differentiation markers used for cytofluorimetric cell sorting [88]. These cells reside in the bone marrow and can be mobilized in response to a variety of signals [89]. Induced quiescence, overexpression of Mdr1 and Abcg2 transporters, glycolysis-mediated ATP generation, telomere shortening, accumulated mitochondrial mutations, and reduced ROS production collectively turn these cells into a senescent state, without sustained abilities of self-renewal and differentiation [90–93]. Intriguingly, although a high number of HSCs is retrieved in the elderly heart, these undergo a drastic lowering of clonal diversity and switch towards the myeloid lineage, consequently impairing their regenerative abilities [94]. Early committed, mobilized HSCs, that is, endothelial progenitor cells (EPCs), might also display senescent features, for example, intensified ROS production prompted by overexpressed angiotensin II and increased induction of apoptosis, which can be both potentially associated with reduced levels of SDF-1 [95, 96].

In support of HSC performance, mesenchymal stem cells (MSCs), which were originally thought to reside in the bone marrow alone, can be found in a variety of different tissues and have been widely studied to perform cell therapies. For example, the control of adipose stromal cell (ASC) fate by epigenetic regulators might be an interesting tool to boost both cardiac commitment and regenerative capacities of these cells. Aged MSCs show a classic senescent phenotype and demonstrate reduced migration capacity, decreased plasticity [97], and alterations in their immunoregulatory abilities, that is, one of the most striking properties of these cells [88, 98–100].

Table 3 presents a summary of the altered characteristics of CSCs, CDCs, HSCs, EPCs, and MSCs during cell senescence.

5. Rejuvenation Biotechnology: Antisenescence Regenerative Therapies and Disease Modeling

Even though senescence was initially depicted as a beneficial program activated by the organism to eliminate aged, dysfunctional mutated cells, scientists are still debating its true biological significance [3, 80, 101, 102].

Cell clearance is the last and most critical stage of senescence progression. As mentioned before, aged cardiovascular tissues are incredibly enriched in differentiated as well as stem/progenitor senescent cells. Moreover, cell clearance has been related to regression of several diseases, as neurodegenerative disorders and cancer [103, 104].

It has been shown that in senescent cancer tissues, cell clearance is impaired due to mutation in cell cycle checkpoint-related genes, for example, p53, with severe deleterious effects on tumor progression [105].

Indeed, it is fascinating that evolutionarily inferior organisms, as the unicellular ciliate protozoa and the multicellular basal metazoans, sponges, cnidarians, and flatworms, do not show senescence and are able to remain in a quasi-immortal state [106], firstly defined by Finch in 1994 as negligible senescence [107]. A few years later, de Grey et al. introduced the concept of engineered negligible senescence, as

Table 3: Senescence in endogenous stem reservoirs of the heart and in cardiac cell therapy.

CSCs and CDCs	
(i) Signs of senescence	
(a) Reduced telomere length	
(b) Decreased telomerase activity	
(c) Reduced expression of $p16^{INKA}$ and $p21^{CIP}$	[80]
(d) Decreased expression of IL-6- and IGFBP7-enriched SASP	
(e) Increased expression of the Wnt inhibitor Sfrp1and of p16 and a peculiar SASP typical in old subjects (65–83 years), differently from younger ones (2–65 years)	[81]
(ii) Known senescent triggers	
(a) Age-related cardiovascular pathologies, compromising heart function	[83–85]
(b) Antitumoral drugs, as anthracyclines	[82]
(c) Aged animals in physiological settings possess a remarkable number of cardiac stem cells, but dysfunctional in several biological activities, as the metabolism of vitamins and tyrosine, the circadian rhythm, and the complement and coagulation cascades. Consequently, cell proliferation, multipotency, and differentiation abilities are impaired	[86]
(d) Epigenetic modifications	[87]
HSC	
(i) Signs of senescence	
(a) Induced quiescence	
(b) Overexpression of Mdr1 and Abcg2 transporters	
(c) Glycolysis-mediated ATP generation	
(d) Telomere shortening	[90–93]
(e) Accumulated mitochondrial mutations	
(f) Reduced ROS production	
(g) Reduced abilities of self-renewal and differentiation	
(h) Although a high number of HSCs is retrieved in the elderly heart, these undergo a drastic lowering of clonal diversity and switch towards the myeloid lineage, consequently impairing their regenerative abilities	[94]
EPCs	
(i) Signs of senescence	
(a) Overexpressed angiotensin II	
(b) Increased induction of apoptosis	
(c) Reduced levels of SDF-1	[95, 96]
(d) Intensified ROS production	
MSCs	
(i) Signs of senescence	
(a) Classic senescent phenotype	
(b) Reduced migration capacity	[97]
(c) Decreased plasticity	
(d) Alterations in immunoregulatory abilities	[88, 98–100]

CSC: cardiac stem cells; CDCs: cardiosphere-derived cells; HSCs: hematopoietic stem cells; EPCs: endothelial progenitor cells; MSCs: mesenchymal stem cells.

to encompass all the biomedical strategies applied to reach a body rejuvenation in complex organisms, as the higher bilaterians [108].

In the quest of heart immortality, several therapeutic approaches have been explored, such as new drug generations, cardiac cell therapies, and tissue engineering strategies (Table 4).

Naturally inspired drugs have been formulated by the direct observation of the molecular pathways involved in senescence. Demaria et al. demonstrated that the administration of recombinant platelet-derived growth factor-AA (PDGF-AA), typically present in SASP secretome, could accelerate wound healing in a mouse model of impaired tissue repair [101].

Moreover, inhibitors of genetic pathways involved in senescence-related apoptosis have revealed senolytic activity. For example, it has been shown that the antitumoral agent panobinostat is able to target accumulated senescent cells for their effective clearance. This inhibitor of the histone deacetylases has received FDA approval for the treatment of several malignancies [109].

Similarly, desatinib and quercetin can act in concert to clear senescent adipocyte and endothelial cells in atherosclerotic lesions, by inhibiting the cell death regulator Bcl-2

Table 4: Current approaches of rejuvenation biotechnology.

Naturally inspired novel drug generations	
(i) rPDGF-AA might accelerate wound healing in a mouse model of impaired tissue repair.	[101]
(ii) FDA-approved antitumoral agent panobinostat is able to target accumulated senescent cells for their effective clearance.	[109]
(iii) Desatinib and quercetin clear senescent adipocyte and endothelial cells in atherosclerotic lesions. BH3 mimetic inhibitors, as ABT-199, ABT-263, and ABT-737, induce the clearance of senescent HSC and consequently increase the proliferation of the healthy ones.	[110, 111]
(iv) Administration of exogenous IL-10 or activators of SIRT-1 is particularly beneficial in the clinical treatment of myocardial infarction, by acting on the macrophages/fibroblast axis.	[112, 113]
(v) Intraperitoneal injection of recombinant GDF11 reverses age-related cardiac hypertrophy.	[114]
(vi) Administration of BPIFB4 increases the production of NO and stimulates relaxation, reversing endothelial cell senescence in atherosclerosis and other pathologies.	[115–119]
Stem cells for senescence protection	
(i) Fetal MSCs secrete bioactive factors promoting proliferation and differentiation in aged MSCs.	[120]
(ii) MSC secretome has beneficial effects in the protection, differentiation, and regeneration of CSCs and ancillary cellular elements.	[121]
(iii) CSCs ameliorate cardiac functionality in the anthracycline-induced cardiomyopathic heart.	[113]
(iv) A significant positive correlation exists between BB treatment of donor patients and both successful CS isolation and CS-forming cells yield from primary explant cultures. A significantly faster and higher CS-forming capacity was detectable in BB explants compared to NBB. A significantly increased percentage of $CD90^+$ cells was observed in NBB CDCs.	[122]
(v) CD90 expression in injected CDCs negatively correlated with infarct scar size reduction (CADUCEUS trial). This study supports the possible predictive and adjuvant role of β-blocker treatment in cardiac cell therapy applications, as recently suggested for MSC-based therapies. It also suggests novel insights on the influence of BB treatments on the quality and abundance of the cardiac reparative cellular pool.	[123, 124]
Stem cell engineering and reprogramming strategies	
(i) Resveratrol/rapamycin induces an epigenetic cellular reprogramming in senescent CSCs derived from decompensated hearts, by increasing cell proliferation.	[125]
(ii) PIM overexpression in senescent CSCs reverses heart aging.	[126]
(iii) Pluripotency reprogramming is feasible in centenarian cardiac fibroblasts by OCT4, SOX2, c-MYC, KLF4, NANOG, and LIN28 overexpression.	[129]
(iv) Treatment with inhibitors of BMP-SMAD signaling, for example, Dorsomorphin, SMAD6, and SMAD7, generates iPSCs in p16-mediated senescent fibroblasts.	[130]
In vitro 3D tissue engineering platforms to model senescence and aging	
(i) Evaluation of the effect of oxidative stress on a bioengineered tissue, constructed with a decellularized, MSC-secreted ECM scaffold and human umbilical cord-derived MSC. By comparison to an artificial matrix layer in fibronectin and collagen I, cells seeded onto decellularized scaffolds are less incline to develop senescence after H_20_2 stimulation, through an effect mediated by SIRT-1 upregulation.	[131]
(ii) Age of cardiac fibroblasts is a determining factor in the electrical and mechanical performance of cocultured cardiomyocytes in an *in vitro* 3D cardiac tissue, composed of a mixture of hydrogel and fibroblasts in molds of polidymethylsiloxane. This model is useful to recapitulate *in vitro* the alterations observed in senescent fibroblasts of the adult heart.	[132]

rPDGF-AA: recombinant platelet-derived growth factor-AA; BPIFB4: bactericidal/permeability-increasing fold-containing family B member 4; MSCs: mesenchymal stem cells; CSCs: cardiac stem cells; CSs: cardiospheres; BB: β-blocker; NBB: non-β-blocker; iPSCs: induced pluripotent stem cells; ECM: extracellular matrix.

[110]. BH3 mimetic inhibitors acting on the same gene, as ABT-199, ABT-263, and ABT-737, induce the clearance of senescent HSC and consequently increase the proliferation of the healthy ones [111].

Administration of exogenous IL-10 is particularly beneficial in the clinical treatment of myocardial infarction. In fact, Jung et al. verified that IL-10, an anti-inflammatory cytokine secreted by M2 macrophages, is able to stimulate the monocyte polarization towards the regenerative profile and the activation of fibroblasts, with consequent positive effects on left ventricle function and dilatation [112]. A similar effect can also be achieved by conditioning with activators of SIRT-1 [113]. Analogously, age-related cardiac hypertrophy has been reversed by intraperitoneal injection of recombinant GDF11, a member of the TGF-β growth factor superfamily [114].

For the reversal of endothelial cell senescence in atherosclerosis and other pathologies, the administration of a bactericidal/permeability-increasing fold-containing family B member 4 (BPIFB4) isoform has demonstrated to increase

the production of NO and stimulates relaxation, as shown by the work of Puca's group [115–119].

Apart from targeting molecular pathways, further strategies to enable senescence protection are represented by the use of different stem cells. Fetal MSC have been confirmed to secrete bioactive factors able to promote proliferation and differentiation in aged MSC [120]. This is particularly relevant in therapies based on adult MSC in order to protect these cells from possible senescence triggers in unfavorable microenvironments. Beneficial effects of MSC secretome are also being exploited in a recent biomedical approach, combining cardiac stem cells and ancillary cellular elements to increase their protection, differentiation, and regeneration [121].

In addition, cardiac stem cells are being infused also in the hostile microenvironment offered by the anthracycline-induced cardiomyopathic heart, whose compromised functions have been ameliorated with this therapeutic approach [113].

We recently described for the first time a significant positive correlation between β-blocker (BB) treatment of donor patients and both successful CS isolation and CS-forming cell yield from primary explant cultures. Our results show profound differences in cell phenotype based on their isolation from either BB- or non-β-blocker-treated (NBB) patients. In fact, a significantly faster and higher CS-forming capacity was detectable in BB explants compared to NBB. Moreover, an immunophenotypical shift of the described CDC marker CD90 was detectable between the two groups, with a significantly increased percentage of $CD90^+$ cells in NBB [122]. In the CADUCEUS clinical trial, CD90 expression in injected CDCs negatively correlated with infarct scar size reduction. This study supports the possible predictive and adjuvant role of β-blocker treatment in cardiac cell therapy applications, as recently suggested for mesenchymal stem cell-based therapies. It also suggests novel insights on the influence of BB treatments on the quality and abundance of the cardiac reparative cellular pool [123, 124].

In order to increase the therapeutic success of cardiac stem cell therapies in elderly patients, genetic engineering might be a valid tool too. Avolio et al. have proven that the resveratrol/rapamycin combined treatment of senescent cardiac stem cells derived from decompensated hearts generates an epigenetic cellular reprogramming that reverses the aged phenotype and increases the proliferation [125]. The overexpression of Pim-1 was shown to have analogous effects in senescent cardiac stem cells [126]. The reprogramming approach to pluripotency is a promising powerful strategy to overcome senescence. Yamanaka and his group demonstrated as first the feasibility to convert somatic differentiated cells into induced pluripotent stem cells (iPSCs) by the overexpression of four transcription factors, that is, OCT4, SOX2, c-MYC, and KLF4 [127, 128]. Strikingly, Lapasset et al. induced the pluripotency state in senescent and centenarian fibroblasts, by adding NANOG and LIN28 to the reprogramming gene cocktail used before [129].

Interestingly, the treatment of p16-mediated senescent fibroblasts with inhibitors of BMP-SMAD signaling (e.g., Dorsomorphin, SMAD6, and SMAD7) is able to favor the generation of iPSCs [130].

Lastly, the creation of platforms to model senescence and aging in a tridimensional (3D) tissue environment is of paramount importance to better simulate cell-ECM interactions and develop clinically relevant therapeutic solutions. Recently, two research groups have demonstrated the feasibility to model senescence in 3D settings. Zhou et al. evaluated the effect of oxidative stress on a bioengineered tissue, constructed with a decellularized, MSC-secreted ECM scaffold and human umbilical cord-derived MSC. By comparison to an artificial matrix layer realized with fibronectin and collagen I, the authors showed that cells seeded onto decellularized scaffolds are less incline to develop senescence after H_20_2 stimulation, through an effect mediated by SIRT-1 upregulation [131].

Li et al. reconstructed *in vitro* a 3D cardiac tissue by injecting a mixture of hydrogel, neonatal rat ventricular cardiomyocytes, and fibroblasts in molds of polidymethylsiloxane. They revealed that the age of cardiac fibroblasts is a determining factor in the electrical and mechanical performance of cocultured cardiomyocytes [132], by recapitulating *in vitro* the alterations observed in senescent fibroblasts of the adult heart.

Conflicts of Interest

The authors declare no conflict of interest.

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 9845175, 9 pages
https://doi.org/10.1155/2017/9845175

Research Article

Increased Levels of sRAGE in Diabetic CKD-G5D Patients: A Potential Protective Mechanism against AGE-Related Upregulation of Fibroblast Growth Factor 23 and Inflammation

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Received 22 June 2017; Revised 21 August 2017; Accepted 7 September 2017; Published 25 September 2017

Academic Editor: Carmela R. Balistreri

Advanced glycation end products (AGEs) may induce cardiac remodeling in kidney disease by promoting fibroblast growth factor 23 (FGF-23) expression. Since AGEs are increased in diabetes mellitus (DM), our first aim was to evaluate the existence of any potential association between AGEs, FGF-23, inflammation, and increased cardiovascular risk in DM patients on dialysis (CKD-G5D). Secondarily, we explored the potential role of the soluble receptor for AGEs (sRAGE) as a marker of heart failure. Levels of glycated albumin (GA), sRAGE, c-terminal FGF-23 (cFGF-23), brain natriuretic peptide (BNP), and inflammatory mediators were compared between DM and non-DM CKD-G5D patients. The levels of sRAGE, cFGF-23, BNP, and proinflammatory markers were over the ranges of normality in both DM and non-DM groups. Only GA and sRAGE levels were increased in DM compared to non-DM patients. Plasma levels of sRAGE and CRP were the only independent predictors of BNP concentration. In conclusion, in DM CKD-G5D patients, sRAGE appeared to be a marker of cardiac remodeling. Indeed, its increase could be a potential protective mechanism against the increased risk of cardiovascular complications related to AGEs and inflammation. The causal relationship between sRAGE and cardiovascular risk in these patients needs to be further confirmed by mechanistic studies.

1. Introduction

End-stage renal disease (ESRD) in patients with chronic kidney disease (CKD) is a condition characterized by including volume overload, hyperkalemia, metabolic acidosis, hypertension, anemia, and mineral and bone disorders (MBDs), and it is considered a clinical model of premature aging. ESRD patients have an increased risk of different diseases, mainly at cardiovascular and cerebrovascular level, and have a mortality rate at least 20–30 times higher than what their healthy age-matched patients have [1]. ESRD is stricly related to cardiovascular diseaes (CVDs) through several mechanisms, which include the inflammatory response, the production of reactive oxygen species, the phosphate toxicity, and the activation of different endocrine pathways, such as the fibroblast growth factor 23 system (FGF-23) [1]. Also, MBDs represent a severe complication and an important mortality risk factor in CKD patients on dialysis (CKD-G5D) [2].

FGF-23 is a 32 kDa glycoprotein secreted by osteocytes which has been receiving great interest as a new risk factor for CVDs and death both in individuals with CKD [3, 4] and in adults with preserved kidney function [5, 6]. In

particular, increased FGF-23 levels have been associated with vascular dysfunction, left ventricular hypertrophy, and the risk of heart failure, stroke, and death [3, 7, 8]. In CKD, FGF-23 levels increase as a compensatory mechanism to keep normal phosphate levels by inhibiting renal phosphate reabsorption and 1-alpha-hydroxilase activity, the key enzyme for calcitriol production [9]. Anyway, although this increase is acknowledged as a physiological protective mechanism, it could directly contribute to the onset and progression of inflammation and CVDs [7, 8, 10].

It has been recently observed that FGF-23 expression may be promoted *in vitro* by advanced glycation end products (AGEs) through the upregulation of NF-κB [11]. Indeed, in a mouse model of renal failure, the activation of the cell-surface receptor for AGEs (RAGE) induced FGF-23 expression in cardiac fibroblasts and promoted cardiac remodeling [12].

Metabolic disorders including diabetes mellitus (DM) are characterized by high levels of AGEs that are key mediators of DM-related complications, inflammation, and aging. These products, generated by nonenzymatic reactions between reducing sugars and protein or lipids, mainly promote reactive oxygen species generation and a proinflammatory response through RAGE activation. Besides the cell membrane form, RAGE also exists as a soluble circulating molecule, sRAGE. This form, by binding the circulating AGEs and preventing their activation of RAGE, plays a role as an important protective agent [13, 14].

In renal diseases, AGEs and sRAGE may accumulate due to their increased formation and reduced elimination [15–19]. Indeed, the RAGE pathway has been suggested as a causal risk factor for both atherosclerosis [20] and left ventricular hypertrophy [21] in these patients.

Although the potential role of sRAGE as a marker for CVDs has been pointed out in different previous studies [22–27], its role in ESRD is less characterized.

To better evaluate the role of the AGEs/sRAGE pathway in ESRD, we firstly evaluated the existence of any potential association between AGEs, FGF-23, inflammation, and increased risk of CVDs in DM CKD-G5D patients. Secondarily, we explored the potential role of sRAGE as a marker of heart failure in CKD-G5D.

2. Materials and Methods

2.1. Source Population. We performed a cross-sectional study in patients on CKD-G5D. We enrolled patients who underwent hemodialysis (HD) or peritoneal dialysis (PD) treatment for at least 3 months with age $\geq$ 18 years and agreement to participate in the study. We excluded patients with missing or incomplete clinical history, incapacity to cooperate to the study, and hepatic encephalopathy. This study was performed in accordance with the ethical principles of the Declaration of Helsinki, as revised in 2013. The protocol was approved by the Ethics Committee of San Bortolo Hospital (N.41/14). All participants were informed of the objectives of the study and signed the informed consent.

2.2. Measurements

2.2.1. Data Collection. Demographic, anthropometric, and clinical data (i.e., age, gender, smoking status, alcohol consumption, hypertension, DM, cardiovascular disease, and cerebrovascular disease) were collected. Screening and diagnosis of DM were performed according to the American Diabetes Association guidelines [28]. Hypertension was defined as values $\geq$ 140 mmHg systolic blood pressure and/or $\geq$90 mmHg diastolic blood pressure [29].

Blood samples in EDTA were collected during outpatient visits in PD patients or prior to dialysis treatment after long interdialytic intervals in HD patients. Samples for nonroutine assays were immediately frozen and stored at −80°C until measurements.

Concerning routine biochemical assays, total bilirubin (reference value (RV): male 0.3–1.5 mg/dL, female 0.2–1.2 mg/dL), calcium (RV: 8.5–10.5 mg/dL), phosphorous (RV: 2.2–4.2 mg/dL), LDL cholesterol (RV: <115 mg/dL), HDL cholesterol (RV: male > 40 mg/dL, female > 45 mg/dL), and total protein (RV: 6.4–8.7 g/dL) were quantified using colorimetric methods on Dimension Vista® 1500 Intelligent Lab System (Siemens, Milan, Italy). The same laboratory equipment was used for urea (RV: 15–50 mg/dL under 70 years old, 19–65 mg/dL over 70 years old), creatinine (RV: male up to 1.3 mg/dL, female up to 0.9 mg/dL under 70 years old and 1.2 mg/dL over 70 years old), uric acid (RV: male 3–8 mg/dL, female 2.4–6.6 mg/dL under 70 years old and 3–8 mg/dL over 70 years old), alanine aminotransferase (ALT) (RV: female < 31 U/L, male < 53 U/L under 70 years old and <34 U/L over 70 years old), and aspartate aminotransferase (AST) (RV: <37 U/L), which were all quantified by enzymatic methods, for total cholesterol (RV: <190 mg/dL) and triglycerides (RV: <150 mg/dL), both measured by kinetic enzyme assays, then for brain natriuretic peptide (BNP) (RV: <50 ng/L under 70 years old, <300 ng/L in the age range 51–75, and <600 ng/L over 70 years old) and C-reactive protein (CRP) (RV: <0.5 mg/dL), which were quantified by an immunochemiluminescent and a turbidimetric method, respectively. Sodium (RV: 35–145 mmol/L), potassium (3.3–5.0 mmol/L), and chloride (95–110 mmol/L) were measured on Dimension Vista System using ion-selective electrodes. Glucose (RV: <100 ng/mL) and albumin (RV: 2.1–4.5 g/dL) were quantified on the ILab650 system (Instrumentation Laboratory, A Werfen Company, Milan, Italy) using an enzymatic and a colorimetric method, respectively. Parathyroid hormone (intact PTH) (RV: 5–35 ng/L), 25-hydroxy vitamin D (25-(OH)D3) (RV: 30–100 μg/L), and β2-microglobulin (β2-microglobulin: 0.8–2.5 mg/L) were measured using the Liaison XL system (DiaSorin, Vercelli, Italy) by immunochemiluminescent methods. The acid-base equilibrium (pH, HCO_3^-) (RV: 7.32–7.42 for pH, 22–29 mmol/L for HCO_3^-) was quantified by the Rapidpoint 405 Blood Gas Analyzer (Siemens).

2.2.2. FGF-23 Quantification. The carboxyl-terminal (C-terminal) portion of FGF-23 (cFGF-23) levels was determined in plasma by two-site enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocol

Table 1: Demographic, anthropometric, and clinical characteristics of CKD patients included in the study.

	All CKD ($n = 76$)	Non-DM CKD ($n = 52$)	DM CKD ($n = 24$)	p
Age (years)	62.41 (52.02–72.05)	61.21 ± 13.94	65.42 (54.83–70.94)	0.80
Male gender (n, %)	55, 72.37%	35, 67.31%	20, 83.33%	0.18
BMI	27.20 ± 5.56	27.38 ± 5.28	24.14 (22.10–31.35)	0.50
HD (n, %)	32, 42.11%	20, 38.46%	12, 50%	0.45
Smoking (n, %)	9, 11.84%	4, 7.69%	5, 20.83%	0.13
Ex-smoking (n, %)	29, 38.16%	20, 38.46%	9, 37.50%	1.00
Alcohol consumption (n, %)	2, 2.63%	2, 3.85%	0, 0%	1.00
Hypertension (n, %)	48, 63.16%	35, 67.30%	13, 54.17%	0.31
Cardiovascular diseases (n, %)	22, 28.95%	12, 23.08%	10, 41.67%	0.11
Cerebrovascular diseases (n, %)	4, 5.26%	2, 3.85%	2, 8.33%	0.59
Therapy with activated vitamin D	45, 59.21%	31, 59.62%	14, 58.33%	1.00
Therapy with paricalcitol	22, 28.95%	17, 32.69%	5, 20.83%	0.05

Data are expressed as median (25th–75th percentiles) or number and proportions. BMI: body mass index; HD: hemodialysis. Comparison between groups was performed by Mann–Whitney U test or Fisher exact test.

(Immutopics Inc., San Clemente, CA). Two hundred microliters of plasma was used to assay the sample in duplicate. Samples with values greater than the highest standard were diluted 1 : 10 or greater with the 0 RU/mL standard or optional sample diluent reagent and reassayed. The lowest concentration of cFGF-23 measurable is 1.5 RU/mL, and the maximum intra- and interassay coefficients of variations were 2.4% and 4.7%, respectively.

2.2.3. Glycated Albumin Quantification. The glycated albumin (GA) and the percentage of glycated albumin (GA%) were determined in plasma by the enzymatic QuantiLab® glycated albumin assay (Instrumentation Laboratory) using the ILab650 system (Instrumentation Laboratory). The ILab analyzer automatically calculates the results of each sample. The GA% is calculated by the GA/albumin ratio and corrected by arithmetic algorithm defined to align the GA% levels to the HPLC method [30–32]. The minimum detectable concentration of GA measurable is 1.15 g/L. The maximum intra- and interassay coefficient of variations were 2.1% and 1.3% for GA and 1.2% and 1.0% for GA%, respectively.

2.2.4. sRAGE and Inflammatory Cytokine Quantification. The quantitative determinations of sRAGE, pentraxin-3 (PTX3), and tumor necrosis factor alpha (TNFα) concentrations were performed by commercial human ELISA kits (R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions. The minimum detectable dose ranged from 1.23 to 16.14 pg/mL for sRAGE, 0.007 to 0.116 ng/mL for PTX3, and 0.5 to 5.5 pg/mL for TNFα. The maximum intra- and interassay coefficients of variations were, respectively, 4.8% and 8.3% for sRAGE, 4.4% and 6.2% for PTX3, and 5.2% and 7.4% for TNFα. The GloMax®-Multi Microplate Multimode Reader was used for photometric measurements (Promega, Milan, Italy).

2.3. Statistical Analysis. Qualitative variables are summarized as numbers and percentages; quantitative variables are expressed as mean with standard deviation (SD) or median and interquartile range (IQR). The normality of data distribution was assessed by the Kolmogorov-Smirnoff test. t-test and Mann–Whitney U test were used for group comparison. To test the univariate association between variables, Pearson (for normal-distributed data) or Spearman (for non-normal distributed data) correlation tests were used, as appropriate. Stepwise regression analysis was performed to evaluate the independent correlates of BNP in CKD-G5D patients. All statistical analyses were performed using STATISTIX 7.0 (Analytical Software, Tallahassee, FL, USA) and GraphPAd Prism 5.0 biochemical statistical package (GraphPad Software, San Diego, CA, USA). A p value < 0.05 was considered significant.

3. Results

3.1. Patient Characteristics. We enrolled a total of 76 CKD-G5D patients (32 HD, 44 PD, median age 62.41 (IQR: 52.02–72.05) years, 55M) of which 24 were with DM (type 2 DM: 22; type 1 DM: 2) (mean age 61.01 (50.94–72.83) years, 35M) and 54 were without DM (65.42 (54.83–70.94) years, 20M). Demographic and anthropometrical data are presented in Table 1. Sixty-seven patients (87%) were under treatment with vitamin D or its synthetic analog. The active vitamin D therapy, which included cholecalciferol and calcitriol, was used in 45 (59.21%) patients. Twenty-two (28.95%) patients were treated with paricalcitol and cinacalcet.

Biochemical characteristics of patients included in the study are shown in Table 2.

3.2. Plasma Levels of GA, FGF-23, sRAGE, and Inflammatory Markers. CKD-G5D patients were classified according to the presence of DM, and the two groups were compared to explore potential differences in the levels of GA, as a marker of protein glycation, sRAGE, cFGF-23, and the proinflammatory molecules CRP, PTX-3, and TNFα.

According to the reference limits of GA [33], which have been very recently documented also in Caucasians

Table 2: Biochemical characteristics of CKD-G5D patients included in the study.

	All CKD-G5D ($n = 76$)	Non-DM CKD-G5D ($n = 52$)	DM CKD-G5D ($n = 24$)	p
Creatinine (mg/dL)	9.40 ± 3.09	9.71 ± 3.39	8.71 ± 2.19	0.13
Uric acid (mg/dL)	5.70 ± 1.29	5.67 ± 1.24	5.76 ± 1.41	0.78
Urea (mg/dL)	124.60 ± 31.41	122.10 ± 31.60	129.90 ± 30.97	0.32
Total bilirubin (g/dL)	0.40 (0–30-0.50)	0.40 (0.30–0.50)	0.42 ± 0.16	0.56
pH venous	7.35 (7.32–7.38)	7.35 (7.32–7.38)	7.36 (7.33–7.38)	0.47
HCO_3 venous (mmol/L)	25.18 ± 4.29	25.02 ± 4.54	25.53 ± 3.76	0.63
K—potassium (mmol/L)	4.46 ± 0.72	4.46 ± 0.71	4.48 ± 0.75	0.91
Na—sodium (mmol/L)	140.00 (138.00–142.00)	140.00 (138.30–142.80)	139.50 ± 3.02	0.33
Cl—chloride (mmol/L)	101.50 (97.00–105.00)	101.10 ± 4.28	101.20 ± 5.74	0.95
Ca—calcium (mg/dL)	8.95 ± 0.47	8.98 ± 0.48	8.88 ± 0.44	0.38
P—phosphorus (mg/dL)	5.15 ± 1.36	5.14 ± 1.34	5.20 ± 1.44	0.86
Total cholesterol (mg/dL)	157.10 ± 40.66	163.90 ± 35.42	142.50 ± 47.76	**0.032**
LDL cholesterol (mg/dL)	80.82 ± 35.92	85.65 ± 32.32	70.33 ± 43.17	**0.016**
HDL cholesterol (mg/dL)	44.00 (38.00–54.00)	45.50 (40.00–60.25)	42.42 ± 11.81	**0.030**
Triglycerides (mg/dL)	131.50 (86.25–201.80)	117.50 (82.75–204.30)	154.00 ± 74.15	0.55
ALT (UI/L)	19.00 (15.00–24.00)	20.12 ± 7.79	20.00 (15.25–31.25)	0.30
AST (UI/L)	11.00 (6.00–15.75)	11.00 (6.00–14.75)	11.42 ± 6.79	0.93
Glucose (mg/dL)	103.50 (92.00–138.50)	99.00 (91.00–110.00)	165.00 (131.80–220.80)	**<0.0001**
Total protein (g/dL)	7.00 ± 0.57	7.02 ± 0.63	6.96 ± 0.41	0.64
Albumin (g/dL)	35.03 ± 4.82	35.01 ± 4.81	34.70 (31.35–39.23)	0.75
GA%	14.00 (12.03–17.15)	13.09 ± 2.05	18.30 (17.13–23.20)	**<0.0001**
iPTH (pg/mL)	103.50 (53.25–211.00)	102.00 (55.00–254.30)	134.30 ± 119.90	0.46
25-(OH)D3 (ng/mL)	17.90 (10.93–23.28)	17.95 (11.45–25.15)	16.32 ± 6.77	0.30
CRP (mg/L)	0.34 (0.29–0.78)	0.31 (0.29–0.77)	0.41 (0.29–1.04)	0.39
β2-microglobulin (ng/mL)	22.80 (16.66–28.98)	22.96 (16.88–29.61)	21.08 ± 7.66	0.24
BNP (pg/mL)	2542.00 (1511.00–10762.00)	2265.00 (1108.00–8272.00)	3064.00 (1795.00–18558.00)	0.148
cFGF-23 (RU/mL)	1441.00 (759.00–3614.00)	1345.00 (508.10–3087.00)	1707.00 (1183.00–4016.00)	0.142
sRAGE (pg/mL)	3089.30 ± 1339.74	2838.00 ± 1163.75	3633.80 ± 1548.45	**0.015**
PTX3 (ng/mL)	1.57 (0.76–2.94)	1.57 (0.75–3.23)	1.89 ± 1.48	0.65
TNFα (pg/mL)	10.71 (4.85–21.34)	12.15 (5.60–24.19)	11.66 ± 10.28	0.134

Data are expressed as mean ± SD or median (25th–75th percentiles). ALT: alanine transaminase; AST: aspartate transaminase; BNP: brain natriuretic peptide; CRP: C-reactive protein; GA: glycated albumin; cFGF-23: c-terminal portion of fibroblast growth factor-23; iPTH: intact parathyroid hormone; 25-(OH)D3: 25-hydroxy vitamin D; PTX3: pentraxin-related protein PTX3; sRAGE: soluble receptor for advanced glycation end products; TNFα: tumor necrosis factor alpha. Comparison between groups was performed by unpaired t-test or Mann–Whitney U test. p values less than 0.05 are indicated in bold.

[34, 35] (upper reference limit: 14.5% (95% CI: 14.3–14.7) [34]; range: 9.0% (90% CI: 8.7–9.5) to 16.0% (90% CI: 15.6–16.4) [35]), in non-DM CKD-G5D patients, GA (95% CI: 12.52–13.66) was within the ranges of normality (Table 1 and Figure 1). Differently, in the DM CKD-G5D group, it reached pathological levels and it was statistically significantly higher than in the non-DM CKD-G5D patients ($p < 0.001$) (Table 1 and Figure 1). According to our previous results on sRAGE concentrations in healthy subjects (mean value 1363.0 ± 693.2 ng/mL) [36], sRAGE levels were above the normal values both in non-DM CKD-G5D and DM CKD-G5D patients and resulted statistically significantly higher in the DM CKD-G5D compared to the non-DM CKD-G5D group ($p < 0.05$) (Table 1 and Figure 1). cFGF-23 levels were higher than the reference value (<180 RU/mL), but we did not find any significant difference between groups (non-DM CKD-G5D: median value, 1345.00, 25th–75th percentiles (508.10–3087.00) RU/mL; DM CKD-G5D: 1707.00, (1183.00–4016.00 RU/mL) (Table 2 and Figure 1)).

As markers of inflammation, we evaluated CRP, PTX-3, and TNFα. According to the existing reference values for healthy subjects (<0.5 mg/L for CRP, <1.18 ng/mL for PTX-3, and 1.12 pg/mL for TNFα, resp.), all the proinflammatory markers evaluated were greatly upregulated in both groups but without significant differences between them (Table 2 and Figure 2).

A univariate association analysis was then performed in CKD-G5D patients to explore potential correlations between the markers previously studied. We did not find any significant correlation between GA and cFGF-23 ($r = 0.073$, $p = 0.529$), between sRAGE and cFGF-23 ($r = -0.056$;

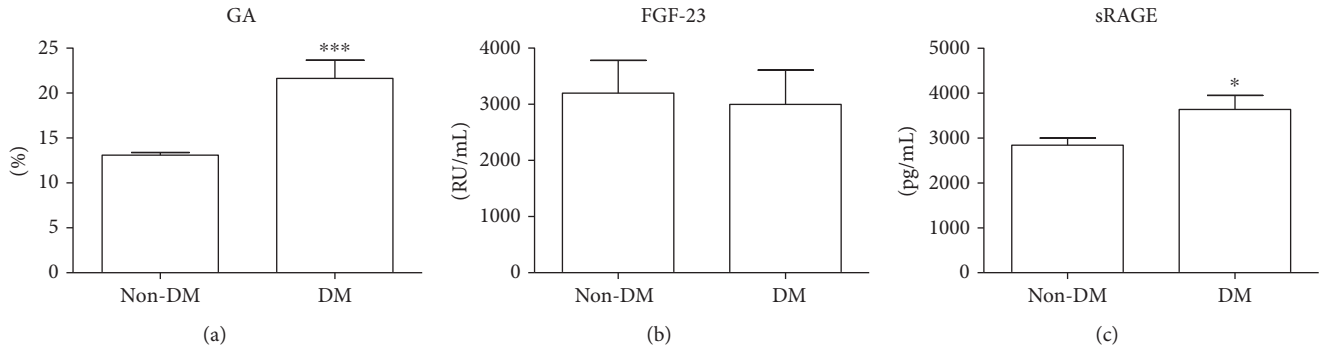

Figure 1: Evaluation of GA, FGF-23, and sRAGE levels in CKD-G5D patients. GA levels (a) and sRAGE (c) were higher in DM CKD-G5D patients than in non-DM CKD-G5D patients ($^{***}p < 0.001$ and $^{*}p < 0.05$, resp.). FGF-23 levels (b) were the same in the two groups.

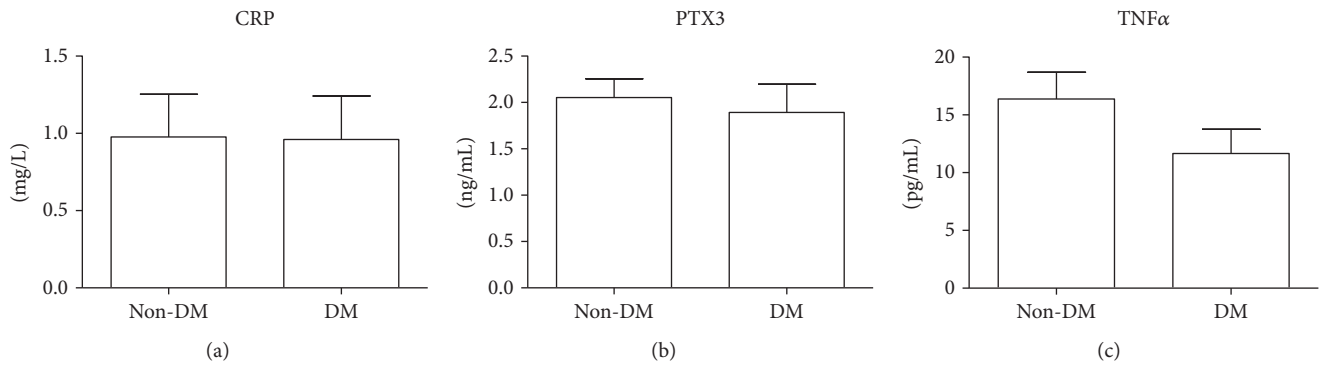

Figure 2: Evaluation of inflammation-related molecules in CKD-G5D patients. CKD-G5D patients were classified into two groups according to the presence of diabetes mellitus (DM). CRP (a), PTX3 (b), and TNFα (c) levels were compared between DM and non-DM groups. No statistically significant differences were observed between the two groups.

Table 3: Stepwise regression analysis (t value) of the association between some independent variables and BNP in CKD-G5D patients.

BNP (pg/mL)	Independent variables					Model R^2
	CRP (mg/L)	Creatinin (mg/dL)	Na (mmol/L)	K (mmol/L)	sRAGE (pg/mL)	
	2.44	0.84	−0.81	1.40	2.72	0.20
p value	**0.017**	0.40	0.42	0.17	**0.008**	
Constant value						
Regression coefficient	1652.06				2.49	
SE regression coefficient	676.00				0.92	

CRP: C-reactive protein; sRAGE: soluble receptor of advanced glycation end products; SE: standard error.

$p = 0.633$), and then between GA and sRAGE ($r = 0.29$, $p = 0.061$).

3.3. Relationships between CVD Risk Factors and BNP. The potential correlations between BNP, a marker used for screening and prognosis of heart failure, and clinical variables in CKD-G5D patients were explored. BNP was significantly positively correlated with creatinine ($r = 0.27$; $p = 0.017$), potassium ($r = 0.247$; $p = 0.031$), CRP ($r = 0.260$; $p = 0.023$), sRAGE ($r = 0.314$; $p = 0.006$), and β2-microglobulin ($r = 0.407$; $p < 0.001$) and significantly negatively correlated with sodium ($r = -0.341$; $p = 0.003$).

In a multivariate stepwise regression model, plasma sRAGE and CRP levels were the only independent predictors of BNP (Table 3). All the other parameters did not enter the model.

4. Discussion

CKD-G5D patients are an interesting model of premature aging. These patients, due to the lack of renal function, show a uremic milieu in which phosphate retention and uremic toxin accumulation, including AGEs, promote oxidative stress and inflammation. These conditions may in turn activate specific cellular mechanisms, such as telomere attrition, DNA damage, and mitochondrial dysfunction, which affect cellular homeostasis, promote premature cellular senescence,

and increase the risk of death mainly due to cerebrovascular and cardiovascular complications [37].

AGEs are recognized as important damaging molecules for the cardiovascular system due to their ability to promote endothelial dysfunction, arterial stiffness, atherosclerosis, immune system alteration, and cardiac fibrosis and remodeling [38–41]. It is known that the generation of AGEs is strongly increased in DM, being AGEs by-products of hyperglycemia. Recent preclinical studies [11, 12] suggested that these molecules, in addition to their known role as proinflammatory agents, are able to increase the production of FGF-23, a key molecule involved in the crosstalk between kidney function, bone metabolism, and the cardiovascular system [7, 8, 42]. To our knowledge, this is the first study investigating in human any potential association between AGEs, sRAGE, cFGF-23, and cardiovascular complications in CKD-G5D patients with DM. Our results indicated that both GA and sRAGE levels were increased in DM CKD-G5D compared to non-DM CKD-G5D patients, but the levels of cFGF-23 did not differ between the two groups. Similarly, the concentrations of the proinflammatory molecules evaluated were almost the same in the two groups, although we expected to observe higher levels in DM CKD-G5D patients, as a consequence of the increased glycated milieu. To our opinion, one possible explanation just deals with the upregulation of sRAGE. Different studies have shown that sRAGE levels are increased in DM as a counteract system against glycated products [26, 43–46]. Assuming the activation of the same mechanism also in our DM CKD-G5D patients, sRAGE, by blocking glycated products, could reduce the activation of various damaging cellular mechanisms, including the stimulation of cFGF-23 and other proinflammatory molecules. Indeed, since AGE accumulation has been associated with the development and progression of heart failure [47, 48], the lack of difference also in BNP levels between the two groups reinforces the idea of a protective role of sRAGE in DM CKD-G5D patients. A further explanation could arise by considering that cFGF-23, which starts to rise early in CKD, in CKD-G5D is up to thousand-fold higher than the normal levels [49]. For this reason, we could not exclude the possibility that in DM CKD-G5D patients, a further stimulation of the FGF-23 system by potential activators, like AGEs [11, 12], is not possible or may not be appreciated.

Concerning AGEs, we focused our attention on GA. As for other AGEs, we expected to observe that GA levels were over the ranges of normality not only in DM CKD-G5D group but also in non-DM CKD-G5D patients, due to the increased oxidative stress and the reduced kidney clearance typical of the disease [15, 17, 18, 50]. Of course, the upregulation of sRAGE at levels above controls in both groups and its further increase in DM seem to suggest the existence of a glycated milieu in all CKD-G5D patients, regardless of the presence of DM. According to these data, the observation that GA levels were over the range of normality only in DM CKD-G5D group strongly reinforces the utility of GA as a useful glycation marker for DM monitoring in CKD-G5D patients in which HbA1c does not work well due to just kidney-related anemia [51, 52].

sRAGE has been regarded as a diagnostic and prognostic marker of cardiovascular outcome in various pathological conditions, that is, obesity, DM, metabolic syndrome, chronic heart failure, and also CKD [20–22, 24, 26]. Concerning heart failure, conflicting results on the relationship between sRAGE and heart failure risk exist. Both lower and higher circulating levels of sRAGE were described as valuable predictors of heart failure, its severity, and mortality, and some studies suggested the existence of a robust association between NT-pro BNP levels, as a diagnostic and prognostic marker of heart failure and sRAGE [27, 48, 53–56]. Also, in our study, we observed a positive correlation between sRAGE and BNP. Indeed, sRAGE emerged as an independent predictor of BNP levels, thus suggesting its potential role as a marker of cardiac remodeling in CKD-G5D patients.

Leonardis et al. [21] studied the relationships between sRAGE and left ventricular hypertrophy in CKD, not in CKD-G5D. They showed that sRAGE levels were increased compared to those of controls but, unlike us, were inversely correlated with functional parameters of cardiac function. Probably, since the two studies have been performed on different populations, they are not easily comparable and further studies in ESRD are therefore necessary to support data herein presented.

The study of Kim et al. [20] has been performed on PD patients but explored sRAGE correlation with carotid atherosclerosis, not parameters of heart failure. Although different in its aim, some data of this study could be useful for a better comprehension also of our results. They observed that CKD-G5D patients had increased sRAGE levels compared to controls but, differently from our results, the subgroup of DM patients had lower sRAGE and higher IL-6 levels, a marker of inflammation, than the non-DM group. To our opinion, this observation seems to reinforce our hypothesis of a protective role of sRAGE against a further increase of the inflammatory status in DM patients. Anyway, the reasons of the different results are not clear but could deal with a different regulation of sRAGE expression at cellular level, the duration of disease, and features of patients included in the study.

In conclusion, in DM CKD-G5D patients, sRAGE appeared to be a marker of cardiac remodeling. Indeed, its increase could be a potential protective mechanism against the increased risk of cardiovascular complications related to AGEs and inflammation. The causal relationship between sRAGE and cardiovascular risk in these patients needs to be further confirmed by mechanistic studies. Also, the evaluation of additional glycated products, the quantification of sRAGE, secreted form of the receptor, and a comparison between HD and PD could help to improve the knowledge of the role of glycated pathways in these patients.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Elena Dozio and Valentina Corradi equally contributed to the manuscript.

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 5719461, 14 pages
https://doi.org/10.1155/2017/5719461

Research Article

Seasonal Variation in Vitamin D in Association with Age, Inflammatory Cytokines, Anthropometric Parameters, and Lifestyle Factors in Older Adults

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Received 14 June 2017; Accepted 9 August 2017; Published 14 September 2017

Academic Editor: Carmela R. Balistreri

Vitamin D deficiency is present even in sunny regions. Ageing decreases pre-vitamin D production in the skin and is associated with altered cytokine profile. We performed a multivariate analysis considering lifestyle factors, anthropometric, and inflammatory markers according to seasonal variation in Mexican healthy older adults. The same cohort was followed during 12 months. Vitamin D deficiency/insufficiency was found in 91.3% of the subjects despite living in appropriate latitude (25°40′0″N). 25(OH)D levels remained below <30 ng/mL through all seasons. Vitamin D deficiency did not correlate to sun exposure or dietary intake. Gender was the strongest associated factor, explaining a variance of 20%. Waist circumference (WC) greater than 88 cm was a risk factor for vitamin D deficiency. Age (>74 years) combined with WC (>88 cm) and BMI (>32.7) showed a high probability (90%) of vitamin D deficiency. Remarkably, an increase in one centimeter in WC decreased 25(OH)D by 0.176 ng/mL, while an increase in one point BMI decreased 25(OH)D by 0.534 ng/mL. A cutoff point of 74 years of age determined probability of vitamin D hipovitaminosis. Vitamin D deficiency was correlated with TNF-α serum levels, possibly increasing the susceptibility of older adults to a proinflammatory state and its related diseases.

1. Introduction

The main source of Vitamin D comes from sun exposure of the skin, from 7-dehidrocholesterol in response to ultraviolet B radiation (UVB), to further be metabolized in the liver to 25-hydroxyvitamin D 25(OH)D which is the metabolite used to assess vitamin D status. It requires further metabolism by the 1-alpha hydroxylase (CYP27B1) in the kidneys to produce the biologically active form 1,25-dihydroxyvitamin D (1,25(OH)2D3). In addition to the kidneys, many other tissues and cells throughout the body, including immune cells, express CYP27B1 and are therefore capable of regulating their own 1,25(OH)2D3 local concentration [1].

While the CYP27B1 in the kidneys is regulated by parathyroid hormone (PTH), fibroblast growth factor-23 (FGF-23), and 1,25(OH)2D3, its production in the immune system cells is driven by inflammatory conditions, either directly by the presence of cytokines [2] or in response to activation through the vitamin D receptor (VDR). Activated VDR binds response elements on DNA that are associated

with antimicrobial [3] and immune regulatory functions [4]. Thus, vitamin D deficiency might result in the impaired function of immune cells [5] and cytokine imbalance [6, 7]. It has also been observed that sufficient 25(OH)D levels are related to increasing concentrations of IL-4 and IL-10 and to low levels of proinflammatory cytokines such as IL-1, IL-6, IL-12, IL-17, IL-23, and IFN-γ [3, 8]. Aging is associated with changes in the immune system, with a decline in the number of naïve T cells in favor of an increase in memory T cells, and with a relative increase of Th2 cell versus Th1 cells [9]. These shifts in the T cell population change the cytokine expression profile. Furthermore, there is a decline in the level of androgens and estrogens, which have been linked with suppression of IL-6, a proinflammatory cytokine [9]. In this regard, vitamin D deficiency has been linked with several diseases such as autoimmune disorders [10], osteoarthritis [11], and cancer [12], in which chronic inflammation might be a causative factor.

In the US, 41.6% of the population is estimated to be vitamin D deficient, with Hispanics being the second ethnic group most at risk (69.2%) behind African Americans (82.1%) [13]. In Mexico, the biggest study to date found a prevalence of 9.8% for vitamin D deficiency and of 20% for insufficiency [14]. However, a more recent survey revealed a contradictory result, indicating a vitamin D deficiency prevalence of 43.6%, while an additional 46.8% were vitamin D insufficient [15].

Some studies have found that vitamin D levels increase in summer and decrease in winter [16–18] due to dependency of vitamin D on sunlight. Studies have also shown that this seasonal variation might depend on latitude, since it has been found that vitamin D production is greater on latitudes close to the equator [19]. However, vitamin D deficiency has even been reported in sunny regions [20].

This deficiency has been linked to many factors. For instance, skin pigmentation has a strong effect on vitamin D status, since it reduces the UVB radiation that effectively reaches the skin [21]. In the same way, sunscreen use decreases vitamin D production [22]. On the other hand, obesity is a risk factor. It has been proposed that this is due to fatty tissue uptake of vitamin D, reducing its bioavailability [23]. BMI and WC in particular have been negatively associated with vitamin D levels and greater prevalence of deficiency [18, 24, 25]. Regarding age, it has been proposed that vitamin D deficiency in the elderly can be attributed to a decrease in the skin capacity to produce vitamin D due to ageing, from a lack of exposure to sunlight, or from a deficient dietary intake [26].

However, there are scarce results examining vitamin D deficiency and its association with anthropometric, inflammatory, and lifestyle factors in the same cohort of patients through 12 months. There are also, to our knowledge, no studies of this type searching for seasonal changes in the same cohort in regions at latitude 25°40′0″N, where sunlight is adequate throughout the year. Therefore, the aim of the current study is to evaluate a 12-month follow-up seasonal variation of vitamin D status and its association with proinflammatory cytokines, anthropometric parameters, and lifestyle factors in Mexican healthy older adults.

2. Materials and Methods

2.1. Study Population. A longitudinal study was done in a sample of healthy adults older than 55 years old (55–86 yo) in the city of Monterrey, in the northeastern part of Mexico (latitude 25°40′0″N). The same cohort of patients ($n = 23$) was followed for the duration of 12 months; all parameters assessed were evaluated every three months according to the seasonal variation. Inclusion criteria required subjects to be older than 55 years old, be generally healthy, and be community living independent. Exclusion criteria included renal disease, hepatic disease, diabetes mellitus, hypertension, cardiovascular diseases, use of corticosteroids, oral contraceptives, anticonvulsant medications, gastrointestinal malabsorption diseases, gastrointestinal resections (bariatric surgery), consumption of supplements containing vitamin D, the use of sunscreen, living in nursing homes, or being institutionalized. Written informed consent was obtained from all of the subjects. Approvals by the Ethics and Research Committees of the School of Medicine, TEC de Monterrey, and by the Secretariat of Health were obtained. This study was registered in clinical trials with the following code: NCT02087683. Each subject was evaluated every three months according to each season of the year.

2.2. Vitamin D Intake and Sun Exposure. Subjects answered a previously validated face-to-face questionnaire [27], which was applied by a registered dietitian. The questionnaire included weekly consumption of foods rich in vitamin D such as milk, yogurt, cheese, fish, egg yolk, fortified fruit juice, margarine, and cereals, as well as vitamin supplements. Subjects were shown portion size kits to determine the number of portions per day and the number of days per week each food was consumed. In addition, subjects were screened for sun exposure, specifically between 11 : 00 AM and 3 : 00 PM in minutes per day and days per week. The use of sunscreen was also included in this screening, as an exclusion criteria. Skin phototypes were assessed according to Fitzpatrick's classification. None of the patients were outdoor workers.

2.3. Anthropometric Measurements. The anthropometric variables were evaluated every three months, according to seasonal variation at the moment of the blood withdrawal for vitamin D and the other laboratory variables. The anthropometric parameters measured were weight, height, waist circumference (WC), and percentage of body fat (BF%) measured by bioimpedance (standardized TANITA 350; BMI was calculated as kg/m^2) according to standardized protocols [28].

2.4. Vitamin D, Metabolic Assessment, and Cytokines. Blood samples were taken from each subject to obtain a total of four samples per patient, one sample for each season of the year. Serum 25(OH)D and parathyroid hormone were measured by chemiluminescence and calcium by spectrophotometry within four hours of venipuncture. Additional blood samples were centrifuged to obtain serum and plasma. Serum and plasma samples were frozen at −80°C and used to measure total calcium, phosphorous, parathyroid hormone, and cytokine profile. Cytokine profile was performed in serum

samples using the Legendplex Human Inflammation panel for a multianalyte cytometric assay (BioLegend, San Diego, CA, USA). The cytokine capture beads measured were IL-1β, IFN-α, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33. Each experiment was performed in triplicate, as per manufacturer's instructions. Data were collected on a flow cytometer FACS-Canto II (Becton Dickinson, USA). The analyte concentration was calculated using the standard curve provided, and serum concentrations of all cytokines were determined using the Legendplex software (BioLegend). According to the guidelines of the Endocrine Clinical Society, vitamin D deficiency was defined as a level of serum 25(OH)D < 20 ng/mL, vitamin D insufficiency between >20 and <30 ng/mL, and vitamin D sufficiency as >30 ng/mL [29].

2.5. Statistical Analysis. An exploratory analysis using principal component analysis (PCA) was performed for continuous variables, considering only subjects who presented no missing values. Pearson correlations and hierarchical clustering analysis were used to assess the relation among cytokines, as well as between 25(OH)D concentration and anthropometric parameters. Confirmation of correlations of 25(OH)D concentration was done using uni- and multivariate linear regressions, blocking by sex. A conditional inference tree [30] was generated using anthropometric parameters to obtain ranges of prediction for 25(OH)D levels.

Finally, a linear mixed effects model was performed, evaluating the effect of seasonal variation in 25(OH)D and cytokines concentration, considering age, sex, and individual as random effects, using maximum likelihood to calculate p values [31] and Tukey all pair comparisons for post hoc grouping. Statistical analyses were done in the R platform (R Core Team) using the packages lme4 [32], multcomp [33], and partykit [34], along with the included stats library.

3. Results

3.1. Demographic, Anthropometric, and Lifestyle Parameters. As a description of the studied population, Table 1 presents the demographic and anthropometric characteristics of the participants. A total of 23 patients were recruited, with a mean age of 68.8 years (range 55–86 years) and a similar number of male (10) and female (13) participants. According to the Fitzpatrick phototyping scale, the majority had type IV skin. Subjects had a mean BMI of 29 (20.1–46.6), a WC of 99.8 cm (78–121), and a BF% of 35.2 (17.5–51.3). The mean vitamin D intake was 112 IU (0–391.4) per day. The most common food source of vitamin D was milk, followed by fatty fish and cheese. The mean sun exposure occurred between 11 : 00 AM and 3 : 00 PM, equaling 82.3 min (0–840) per week.

3.2. Unsupervised Multivariate Analysis. Gender was the parameter that could explain the most variance (20%, principal components 3 and 4; Figure 1(a)). The main anthropometric and clinical parameters affecting this separation were body fat, BMI, and 25(OH)D levels, which were higher in women. Height, WC, weight, and minutes of sun exposure were higher in men (Figure 1(b)). Thus, blocking by gender was necessary and was expected to attenuate sampling effects due to these parameters. None of the other analyzed factors appeared to have an effect on the data set.

Table 1: Anthropometric parameters.

Parameter	Mean (range)
Age (years)	68.8 (55–86)
Sex (F/M)	F (13) M (10)
Skin (I–VI)	II (1) III (4) IV (17) V (1)
Smoker (Y/N)	Yes (4) No (19)
Cigarettes per day	1.3 (0–20)
Vitamin D (ng/mL)	20.7 (5–41.5)
Weight (kg)	77.7 (54.8–102.1)
Height (m)	1.64 (1.48–1.81)
Fat (%)	35.2 (17.5–51.3)
Waist (cm)	99.8 (78–121)
BMI (kg/m^2)	29 (20.1–46.6)
Vitamin D intake (IU)	112 (0–391.4)
Sun exposure (min)	82.3 (0–840)
PTH (pg/mL)	83.7 (21.8–282.3)
Calcium (pg/mL)	9.0 (7.6–10.1)

3.3. Anthropometric Parameters and Age but Not Lifestyle Factors Are Related to Vitamin D Levels. As shown in Figure 2, only one cluster with three parameters showed a significant ($p < 0.01$) negative correlation with 25(OH)D concentration, namely, weight, WC, and BMI, which were highly correlated to each other. Interestingly, neither dietary vitamin D intake nor sun exposure affected 25(OH)D levels. Focusing on these correlated parameters, a general linear regression was performed, blocking by sex, which revealed that indeed there was a significant negative correlation between 25(OH)D levels and WC ($p < 0.001$), BMI ($p < 0.001$), and weight ($p < 0.001$). We found a significant negative correlation between vitamin D concentration and age for women but not for men (Figure 3). When analyzing data separately, only BMI remained a significant ($p < 0.01$) predictor in the male population, with a determination coefficient of 0.24 (data not shown). Interestingly, WC explained 48.4% of the variation in 25(OH)D levels in women ($p < 0.001$), while it was not significant in men ($p > 0.2$) when analyzed separately. A multivariate lineal model, using sex as a covariate, showed that in women an increase in one centimeter in WC translates to a decrease in 25(OH)D concentration of 0.176 ng/dL. An increase in one BMI unit decreased vitamin D by 0.534 ng/dL (Table 2).

In our effort to generate predictors of 25(OH)D levels using anthropometric parameters, a conditional inference

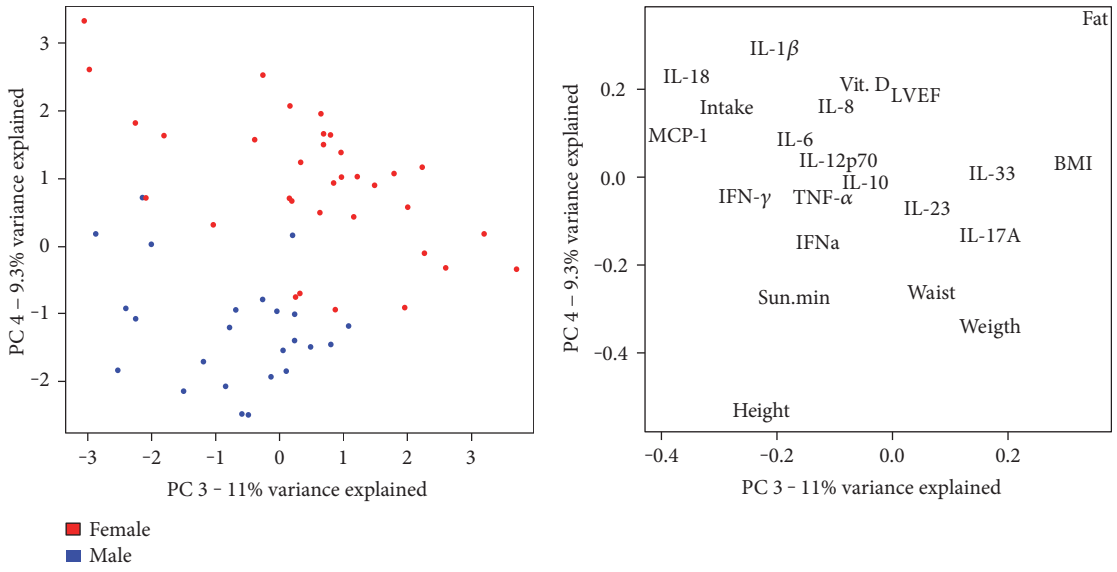

Figure 1: Principal component analysis of the continuous variables. Score plot (a) showing the separation of the female (red) and male (blue) population; and loadings plot (b) showing the variables responsible for the separation.

tree was used to generate simple classification rules to aid diagnosis (Figure 4). The model, which first considered all anthropometric measures, only found WC, BMI, and age to be of relevance in classifying patients by vitamin D level; this agreed with the correlations and linear model. The decision tree correctly classified 73.7% of the patients according to vitamin D status, with only 8% of false positives for sufficiency (Table 3). The main predictor was WC. A WC exceeding 88 cm was considered as a risk factor for vitamin D insufficiency and deficiency. This WC combined with a BMI greater than 32.702 indicated an extremely high probability (90%) of having vitamin D deficiency. On the other hand, a cutoff point of 74 years of age is a determinant of vitamin D status. A BMI ≤ 32.7 and an age > 74 years also predict a 90% probability of vitamin D deficiency and 10% of insufficiency, while an age < 74 years is a predictor of 70% insufficiency versus 20% deficiency status (Figure 4).

3.4. Vitamin D Seasonal Variations. The results shown in Figure 5(a) indicate that winter is the season in which the subjects had the lowest levels of 25(OH)D (18.8 ± 7.5 ng/mL), with concentrations recovering in spring (20 ± 7.3 ng/mL), to reach maximum levels in both summer (21.1 ± 7.3 ng/mL) and autumn (21.9 ± 7.5 ng/mL). 25(OH)D concentrations were significantly lower in winter compared to summer ($p < 0.05$) and autumn ($p < 0.001$). Figure 5(b) shows the prevalence of vitamin D deficiency and insufficiency, which follows a trend agreeing with the previous results, with more than 60% of patients having a deficiency of vitamin D in winter, compared to less than 40% having a deficiency in summer. During winter and autumn, 87.5% of the subjects had 25(OH)D levels <30 ng/mL, while in summer and spring, 91.3% had.

3.5. Cytokine Seasonal Variation. After blocking by gender, the panel of cytokines (IL-1β, IL-6, IL-10, IL-18, MCP1, and TNF-α) significantly varied with the season, considering age, sex and individual as random effects. As shown in Figure 6, all of these cytokines have the highest discernible levels in autumn, winter, or both, while the lowest discernible concentration is invariably in spring. This variation is independent from 25(OH)D concentrations, except for TNF-α, the levels of which are exacerbated in patients with vitamin D deficiency (Figure 7). A hierarchical clustering was performed for the cytokine measurements, and the results of which are displayed in Figure 8. Cytokines are therefore grouped in two main clusters, the bigger being a set of five significantly ($p < 0.01$) positive correlated cytokines: IL-17A, IL-33, IL-23, IL-10, and IL-12p70. The second cluster conformed by TNF-α, IFN-α, and IL-6 correlated among them ($p < 0.05$), but not with 25(OH)D levels. The remaining cytokines are not significantly correlated with one another, although IL-18 is correlated to the main cluster via positive, significant ($p < 0.05$) correlations with IL-17A, IL-33, and IL-23; similarly IL-8 is significantly ($p < 0.05$) correlated to all members of the second cluster (TNF-α, IFN-α, and IL-6); and finally, IFN-γ is significantly correlated with IL-33. When analyzing the periodicity of proinflammatory compound expression separating by gender, some of the cytokines remained significantly different for men and for women (IL-18, IL-6, and IL-1β; 0.012 and 0.0002, 0.008 and 0.006, 0.003 and 0.007, resp.), while others remained significant only in men (MCP-1 and TNF-α; 0.044 and 0.002, resp.).

4. Discussion

This was a longitudinal 12-month follow-up study of the same cohort to determine vitamin D seasonal changes and

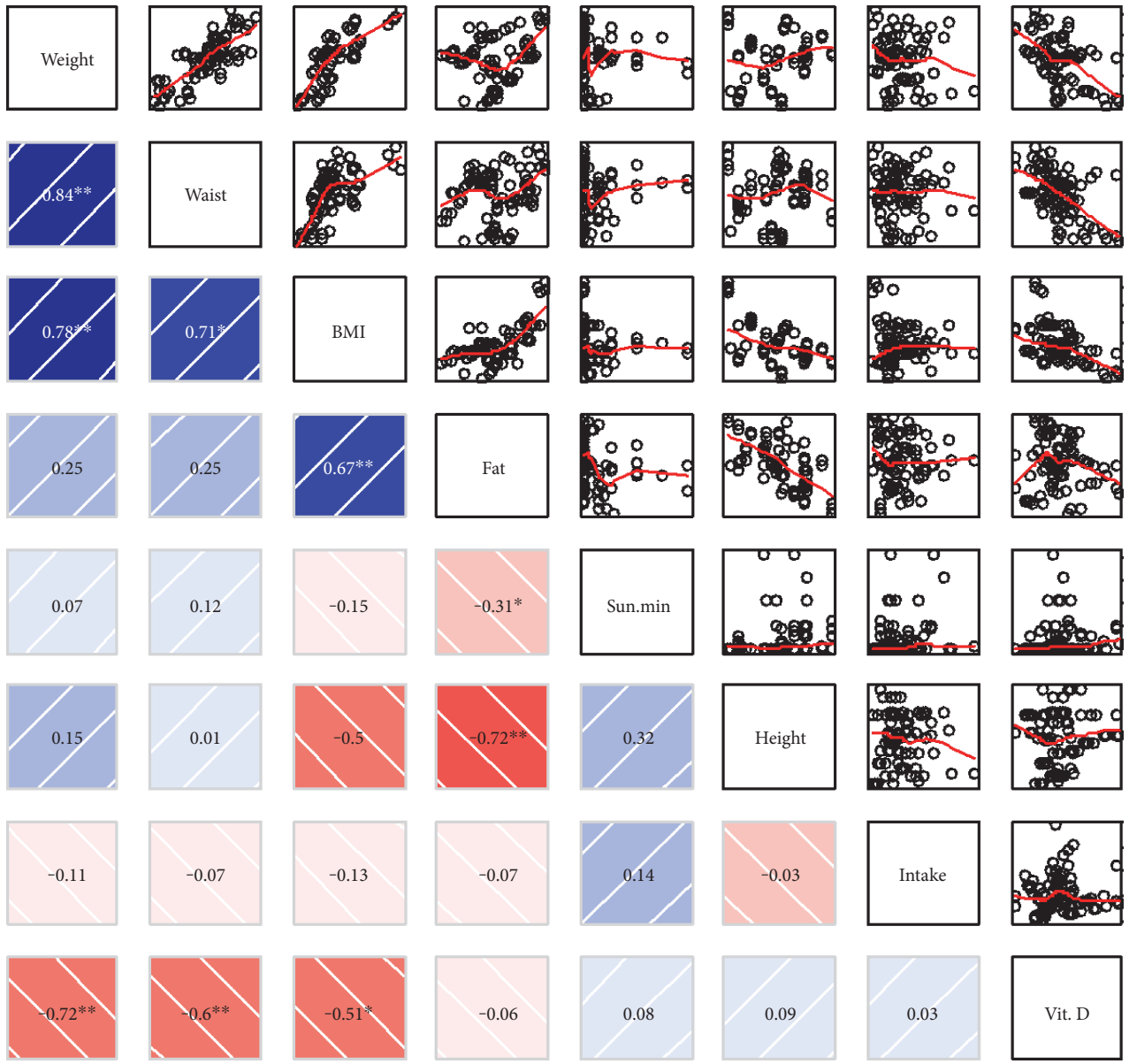

Figure 2: Correlation of anthropometric parameters and 25(OH)D. Upper panels show the raw data (circles) and locally weighted smoothing (red line) and lower panels show the Pearson correlation coefficient (r). Significant correlations are shown in bold ($^{*}p < 0.05$, $^{**}p < 0.01$).

their association with anthropometric parameters, lifestyle factors, and proinflammatory cytokines in an older adult Mexican population. Our results show a great prevalence of vitamin D deficiency and insufficiency across all seasons, with significantly greater prevalence of deficiency in winter compared with summer and autumn. Vitamin D levels were negatively correlated with BMI, WC, and weight, as well as with gender differences and TNF-α levels. While WC explained almost half of the variations in vitamin D levels in women, BMI was the second significant predictor of vitamin D. However, neither dietary vitamin D intake nor sun exposure affected 25(OH)D levels.

Vitamin D deficiency or insufficiency is very common worldwide. Our results in this sample of Mexican older adults show that 25(OH)D levels were <30 ng/mL through the four seasons with significant seasonal variations as well as in the prevalence of deficiency/insufficiency which was around 90%. 25(OH)D levels were significantly lower in winter than in summer and autumn. Other countries have also shown a high level of vitamin D deficiency, but not as high as ours. A study performed in seven different cities of Canada showed that about 60% of subjects > 35 years had levels of 25(OH)D below 30 ng/mL [16]. In Europe, vitamin D deficiency was estimated to be 40.4% where ethnic groups with more skin pigmentation were found to have higher prevalence of vitamin D deficiency [35]. In Mexico, a recent survey in an open population (≥14 years old) showed a deficiency prevalence of 43.6%, with 46.8% of the population in the insufficiency range [15]. These findings are similar to those reported in Canada and Europe, despite Mexico's more favorable latitudinal position. These results could be explained by the characteristics of our subject population (skin type IV and

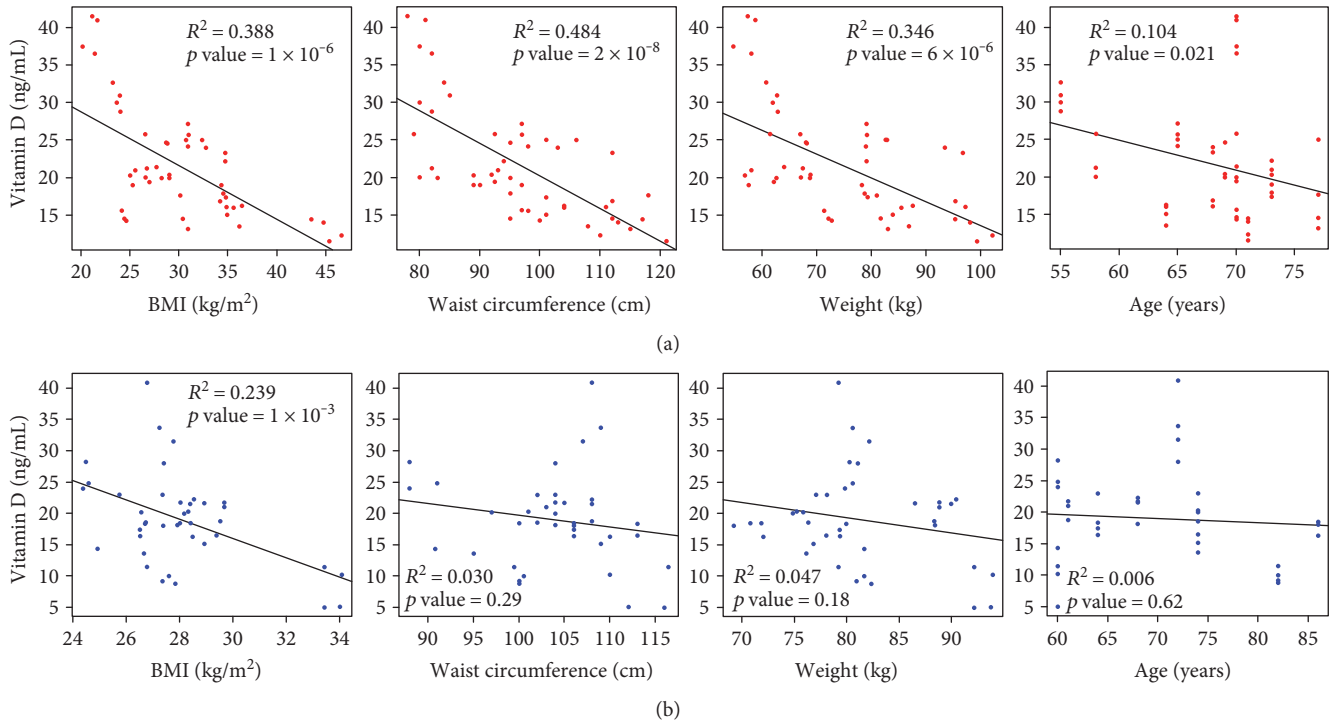

Figure 3: Individual regressions, separated by sex. Linear regressions between 25(OH)D levels and BMI, WC, weight, and age from left to right are shown as a black line in female (a) and male (b) populations. Coefficients of determination (R^2) and p values are shown inside the plots in bold for significant correlations.

Table 2: Stepwise regression of correlated parameters versus 25(OH)D level serum concentration, in ng/mL.

Variable	Effect and significance (B/p) Model		
	1	2	3
Adjusted R^2	0.27	0.32	0.31
Waist (cm)	−0.38/2.8 × 10−7	−0.18/0.09	−0.20/0.07
BMI (kg/m^2)	—	−0.53/0.01	−0.65/0.02
Weight (kg)	—	—	0.08/0.50

increased age), since as we observed, age could also be a factor for insufficiency predictor. In agreement with our findings, data from NHANES 2007–2010 found that 75% of the USA population had 25(OH)D levels below 30 ng/mL and 35% had levels below 20 ng/mL. When stratified by race, 83% of Hispanics had levels below 30 ng/mL, and 36% below 20 ng/mL [36].

4.1. Seasonal Variations. There are some studies that also take into consideration seasonal variations of vitamin D status; however, none of them performed a follow-up seasonal variation of the same cohort as we did. In this regard, in studies where only one sample was taken for each subject (some of them being taken in summer and others in winter), a significant seasonal variation in vitamin D status was seen in Caucasians [17] and Swedes [18], but not in subjects from Iran [37]. A study in Australia, on the contrary, found vitamin D deficiency prevalence in summer that rose in spring but not in winter [38]. The seasonal variation found in most studies might be due to the fact that during winter in the different countries, not only is there less sunlight but also the UVB rays enter the earth in a tangential position that do not reach the skin at the right angle, and pre-vitamin D production in the skin is greatly reduced [39]. Nevertheless, it does not explain why even when this study took place in a city in the northeastern part of Mexico at an appropriate latitude (25°40′0″N), the prevalence of vitamin D deficiency and insufficiency was even greater than at other sites where the latitude is not appropriate, even during sunny seasons when the maximum pre-vitamin D production is in the skin.

4.2. Sun Exposure, Gender, Age, and Anthropometric Parameters. In agreement with our findings, a worldwide meta-analysis found no association between latitude and serum 25(OH)D levels [40]. Nevertheless, we must also take into consideration that during winter, there are fewer people who exercise or are outdoors due to the cold weather in our city. In this sense, a cross-sectional study indicated that outdoor exercise reduced the risk of vitamin D deficiency/insufficiency, since patients who did outdoor exercise had less prevalence of hypovitaminosis D than those who did not exercise outdoors [41]. Taking this in consideration, we analyzed sun exposure and dietary intake and found that neither of them affected 25(OH)D levels in our population. The dietary vitamin D intake was about 112 IU per day, way below the recommendations of the IOM (600–800 IU; IOM2011), while the Endocrine Society Clinical Practice Guideline

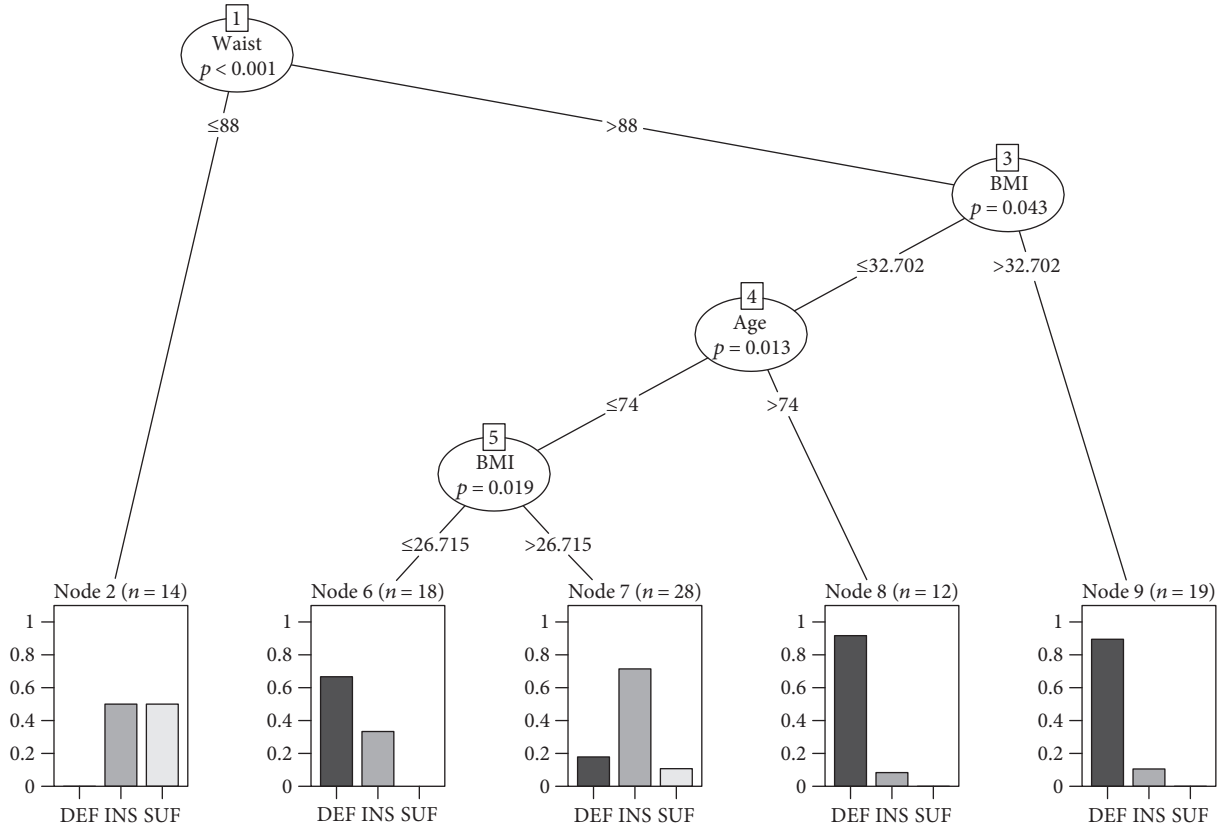

Figure 4: Decision tree relating vitamin D levels and anthropometric parameters. Variable name and p values are shown inside the bubbles, and cut-off values at the corresponding line. Plots at the bottom show the sample distribution, when rules are followed: for example, of all patients with WC >88 cm and BMI >32.702 kg/m^2 (rightmost plot), 90% have vitamin D deficiency and 10% have insufficiency. DEF = deficiency; INS = insufficiency; SUF = sufficiency; WC = waist circumference; BMI = body mass index.

Table 3: Confusion table. Predicted versus actual 25(OH)D levels.

Original	Predicted			
	Deficient	Insufficient	Sufficient	Recall
Deficient	40	5	0	88.9%
Insufficient	9	23	7	59.0%
Sufficient	0	4	7	63.6%
Precision	81.6%	71.9%	50.0%	73.7%

Predicted vitamin D levels results after following the decision tree in Figure 3 and actual vitamin D status (rows) of the patients (women) in this study.

agrees that adults need an intake of 1500–2000 IU [29]. This could partially explain the low vitamin D levels and high prevalence of deficiency of the population, but sun exposure continues to be the main source of vitamin D.

Even though our population's sun exposure between 11 : 00 AM and 3 : 00 PM was 83.4 minutes per week, exceeding the sun exposure recommendations for UBV radiation and vitamin D production (10–15 minutes three times a week between these hours) [42], we must emphasize that most of the population was skin type IV, which lessens the previtamin D production in the skin. Similar to our results, a cross-sectional study with healthy subjects aged 65 years and older concluded that differences in sun exposure do not explain the differences in vitamin D status [43].

When we performed a deeper analysis, taking into consideration other parameters that have been associated with vitamin D deficiency observed in our studied population, we found that gender explained the most variance of 25(OH)D levels, with women having higher 25(OH)D levels than men. We found a significant negative correlation between vitamin D concentration and age for women but not for men. Although there is no clear explanation for this gender difference, the fact that in women, BMI, WC, and weight had a negative correlation with vitamin D levels, while in men, only BMI did, might be related. In addition to this, women presented higher body fat and BMI, while men demonstrated higher height, WC, weight, and minutes of sun exposure. These results agree with some other studies.

In Canada, gender differences were significant, with deficiency prevalence being 30% for men and 24% for women [44]. In this regard, a large meta-analysis across the world also showed that 25(OH)D levels were higher in women than in men [40]. On the contrary, in Iranian subjects, women had slightly lower levels of 25(OH)D than men (20.6 versus 23.2 ng/mL) [37]. A large study in obese adults suggested that subcutaneous fat in women allow them to store vitamin D, which would be released when cutaneous production

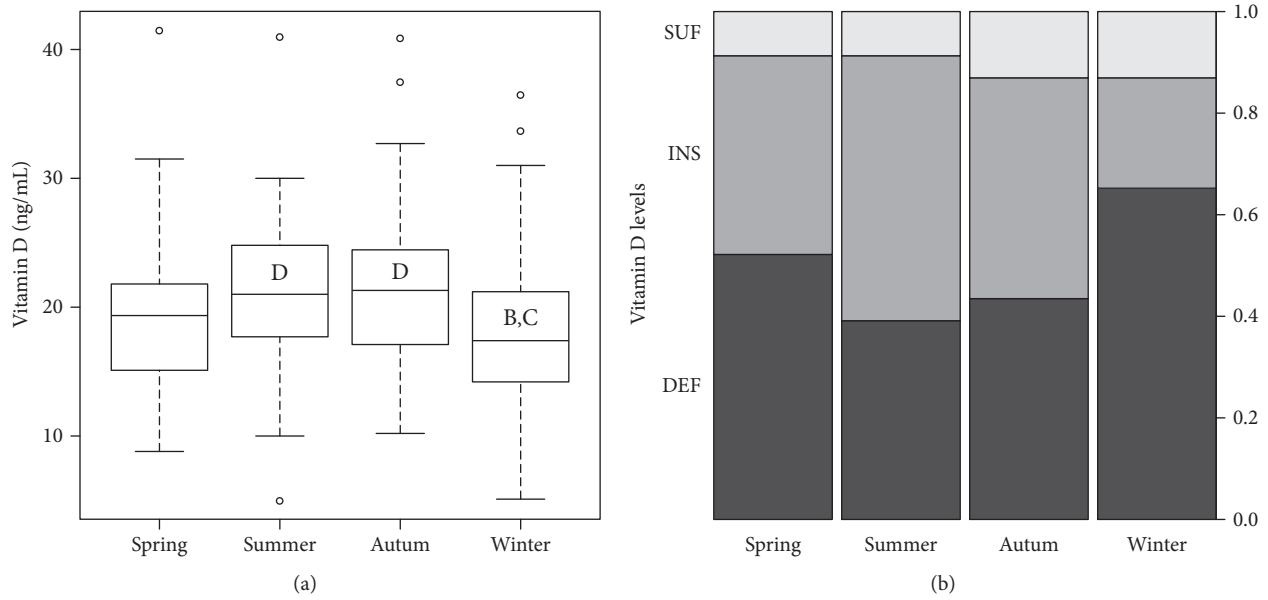

Figure 5: Seasonal variation of vitamin D. (a) 25(OH)D concentrations in the four seasons; the bold line represents the median, the box encloses the first and third quartiles, and the whiskers, the minimum and maximum value; points are outliers (still considered in the statistical analysis). Letters denote statistical difference between group resulting from Tukey's all pair comparisons ($p < 0.05$). (b) Prevalence of the different 25(OH)D levels and vitamin D status during the four seasons: deficiency, dark gray; insufficiency, gray; sufficiency, light gray.

decreased [25]. Given that gender is a strong predictor, when we performed the analysis after blocking by gender, we found a negative correlation between 25(OH)D levels and WC, BMI, weight, and age, but not with the % of body fat. A possible explanation for the lack of correlation of vitamin D levels with body fat percentage might be that BMI, weight, and WC were correlated among themselves, while body fat percentage was not. Although most studies agree that vitamin D levels are negatively correlated with body fat percentage because of its sequestration by fat cells, one study attributes a lack of correlation to a volumetric dilution instead, which might in part explain our results (46). Another explanation might be the wide range of body fat percentage of the participants, from very low (17.5) to very high (51.3) which might have influenced the result. One more possibility would be that we assessed fat percentage but not fat mass in kg, which represents better the total adipocyte mass.

Similarly, observational studies have linked an increase in BMI with lower levels of vitamin D [24, 25]. A negative correlation between BMI and serum vitamin D levels has been found in obese [25] and in nonobese populations [23]. In another study, a significant negative correlation was found between body fat and vitamin D serum levels [45]. In this sense, a cross-sectional study showed that weight, body fat mass, and BMI were negatively correlated to 25(OH)D levels [46], as well as WC and other obesity markers [47, 48].

It has also been observed that in women, even after adjusting for BMI, ethnic group, age, and season, vitamin D remained associated with total abdominal adipose tissue, VAT, SAT, and body fat percentage. It also remained associated with VAT and body fat percentage in men [49]. Remarkably, when our data was analyzed separately, BMI was a significant predictor of 25(OH)D levels in the male population. In women, WC explained 31% of the variation in 25(OH)D levels and showed that for each centimeter increase in WC, there was a decrease of 0.176 ng/mL in the level of 25(OH)D. As well, for each one increase in BMI, concentration of 25(OH)D decreased by 0.534 ng/mL. Furthermore, the decision tree established that the main predictor of vitamin D levels is WC and that in combination with BMI and age, the degree of possibility of vitamin D deficiency can be predicted. Similar to our results, a cross-sectional study showed that weight was the variable most related to vitamin D levels, followed by body fat mass and BMI. Total weight combined with fat mass was the strongest linear fit, which explained 10.4% of the variation in vitamin D levels [46].

A retrospective study in Turkey found that the highest 25(OH)D levels were found in the age group 0–10 and the lowest in the 10–40 years old age group. The highest prevalence of vitamin D deficiency was found in the age group 20–30 years old. The authors suggested that older adults may be taking supplements. About 80% had 25(OH)D levels below 30 ng/mL from February to May, but only 58% remained with such levels in summer. A negative correlation ($p < 0.001$) was found between 25(OH)D and PTH levels [50]. However, this study did not take into account information about vitamin D supplementation nor about any lifestyle (e.g., diet, vitamin D intake, and sun exposure) or anthropometric variables. As the authors suggested, older adults might have been taking supplements, and this would be the reason why younger people had lower values. In our study, vitamin supplementation was an exclusion

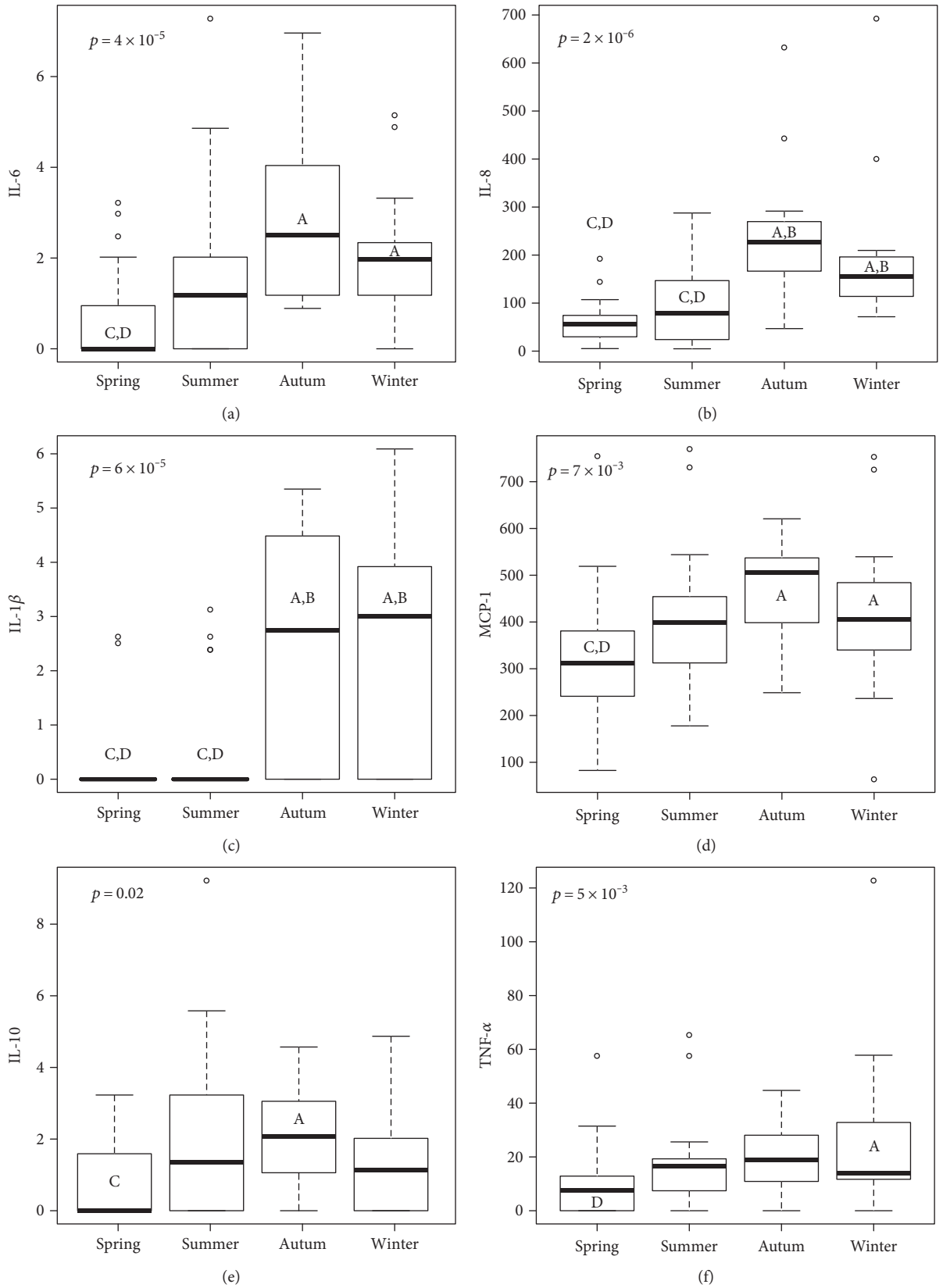

Figure 6: Seasonal variation of the cytokine panel. Concentrations of IL-6 (a), IL-18 (b), IL-1β (c), MCP-1 (d), IL-10 (e), and TNF-α (f) during the four seasons. The bold line represents the median, the box encloses the first and third quartiles, and the whiskers, the minimum and maximum value; points are outliers (still considered in the statistical analysis). Letters denote statistical difference between groups resulting from Tukey's all pair comparisons ($p < 0.05$).

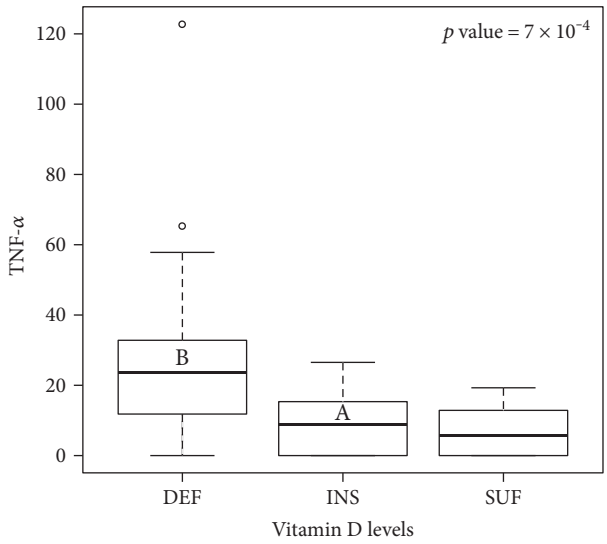

Figure 7: Relation of TNF-α with vitamin D. Concentrations of TNF-α are plotted against the 25(OH)D level. The bold line represents the median, the box encloses the first and third quartiles, and the whiskers, the minimum and maximum value; points are outliers (still considered in the statistical analysis). Letters denote statistical difference between groups resulting from Tukey's all pair comparisons ($p < 0.05$). DEF = deficiency; INS = insufficiency; SUF = sufficiency.

criteria and we did consider vitamin D intake in foods as well as sun exposure. Thus, our results are not influenced by vitamin D supplementation.

The skin content of 7-dehidrocholesterol as well as the response to UV radiation decreases with aging, resulting in a 50% decrease in the cutaneous production of pre-vitamin D [26]. Another author states that the capacity of skin production of vitamin D in a 70-year-old is reduced by 75% compared to the capacity of a young adult [51]. Based on extrapolations from other studies, it is estimated that the average adult older than 60 years living in southern (35°N) United States produces less than 600 IU/day of vitamin D in response to sunlight [52]. On top of the decrease in levels of 25(OH)D, elders have a decrease in the renal production of 1,25-dihydroxyvitamin D due to the generalized decline in renal function associated with aging, a decrease in intestinal calcium absorption, resistance to the action of 1,25(OH)D in the bowel, and a decrease in the number of cellular receptors of vitamin D (VDR) [26]. Furthermore, elders are more likely to take medications that may interfere with vitamin D metabolism [53].

We recognize that one limitation of this study is the small number of subjects included. However, a study that analyzed the causal relation between vitamin D status and obesity within 21 cohorts (42,024 subjects) agreed with our data, finding that for every increase of 1 kg/m^2, there was a reduction of 1.15% of serum 25(OH)D. Here, we found a 1.27% reduction associated to BMI in our population. In this remarkable work, the single nucleotide polymorphism that they chose as marker for obesity was related to an increase in BMI and a reduction in 25(OH)D, reinforcing the idea that obesity causes vitamin D serum levels to drop but that vitamin D status does not affect BMI [54]. Regarding the potential mechanism to explain the association between obesity, BMI, and body fat, vitamin D deficiency is determined less by intestinal absorption and more by sequestration by adipose tissue [55–57]. Some others have indicated that the association between obesity and vitamin D deficiency may be explained almost completely by a simple dilutional effect [46]. It has been also observed that obese people have a 57% lower increase in vitamin D in response to UVB radiation or oral supplementation [23]. Adipose cells have VDR and thus may be influenced by vitamin D status [58].

4.3. Inflammatory Status. Finally, given the crescent data that correlates vitamin D deficiency with many diseases, we performed an analysis of the inflammatory status of these subjects by analyzing their serum cytokine profile. We found that IL-1β, IL-6, IL-10, IL-18, MCP-1, and TNF-α significantly varied with the season, considering age, sex, and individual as random effects, with the highest levels occurring during autumn and/or winter, and the lowest levels occurring in the spring.

However, TNF-α was the only cytokine that was dependent on 25(OH)D concentrations. TNF-α levels were exacerbated in subjects with vitamin D deficiency. This demonstrates that vitamin D modulation by the inflammatory system depends on the immune system activation for the signaling pathway to allow VDR and vitamin D regulators like CyP27B1 to be expressed. Taking this into consideration, we ensured that all subjects included in this study were healthy. We were able to perform an analysis that correlated perfectly with the cytokine families and their influences between each other, validating our data. Furthermore, in agreement with our data, Peterson and Heffernan reported a negative correlation between vitamin D and TNF-α levels in healthy Caucasian women (aged 25–62), a relationship that remained after controlling body fat percentage, menopausal status, age, serum estradiol, serum cortisol, and hormonal contraceptive use [10]. Others found no relationship with IL-6 or IL-10 [6].

Alike our study, another study performed in younger women (aged 19–47) from Kuwait analyzed a wide spectrum of cytokines (IL-1 β, IL-6, IL-8, IL-17, IFN-γ, TNF-α, IL-4, IL-10, and IL-13) and only found a negative correlation between deficient levels of 25(OH)D with TNF-α when C-reactive protein (CRP) was elevated [59]. Moreover, a cross-sectional study in adults (aged 20–59) in Brazil showed a negative association between plasma IL-6 and TNF-α levels and vitamin D in normal-weight participants [60]. A very recent analysis in three studies within the Human Functional Genomics Project that also supports our data found that despite the impact of seasonality on cytokine production, there is indeed a limited dependency on vitamin D levels [61].

Regarding aging, one study reported an increase of IL-4-producing CD4$^+$ cells and a decline in TNF-α and interferon-γ-producing CD4$^+$ cells in older adults [62]. However, another study showed naïve, cytotoxic, and memory CD8$^+$ T cells increased the production of type 1 cytokines (interferon-γ, TNF-α, and IL-2) with age. There was also an

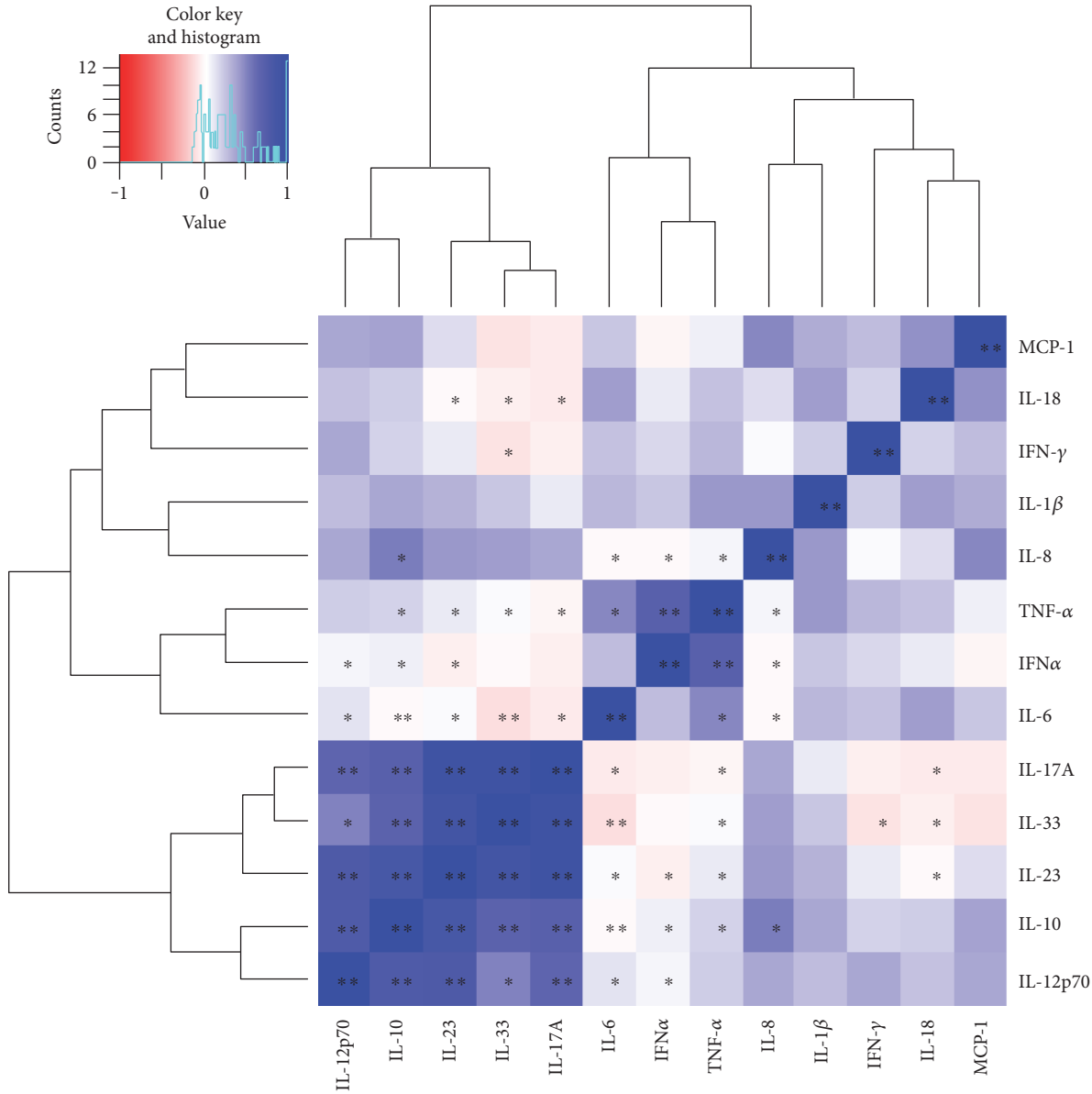

Figure 8: Correlations among cytokines. Pearson correlation coefficients are shown in the heatmap as a color gradient, from red ($r = -1$) to white ($r = 0$) to blue ($r = 1$). Significant correlations are shown as $^{*}p < 0.05$, $^{**}p < 0.01$.

increase with aging in the memory $CD8^{+}$ T cell-producing type 2 cytokines (IL-4, IL-6, and IL-10) [63].

For older adults, several studies are in line with the relation between the inflammatory status and 25(OH)D levels found in the present study. For instance, Liu et al. reported that 25(OH)D 1alpha hydroxylase knockout (CYP27b1−/−) mice, when compared with wild-type mice, had more DNA damage, reactive oxygen species production, inflammatory infiltration of the colon, and production of inflammatory cytokines related with SASP [64]. Accordingly, a previous report demonstrates that 1,25(OH)2D deficiency causes erosion of articular cartilage by inducing DNA damage and the production of senescence-associated inflammatory cytokines [65]. Studies in humans have shown that vitamin D deficiency has also been linked with telomere shortening [66, 67]. Telomere shortening seen in women with 25(OH)D < 16 ng/mL (lowest tertile) was equivalent to 5 years of cellular aging when compared with the telomere length of women with >50 ng/mL (highest tertile) [66]. The high prevalence of vitamin D in our population may thus have implications regarding the production of SASP inflammatory cytokines, mainly TNF-α.

Finally, recent research suggests that vitamin D exhibits anti-inflammatory effects that might contribute to its beneficial impact on several chronic diseases such autoimmune disorders, osteoarthritis, or cancer [10–12]. For instance, studies in cancer cells reveal that vitamin D regulates several of the key molecular pathways involved in procarcinogenesis inflammation such as prostacyclin synthesis, activation of kinase signaling, and nuclear factors involved in angiogenesis [68]. Our findings suggest that the high prevalence of vitamin D deficiency might place our population at increased

risk for a variety of chronic inflammatory diseases. Thus, strategies to increase levels of 25(OH)D levels could translate into a better prognosis and improve the outcomes in the older adult population.

5. Conclusions

In this 12-month follow-up study in older individuals, 25(OH)D levels were below 30 ng/mL, and the prevalence of deficiency was high through all four seasons, although it was higher in winter than in autumn and summer, despite the fact that the study took place in a region with a latitude that provides adequate sunlight in all seasons except winter. 25(OH)D levels showed a negative correlation with BMI, WC, and weight. These anthropometric markers are predictors of vitamin D deficiency. A BMI > 32 and a WC > 88 cm can predict a 90% probability of vitamin D deficiency, independent of age, with a confidence level of 73%. When BMI is <32.7, age is a determinant of vitamin D status, with a cut-off point of 74 years. Given the prediction value, these anthropometric and age parameters allow us to generate a simple way to predict vitamin D deficiency that could aid clinicians in better approaching the management of patients with diseases that are associated with this condition. More studies are needed, especially in regions near the equator, to corroborate our results and to elucidate potential mechanisms for such an extraordinary vitamin D deficiency.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Leticia Elizondo-Montemayor and Gerardo García-Rivas conceived and designed the experiments. Elena C. Castillo performed the experiments. Merit Gómez-Carmona, José R. Villarreal-Calderón, Sofia Tenorio-Martínez, and Bianca Nieblas performed the fieldwork with patients. Carlos Rodríguez-López and Elena C. Castillo analyzed the data. Leticia Elizondo-Montemayor and Gerardo García-Rivas contributed reagents/materials/analysis tools. Leticia Elizondo-Montemayor, Elena C. Castillo, Carlos Rodríguez-López, and Gerardo García-Rivas wrote the paper. Leticia Elizondo-Montemayor and Elena C. Castillo, both authors contributed equally.

Acknowledgments

This work was partially supported by Xignus Research Fund (Leticia Elizondo-Montemayor as well endowed Chair in Cardiology/Grupo de Enfoque Medicina Molecular-TEC de Monterrey) and CONACYT grants CB-256577 and Fronteras de la Ciencias 0682 (Gerardo García-Rivas). Elena C. Castillo was supported by Postdoctoral Fellowship program from CONACYT.

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 6412531, 8 pages
https://doi.org/10.1155/2017/6412531

Research Article

Vitamin D Deficiency Is Associated with Increased Osteocalcin Levels in Acute Aortic Dissection: A Pilot Study on Elderly Patients

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Received 27 April 2017; Accepted 6 June 2017; Published 2 July 2017

Academic Editor: Carmela R. Balistreri

An imbalance between degradation and reconstruction of the aortic wall is one of the leading causes of acute aortic dissection (AAD). Vitamin D seems an intriguing molecule to explore in the field of AAD since it improves endothelial function and protects smooth muscle cells from inflammation-induced remodeling, calcification, and loss of function, all events which are strongly related to the aging process. We quantified 25-hydroxy vitamin D, calcium, parathormone, bone alkaline phosphatase, and osteocalcin levels in 24 elderly AAD patients to identify a potential pathological implication of these molecules in AAD. Median 25-hydroxy vitamin D (10.75 ng/mL, 25th–75th percentiles: 6.86–19.23 ng/mL) and calcium levels (8.70 mg/dL, 25th–75th percentiles: 7.30–8.80 mg/dL) suggested hypovitaminosis D and a moderate hypocalcemia. Thirty-eight percent of AAD patients had severe (<10 ng/mL), 38% moderate (10–20 ng/mL), and 24% mild 25-hydroxy vitamin D deficiency (20–30 ng/mL). A significant inverse correlation was observed between 25OHD and osteocalcin levels. All the other molecules were unchanged. A condition of hypovitaminosis D associated to an increase in osteocalcin levels is present in AAD patients. The identification of these molecules as new factors involved in AAD may be helpful to identify individuals at high risk as well to study preventing strategies.

1. Introduction

Increasing data suggest that vitamin D, in addition to its role in bone metabolism, may exert important extraskeletal effects. Among different tissues and organs, the cardiovascular system has been regarded as one of the main targets of vitamin D actions. Epidemiological studies indicated that serum 25-hydroxy vitamin D (25OHD) levels are inversely associated with atherosclerosis, hypertension, coronary artery disease, peripheral artery disease, and stroke as well as with most of the main traditional cardiovascular risk factors [1–7]. On the other end, clinical trials on vitamin D supplementation failed to demonstrate a reduction in cardiovascular disease events leaving open the question whether hypovitaminosis D is an epiphenomenon rather than an etiological factor [8].

Recent studies suggested the existence of an inverse relationship between 25OHD levels and the presence and size of aortic aneurysms and thoracic aortic dilatations [9–11]. The underlying mechanisms explaining these associations are not fully understood but probably deal with processes which may be affected by hypovitaminosis D, such as wall vessel

inflammation, endothelial dysfunction, and artery wall integrity. In fact, in this regard, the potential anti-inflammatory properties of vitamin D [12, 13] as well as its ability to improve endothelial function and to protect smooth muscle cells from inflammation-induced remodeling, calcification, loss of function, and senescence have been suggested in different studies [14–18].

An imbalance between degradation and reconstruction of the aortic wall is also one of the leading causes of acute aortic dissection (AAD). AAD is an acute event caused by the formation of a tear within the artery wall and the creation of a false lumen within aortic wall between the intima and the media. It is associated with high morbidity and mortality if undiagnosed or not properly treated and is mainly characterized by cystic medial necrosis, elastic layer degradation, smooth muscle cell apoptosis, and inflammation [19]. Any condition which may affect aortic wall integrity may thus promote AAD.

Today, no studies assessed the vitamin D status in AAD patients. Thus, our aim was to evaluate whether vitamin D and vitamin D-related bone markers, which have also been related to metabolic and cardiovascular diseases, namely, osteocalcin (OC), parathormone (PTH), and bone alkaline phosphatase (BAP) [20, 21], are altered in AAD, also according to dissection localization at the ascending or descending aorta. The identification of new factors and pathways potentially associated and involved in AAD may be finally helpful to identify individuals at high risk as well to study preventing strategies.

2. Materials and Methods

2.1. Patients. This is a retrospective investigation study of 44 consecutive AAD patients who attended the I.R.C.C.S. Policlinico San Donato from 2005 to 2012 and were included in the International Registry of Aortic Dissection (IRAD) [22], according to the inclusion and exclusion criteria expected by the register. The study was conducted with the approval of local Ethics Committee (ASL Milano 2, Reference number 1409), and informed consent was signed by each patient. To reduce the effect of potential confounding factors, the following exclusion criteria were further applied: the known presence of generalized bone diseases (including hyperparathyroidism, rheumatoid arthritis, and Cushing's syndrome), malignant disease, recent major abdominal surgery, renal and liver diseases, Marfan syndrome or other genetic disorders, lack of clinical and biochemical data, and insufficient blood samples. Thus, a total of 24 patients were finally studied. According to Stanford classification, 19 were type A patients, having a dissection in the ascending aorta (proximal dissection), and 5 were type B, showing a dissection limited to the descending aorta (distal dissection).

AAD were repaired using deep or moderate hypothermic circulatory arrest and selective antegrade cerebral perfusion. All patients were managed with open distal anastomosis. Height, weight, medication intake, and risk factors were recorded. Body mass index (BMI, kg/m^2) was calculated.

2.2. Biochemical Assays. Serum sample was obtained after centrifugation at 1000g for 15 min and then immediately stored at −20°C until analysis. Serum 25OHD levels were measured by a chemiluminescence assay (Total 25OHD assay, DiaSorin, Saluggia, Italy). The lower detection limit was 4 ng/mL; the intra- and interassay coefficients of variation (CVs) were 3.7 to 7.7% and 5.8 to 10.9%, respectively. BAP was quantified by a direct, 2-site sandwich-type immunoluminometric assay utilizing two monoclonal antibodies (BAP OSTASE, DiaSorin). The lower detection limit was 1.5 μg/L; the intra- and interassay CVs were 3.2 to 4.0% and 6.5 to 8.1%, respectively. A direct, 2-site, sandwich-type immunoluminometric test which utilizes directly coated microparticles was used for OC detection (Osteocalcin, DiaSorin). The lower detection limit was 0.5 ng/mL; the intra- and interassay CVs were 3.0 to 8.0% and 4.0 to 9.0%, respectively. 1–84 PTH was quantified with a 2-site, sandwich-type immunoluminometric test using directly coated microparticles (1–84 PTH assay, DiaSorin). The lower detection limit was 4.0 pg/mL; the intra- and interassay CVs were 3.0 to 5.9% and 5.5 to 9.0%, respectively.

All analyses were performed using the LIAISON Analyzer (DiaSorin). Calcium was quantified by a colorimetric method using Vitros 5600 System (Ortho Clinical Diagnostics, Rochester). The lower detection limit was 4.0 mg/dL; the intra- and interassay CVs were 0.04 to 0.12% and 0.9 to 1.9%, respectively.

2.3. Statistical Analysis. The normality of data distribution was assessed by the Kolmogrov-Smirnoff test. Data were expressed as mean ± standard deviation (SD), median (25th–75th percentiles), or number and percentage. *T*-test, for normal variables, or Mann–Whitney *U*-test, for nonparametric variables, was used to compare two groups. Chi-square test was used for categorical outcomes. For multiple comparison (three groups), the Bonferroni correction was used. Pearson (for normal distributed data) or Spearman (for non-normal distributed data) correlation tests were used to test the univariate association between variables. All statistical analyses were performed using GraphPad Prism 5.0 biochemical statistical package (GraphPad Software, San Diego, CA). A p value <0.05 was considered significant.

3. Results and Discussion

3.1. Results. The demographic, anthropometric, and clinical characteristics of patients enrolled in the study are reported in Table 1. In AAD patients, the median serum 25OHD levels (10.75 ng/mL) suggest an overall condition of hypovitaminosis D. None of the patients had 25OHD levels higher than 30 ng/mL, the threshold for optimal vitamin D status. The levels of 25OHD < 10 ng/mL, 10–20 ng/mL, and 20–30 ng/mL were designated as severe deficiency, moderate, and mild deficiency, respectively. According to this classification, the 38% of AAD patients had severe 25OHD deficiency, 38% moderate 25OHD deficiency, and 24% mild 25OHD deficiency.

Relative to the other vitamin D-related bone turnover markers, OC levels (17.95 ± 8.1 ng/mL) as well as BAP

Table 1: Characteristics of participants included in the study.

	AAD patients ($n = 24$)	25OHD < 10 ng/mL ($n = 9$)	10 ng/mL < 25OHD < 20 ng/mL ($n = 9$)	25OHD > 20 ng/mL ($n = 6$)
Age (years)	62.67 ± 12.11	58.00 ± 14.21	61.11 ± 8.34	72.00 ± 9.59
Gender				
Male (n, %)	15, 62.5	7, 77.78	8, 88.89	4, 66.66
Female (n, %)	9, 37.5	2, 22.22	1, 11.11	2, 33.33
BMI (kg/m^2)	23.09 ± 6.09	23.23 ± 9.77	23.57 ± 2.68	25.28 ± 3.88
Type of dissection				
A	19, 79.17	9, 100.00	6, 66.67	4, 66.67
B	5, 20.83	0, 0.00	3, 33.33	2, 33.33
First SBP	130.00 ± 50.88	117.22 ± 27.05	141.22 ± 70.15	79.00 ± 27.02
First DBP	60.00 (60.00–92.50)	66.00 (55.00–80.00)	83.75 ± 38.15	60.00 (60.00–92.50)
Risk factors (n, %)				
Hypertension	18, 75.0	6, 66.67	6, 66.67	6, 100.00
Diabetes	—	—	—	—
Atherosclerosis	6, 25.0	2, 22.22	1, 11.11	3, 50.00
Known aortic aneurism	2, 6.3	1, 11.11	1, 11.11	0, 0.00
Prior aortic dissection	—	—	—	—
Mitral valve disease	1, 4.2	0, 0.00	1, 11.11	0, 0.00
Bicuspid aortic valve disease	3, 12.5	3, 33.33	0, 0.00	0, 0.00
Aortic valve disease	1, 4.2	1, 11.11	0, 0.00	0, 0.00
Tricuspid valve disease	—	—	—	—
Peripartum state	—	—	—	—
Other aortic disease	—	—	—	—
Cocaine abuse	1, 4.2	1, 11.11	0, 0.00	0, 0.00
Smoking	6, 25.0	3, 33.33	1, 11.11	2, 33.33
ACE inhibitors	4, 16.7	1, 11.11	2, 22.22	1, 16.67
ARB	1, 4.2	0, 0.00	0, 0.00	1, 16.67
Beta blockers	13, 54.2	6, 66.67	4, 44.44	3, 50.00
Ca-channel blockers	4, 16.7	1, 11.11	2, 22.22	1, 16.67
Diuretic	3, 12.5	0, 0.00	1, 11.11	2, 33.33
Nitroprusside	6, 25.0	0, 0.00	4, 44.44	2, 33.33
Other vasodilators	7, 29.2	4, 44.44	2, 22.22	1, 16.67
Vasopressors	1, 4.2	0, 0.00	0, 0.00	1, 16.67
25OHD (ng/mL)	10.75 (6.86–19.23)	6.29 ± 1.64[a,b]	10.60 (11.00–13.60)[b]	23.02 ± 2.55
Osteocalcin (ng/mL)	17.95 ± 8.14	22.26 ± 10.39[c]	17.86 ± 4.85	11.62 ± 3.57
BAP (mg/L)	7.39 ± 3.24	6.33 ± 1.78	7.56 ± 4.06	8.27 ± 3.84
PTH (pg/mL)	24.40 (14.10–39.68)	31.91 ± 19.65	25.32 ± 18.22	30.88 ± 22.09
Ca (mg/dL)	8.70 (7.30–8.80)	7.77 ± 0.99[d]	8.70 (8.25–8.78)[c]	10.75 ± 2.03

Data are expressed as mean ± SD, median (25th–75th percentiles), or number and proportions. BMI: body mass index; NW: normal weight; OW: overweight; OB: obese; SBP: systolic blood pressure; DBP: diastolic blood pressure; ACE: angiotensin-converting enzyme. ARB: angiotensin II receptor blocker; 25OHD: 25-hydroxy vitamin D; BAP: bone-specific alkaline phosphate protein; PTH: 1–84 parathormone; Ca: calcium. [a]$p < 0.001$ versus 10 ng/mL < 25OHD< 20 ng/mL; [b]$p < 0.001$ versus 25OHD > 20 ng/mL; [c]$p < 0.05$ versus 25OHD > 20 ng/mL; [d]$p < 0.01$ versus 25OHD > 20 ng/mL.

(7.39 ± 3.2 mg/L) and PTH (24.40, 14.10–39.68 pg/mL) were within the ranges of normality. After AAD patient classification according to 25OHD status, we observed a significant increase in OC concentrations ($p < 0.05$ for 25OHD < 10 ng/mL versus 25OHD > 20 ng/mL) at decreasing 25OHD level.

Differently, statistically significant differences were observed neither in BAP and PTH levels among groups (Figure 1) nor in any other clinical or demographic parameters studied (Table 1). The vitamin D status of AAD patients was further evaluated with regard to cut-off value of 15 ng/mL, which has been suggested as a more accurate limit below which cardiometabolic dysfunction may be observed [23–25]. In patients with 25OHD levels lower than 15 ng/mL, OC was higher (18.60, 17.13–23.38) compared to the other group (14.80 ± 4.52, $p < 0.05$),

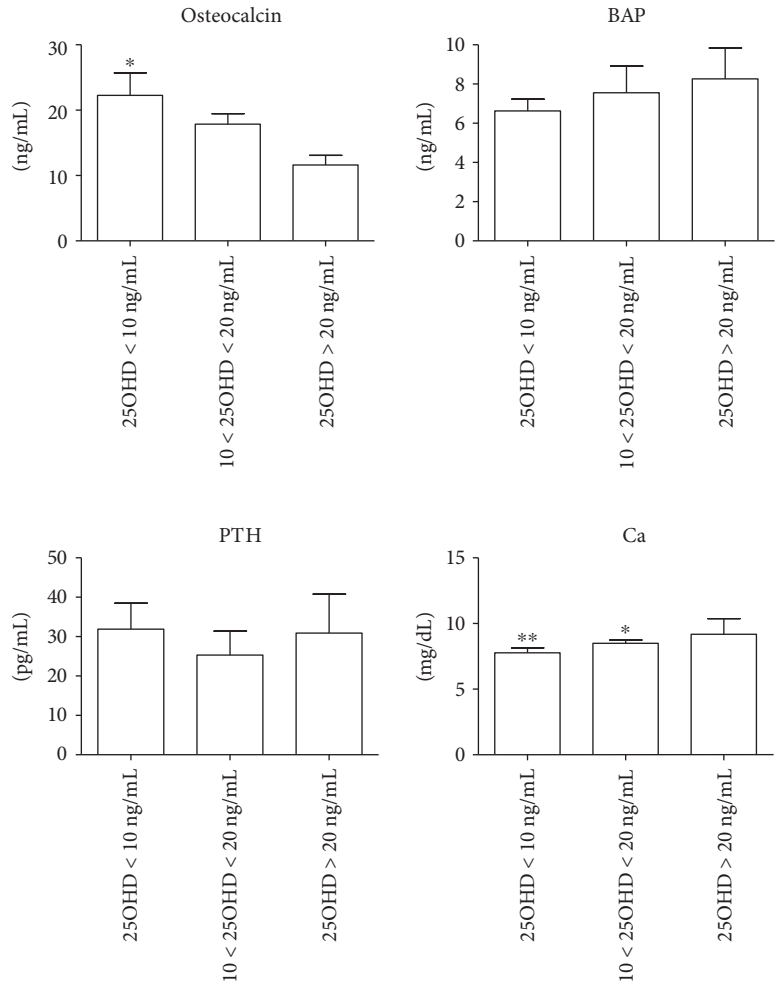

Figure 1: Evaluation of osteocalcin, bone alkaline phosphatase (BAP), parathormone (PTH), and calcium levels in patients with acute aortic dissection (AAD) according to plasma 25OHD concentration. AAD patients were stratified into three groups (severe deficiency: 25OHD < 10 ng/mL; moderate deficiency: 10–20 ng/mL; mild deficiency: >20 ng/mL) according to plasma 25OHD concentration. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus 25OHD > 20 ng/mL.

whereas no differences were observed in the levels of all the other markers (data not shown). Univariate correlation analysis clearly showed the existence of an inverse association between 25OHD and OC ($r = -0.610$, $p = 0.016$). No correlation was observed with the other parameters, instead.

Mean calcium levels suggested a mild condition of hypocalcemia in AAD patients. As expected, calcium levels decreased at decreasing 25OHD levels ($p < 0.01$ for 25OHD < 10 ng/mL versus 25OHD > 20 ng/mL; $p < 0.05$ for 10 ng/mL < 25OHD < 20 ng/mL versus 25OHD > 20 ng/mL). Anyway, univariate correlation analyses with 25OHD and the other parameters of calcium metabolism did not reach the statistical significance (25OHD: $r = -0.266$, $p = 0.245$; OC: $r = -0.126$, $p = 0.586$; BAP: $r = -0.041$, $p = 0.859$; PTH: $r = -0.049$, $p = 0.834$).

AAD patients were further classified according to dissection localization. Levels of 25OHD and OC were, respectively, higher and lower in type B compared to type A patients, although just over the limit of statistical significance (Figure 2). None of type B patients displayed 25OHD severe deficiency. BAP and PTH levels were almost the same in the two groups, as well as calcium.

4. Discussion

The main observation of our study is that AAD patients displayed hypovitaminosis D and there was an inverse relationship between 25OHD and OC levels. Otherwise, no changes in the concentrations of the other bone-related molecules, according to vitamin D status, have been observed in these patients.

Adequate vitamin D levels are first of all important for calcium absorption and thus the regulation of calcium homeostasis. Since low concentration of 25OHD is one of the main cause of hypocalcemia, it is not surprising to observe a trend of decrease in calcium concentration according to the vitamin D status of AAD patients. Otherwise, we did not observe an increase in PTH secretion as a compensatory mechanism to keep calcium concentration normal, as well as any significant correlation between

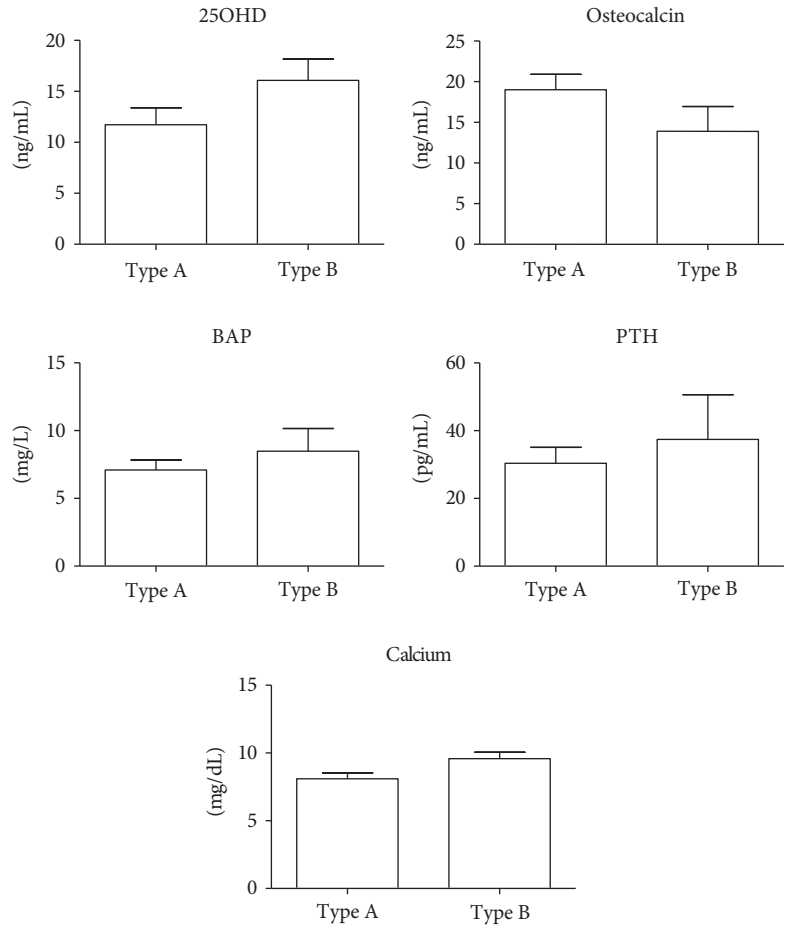

Figure 2: Evaluation of 25OHD, osteocalcin, bone alkaline phosphatase (BAP), parathormone (PTH), and calcium levels in patients with acute aortic dissection (AAD) classified according to dissection localization: types A and type B.

calcium and vitamin D levels. Since PTH secretion is promoted by a decrease in free calcium level, it is possible that, despite the decrease in total calcium level, free calcium did not change. Unfortunately, in the present study, we could measure total but not free calcium.

The unchanged levels of the bone turnover marker BAP seemed also to suggest the lack of activation of bone-related compensatory mechanisms in the regulation of calcium homeostasis in AAD patients. BAP is mainly produced by ostoblasts and plays important roles in bone matrix calcification [26]. Increased BAP levels are indicative of a higher osteoblastic activity as a compensatory mechanism towards increased osteoclastic activities, as observed, in osteomalacia, a condition characterized by a slight increase in BAP levels which are usually normalized after vitamin D treatment [27]. Due to vitamin D deficiency, one could thus expect to observe the activation of bone pathways promoting bone-calcium resorption but this seems not to be the main consequence of hypovitaminosis D in AAD patients.

In the present study, we also evaluated another bone matrix protein, OC, a vitamin D-related product which is released by osteoblasts and has potential role not only in osteoblast-osteoclast interaction and bone resorption but also in metabolism. Concerning OC, we observed the existence of an inverse correlation between this molecule and vitamin D. The fact that we could not observe any association between hypovitaminosis D and bone turnover did not exclude the possibility that reduced vitamin D levels may have consequences on vascular and cardiovascular health. Patients evaluated in this study, in fact, displayed an acute vascular event which was the result of inflammation-related processes and alteration in the composition of the media tunica of the vascular wall. Recent reports indicated that OC is associated to arterial diseases, thus being a molecule able to exert important effects also on vascular tissues which is similar to bone tissue in term of regulation of mineral homeostatsis [21]. In addition to OC, another molecule mainly produced by the bone, osteopontin, emerged as a novel player in arterial remodeling, inflammation, and the promotion of fibrosis, thus suggesting the same ability of these bone-derived molecules to target the cardiovascular system [28, 29]. Previous studies indicated a strong association between hypovitaminosis D and the presence and extend of arterial diseases, regardless of traditional cardiovascular risk factors and different cardiovascular pathologies, from coronary artery disease, to peripheral artery disease and aneurysms [1–7]. The association with the presence and the extent of aneurysms has also been observed regardless of

atherosclerosis which is considered one leading cause of aneurysm formation, thus suggesting a potential direct effect of vitamin D action on artery walls [9–11]. In this regard, vitamin D receptor is expressed both on endothelial as well as on smooth muscle cells where vitamin D can control cellular survival and local inflammation by preventing macrophage infiltration and the consequent production of proinflammatory and detrimental mediators [30, 31]. This means that one of the main vitamin D roles at vascular level may be its ability to play as an anti-inflammatory agent. Reduced vitamin D levels have also been associated to increased arterial stiffness and vascular calcification [32]. Since endothelial dysfunction, inflammation, and degeneration of the tunica media are key event not only in simple aneurysm formation but also in AAD, we found it interesting to explore vitamin D and OC levels in AAD. Our study did not describe the molecular mechanisms that could link hypovitaminosis D to vascular degeneration and AAD but indicated that both vitamin D and OC may play an important pathogenetic role which needs to be further explored, anyway. According to our data, variation in OC levels related to that of vitamin D seemed to reflect the activation of local mechanisms at vascular level rather than an alteration in bone metabolism. In fact, this inverse correlation between vitamin D and OC without changes in the levels of other bone-related molecules strongly reinforced the role of OC as an "extrabone" molecule. OC is mainly produce by osteoblasts where its synthesis is just regulated by vitamin D and where it plays important activities in bone matrix mineralization. OC exists in two main form: carboxylated, promoted by vitamin K, and undercarboxylated. In this latter form, it has less affinities towards calcium and hydroxyapatite and it is easily secreted and plays important endocrine activities, such as the stimulation of insulin secretion, the increase in insulin sensitivity, and the reduction of blood glucose levels [21]. Both forms are anyway secreted. The quantification of total OC, as done in our study, is an overall index of both bone- and nonbone-related effects. We also know that OC may be produced by endothelial progenitor cells [33] which may differentiate into OC-producing cells with a potential involvement in vascular dysfunction and in the pathogenesis of AAD. Also, the vascular smooth muscle cells can differentiate into osteoblast-like cells [34, 35].

This has been mainly observed in the presence of inflammation [36] that, as suggested in different previous papers, may be promoted by hypovitaminosis D [12, 37, 38]. To better define the potential pathogenetic role of OC in AAD, it would be interesting to explore on aortic tissues from AAD patients the relationship among OC expression and vitamin D and its receptor.

In our study, we also compared the type of AAD according to dissection localization. Usually, type A AAD, that is, at ascending aorta, is associated to worsen prognosis. By comparing the two types, we could not observe any difference in the levels of the evaluated molecules, although in type B patients, levels of 25OHD were higher and OC lower compared to type A, but at the limit of the statistical significance, probably due to the reduced number of type B patients.

5. Conclusions

Our study suggests that in AAD hypovitaminosis D is not associated to changes in bone-related metabolic pathways but is inversely related to OC which could be an interesting molecule able to mediate the effect of inadequate 25OHD level at vascular level. Future studies exploring local vascular mechanisms of vitamin D and OC could be helpful to better highlight the role of these molecules in this disease. Whether maintaining an optimal vitamin D status could be a preventing strategy needs also further analyses.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors thank Dr. Elena Costa, I.R.C.C.S. Policlinico San Donato, Milan, Italy, for the clinical chemistry data. The study was supported by the funds from the Italian Ministry for Health "Ricerca Corrente" I.R.C.C.S. Policlinico San Donato (no. 9.14.2).

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